EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.6, No.6, Nov–Dec 2000

Update from Japan

Mass die-off of Caspian seals

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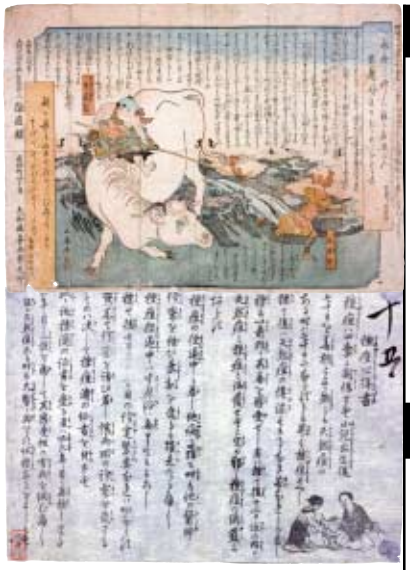

Cover: Detail from an advertising poster promoting smallpox vaccination (circa Kaei 3/1850), by Sunntei (thought to be the pseudonym of Dr. Kuwata). Reproduced with permission from the Nihon University Medical Library, Iidamachi, Tokyo.
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International Editor's Update—Japan

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For 20 years after the end of World War II, infectious diseases were endemic throughout Japan, which served during this first postwar phase almost as a museum of communicable diseases. Improvements in socioeconomic conditions, infrastructure (especially water and sewerage systems), and nutrition brought about a rapid reduction in rates of acute enteric bacterial and parasitic infections. The development and clinical application of antibiotics also contributed to this decrease.

During the second postwar period (1965-1985), further advancement in the use of antibiotics led to control of acute enteric bacterial diseases. However, medical advances such as cancer chemotherapy and organ transplantation, along with an increasing elderly population, created a large immunocompromised population and widespread opportunistic infections. The development of new antibiotics was followed by the emergence of pathogens resistant to drugs.

Since 1975, chemicals used in agriculture have been reevaluated to exclude toxic substances; however, decreased use of chemicals in agriculture has led to the reappearance or emergence of ticks and the rickettsia they transmit.

In the third postwar period (1985-present), increased international travel has led to an increase in imported infectious diseases. Travelers returning from other Asian countries and other continents have become ill with foodborne and insect-borne infections, including shigellosis, cholera, and typhoid fever; several thousand cases are reported each year. In addition, contaminated imported foods have been responsible for sporadic illnesses or small outbreaks.

Misuse or overuse of antibiotics has led to the emergence of methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, fluoroquinolone-resistant *Pseudomonas aeruginosa*, and vancomycin-resistant enterococci. All hospitals in Japan must now be alert to nosocomial infections caused by these drug-resistant pathogens.

The most important public health problems in modern Japan are massive outbreaks of acute enteric bacterial diseases. These outbreaks are caused by foods prepared commercially on a large scale for school lunches and chain stores. Contamination in a single aspect of preparation has resulted in large single-source foodborne outbreaks. More than 20,000 cases of infections caused by vibrios, Staphylococcus, pathogenic *Escherichia coli*, and Campylobacter have been reported in the past 5 years.

Concerning viral diseases, immunization programs against measles, rubella, and mumps have been mounted, in addition to the successful campaign against polio in the mid-1970s. However, except for polio, the coverage rate for individual vaccines is lower than rates in the United States and Europe, and vaccine-preventable viral illnesses remain at unsatisfactory levels. Viral diarrheal enteritis transmitted through foods such as oysters has also been increasing.

Trends in infectious diseases have changed rapidly in Japan during the past 50 years. Three reports are included in this issue that update the status of tuberculosis, flavivirus infection, and antibiotic resistance in Japan.

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Recent Trends in Tuberculosis, Japan

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Despite a decline after World War II, the rate of tuberculosis in Japan remains high. Infection is heavily concentrated in the ≥60-year age group, and 82% of patients are ≥40 years of age. The success rate for treatment of smear-positive patients is 78%. Multidrug-resistant strains of *Mycobacterium tuberculosis* are rare.

Before World War II, tuberculosis (TB) was highly prevalent in Japan. After the war, TB control measures came into widespread use, and both the incidence and death rate decreased rapidly, as in the United States and other industrialized nations (Figure 1). Because of the high rate in the postwar period, however, the TB case rate remains higher in Japan than in other countries. In 1998, the incidence of all forms of TB was 34.8 per 100,000 population in Japan, compared with 6.4 in the United States, 9.5 in the Netherlands, and 4.7 in Norway (the data from Norway are for 1997). The annual risk for infection was estimated to be 4% in the 1950s and 0.05% in the 1990s, reflecting an 11% annual rate of decrease from the 1950s to the 1970s, with a less steep decline thereafter, in parallel with the decrease in death and case rates.

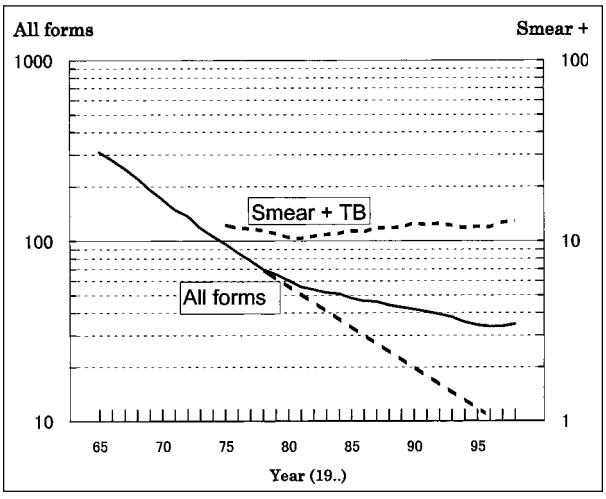

Figure 1. Tuberculosis (rate per 100,000 population) in Japan, 1965-1995.

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Epidemiologic Characteristics

Epidemiologic data show that after years of continuous decline, TB incidence is changing in Japan. In 1950, more than half of adolescents ages ≤20 years are estimated to have been infected, which resulted in a peak of TB incidence and deaths in this age group (Figure 2). In 1995, 1% of those ages ≤20 years and 2% of those aged ≤30 years were infected. A high TB rate among persons ages ≥60 years reflects infection acquired during youth.

Along with the shift in age-specific prevalence of infection, aging of the population during this period contributes to the disproportionate TB rate in older age groups. The proportion of newly reported patients ages ≥40 years was 53% in 1950 and 82% in 1998. These data indicate that the elderly, as well as the physically and socioeconomically disadvantaged, are at high risk for TB.

HIV infection, an important risk factor for TB worldwide, has not been widespread in Japan. However, a voluntary reporting system for TB

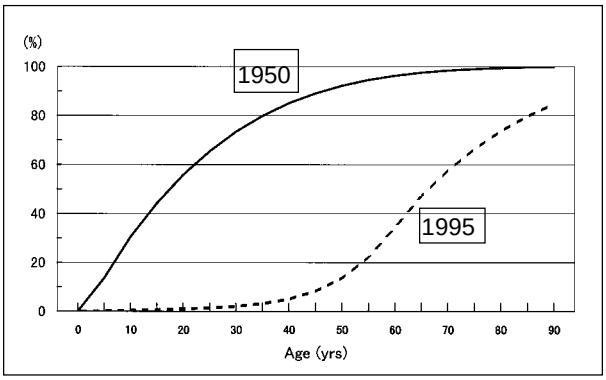

Figure 2. Estimated prevalence of tuberculosis infection by age in Japan, 1950 and 1995.

cases in HIV-infected persons, involving 14 physicians throughout the country, indicates that 90 such cases had been reported as of 1998 and that this number is increasing.

As in the United States, TB is becoming more common in economically disadvantaged households and workers in certain small businesses. The case rate among the disadvantaged and homeless in metropolitan areas is 50 to 60 times higher than the rate among the general population. TB among immigrants has been a problem in Japan, but in 1996 the proportion of TB patients who had entered Japan within the previous 5 years was only 1% of all newly reported cases.

Clinical Characteristics

The proportion of TB cases that are bacteriologically confirmed among all newly reported pulmonary cases has been increasing, from 19% in 1975 to 51% in 1998. Part of this change can be attributed to reporting that favors bacteriologic evidence over X-ray findings. However, data confirm that cases with severe symptoms or extensive disease at onset have increased. The number of patients with newly reported cases who die within a year of reporting has slowly increased, from 1.6% in 1975 to 2.6% in 1997.

According to a nationwide survey of TB treatment programs conducted in 1995, among smear-positive patients, 78% were cured, 12% died within 9 months, 7% were lost to follow up, and 3% did not respond to treatment. Throughout Japan, drug resistance has been generally low, as shown by a nationwide survey conducted at 5-year intervals (Table). Resistance to both isoniazid and rifampicin among previously untreated patients, i.e., multidrug resistance, is still low.

Table. Percentage of drug resistance in tuberculosis patients without prior treatment, Japan, 1997

Drug	Frequency (95% CI)
Isoniazid	4.4 (3.4-5.7)
Rifampicin	1.4 (0.8-2.2)
Streptomycin	7.5 (6.2-9.0)
Ethambutol	0.4 (0.2-0.9)
Any drug	10.2 (8.7-11.9)
Resistant to both Isoniazid and Rifampicin	0.8 (0.4-1.4)

CI = confidence intervals

Recent Slowing of Decline in TB Cases

The rate of decline in TB cases of all forms has slowed from 11% to 3% since 1980, and after 1996, the trend reversed slightly. The case rates for 1996, 1997, and 1998 were 33.7, 33.9, and 34.8, respectively. The reversal was most obvious in persons ≥70 years of age, but the plateau after 1980 is seen in all age groups, including adolescents and children ≤14 years of age. For smear-positive pulmonary TB, the downward trend reversed earlier, especially among the elderly. This reversal may be attributable to the rapid growth of the older age groups, who have high rates of past TB infection and are affected by conditions (e.g., diabetes, corticosteroid therapy, or hemodialysis) that increase their risk for TB. Patients in this older age group are a source of infection, transmitting TB to younger generations and leading to the plateau in case rates.

Another consequence of epidemiologic changes is an increase in small outbreaks of TB. While outbreaks used to be seen primarily in schools, now they are also seen in settings frequented by the young, including places of work and entertainment (e.g., bars, karaoke houses, public saunas). The main epidemiologic basis for the increase in outbreaks is the gap in infection prevalence between age groups, e.g., young, susceptible persons living in close contact with the elderly, who are vulnerable to TB infection (Figure 2).

Nosocomial outbreaks of TB are also a problem. Nationwide surveillance data show that during 1987 through 1997, the case rate of all forms of TB among female nurses was 2.3 times higher than that for the general female population. The relative risk among nurses was highest at 3.3 in the 20- to 29-year-old age group; risk declined with age but is still substantially higher for those <60 years of age.

Declaration of National TB Emergency

Control programs supported by government and the medical profession influence TB trends in Japan. An indicator of TB awareness is physician delay in case detection, i.e., the time from a patient's first visit until diagnosis of TB. According to national surveillance, 62% of all pulmonary cases are detected within 1 month of the first visit, but 15% of cases are detected after 2 months of medical attention. This interval

before diagnosis is expected to increase when TB becomes less frequent, with an accompanying decline in physician awareness of the disease. Because of the recent increase in TB rates, the Japanese minister of health and welfare has declared a TB emergency, urging the public, as well as the medical profession and local governments, to be on the alert for TB so further increases can be prevented.

Trends in Flavivirus Infections in Japan

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Although Japanese encephalitis has declined as an important cause of illness and death in Japan, infection with other flaviviruses has become a public health concern. Recently, reports of imported dengue cases, as well as isolations of tick-borne encephalitis virus, have increased.

The family *Flaviviridae* consists of approximately 70 viruses, nearly 40 of which cause human disease (1). Japanese encephalitis virus (JEV) was an important cause of illness and death in Japan for many years, with >1,000 Japanese encephalitis (JE) cases reported annually in the late 1960s. The number of JE cases has decreased dramatically, and fewer than 10 cases have been reported annually since 1992 (2). Infection by other flaviviruses, including an increase in imported dengue cases and isolations of tick-borne encephalitis (TBE) virus, is becoming a public health threat.

Imported Dengue Cases

Dengue viruses are transmitted by infected mosquitoes, mainly *Aedes aegypti* and *A. albopictus* (3,4). The clinical manifestations of dengue virus infections range from asymptomatic infection to dengue fever and dengue hemorrhagic fever (5). Dengue epidemics caused by dengue virus type 1 occurred in Nagasaki, Osaka, Kobe, and Hiroshima from 1942 to 1945 (6,7). No outbreaks of dengue virus infection have been reported since then in Japan, and no domestic dengue virus infections have been identified. However, during this period there have been imported dengue cases (8,9) in persons who visited dengue epidemic areas, were infected with dengue viruses, and became ill after returning to Japan. In addition, some foreign visitors who were infected in their own countries became ill with dengue while in Japan.

Dengue virus infections were diagnosed in serum specimens of suspected dengue cases submitted from hospitals and clinics by IgM-capture enzyme-linked immunosorbent assay, hemagglutination inhibition tests, and reverse transcriptase-polymerase chain reaction. Neutralization tests and virus isolation were also performed for some specimens. Dengue cases were confirmed by these laboratory tests at the National Institute of Infectious Diseases, Japan, from 1985 to 1999 (Table). The number of imported dengue cases has recently increased. Only two dengue hemorrhagic fever cases were identified, one each in 1990 and 1991; all the other cases were dengue fever. Most of these Japanese dengue patients became infected in Southeast Asia (Thailand, India, Philippines, and Indonesia), although some patients became infected in Central America and Africa in recent years. We believe that these dengue cases account for only a fraction of the total imported cases, although the exact number of imported cases is not known. Under a new infectious disease control law, which took effect on April 1, 1999, dengue fever/dengue hemorrhagic fever is a reportable disease. Thus, an accurate annual number of imported dengue cases will be known in the near future.

Tick-Borne Encephalitis

Central European encephalitis and Russian spring-summer encephalitis (RSSE) viruses are TBE viruses prevalent in Eurasia (1). The presence of TBE virus in Japan was first confirmed when Negishi virus was isolated from an encephalitis patient in 1948. This virus was later determined by antigenic analysis to be TBE virus (10). No further cases of tick-borne encephalitis were identified in Japan until 1993, when Takashima et al. reported a tick-borne encephalitis case in Hokkaido, the northern island of Japan (11). The patient, a dairy farmer, had high fever, double vision, convulsions, and

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Table. Cases of imported dengue and countries that persons visited before onset of symptoms, by year, Japan

Countries	'85	'86	'87	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	Total
No. of cases[a]	4	1	4	4	1	11[b]	6[b]	14	7	11	16	15	6	42	11	153
Asia																
Thailand	3	0	3	1	0	7	3	7	1	6	8	4	1	19	2	65
India	0	0	2	0	0	2	1	2	1	1	4	5	0	6	3	27
Philippines	2	1	1	1	0	1	3	1	3	1	1	1	1	8	1	26
Indonesia	0	0	0	1	0	0	0	3	1	2	2	3	1	5	0	18
Malaysia	0	0	1	0	0	2	0	0	0	0	1	2	0	1	1	8
Myanmar	0	0	0	0	0	0	0	0	0	0	1	0	0	3	4	8
Cambodia	1	0	0	0	0	0	0	0	1	0	1	0	0	3	1	7
Singapore	0	0	0	0	1	0	0	1	0	0	1	1	1	2	0	7
Nepal	0	0	1	0	0	0	0	1	0	1	0	0	1	1	0	5
Laos	0	0	0	0	0	0	1	0	0	1	0	0	1	2	0	5
Vietnam	0	0	0	0	0	0	0	0	0	1	0	0	1	2	0	4
Bangladesh	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	3
Maldives	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	3
Taiwan	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
China	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2
Sri Lanka	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Oceania/South Pacific																
Australia	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Fiji	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
New Caledonia	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Tahiti	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Central America																
Dominica	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
Guatemala	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Africa																
Nigeria	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Liberia	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Côte d'Ivoire	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1

[a]Some patients visited more than one country.
[b]In 1990 and 1991, one patient each year had dengue hemorrhagic fever.

motor paralysis. The 6- and 43-day serum samples showed highest neutralizing antibody titer to RSSE virus, but the level of neutralizing antibody to Japanese encephalitis virus was low. Serologic tests indicated that the encephalitis was caused by TBE virus. Takashima et al. demonstrated that sentinel dogs were seropositive for RSSE virus (11). They then isolated viruses from the dogs' sera and demonstrated that the isolated virus was related most closely to RSSE virus by nucleotide sequencing.

Takeda et al. isolated TBE virus from *Ixodes ovatus* ticks collected in the region and demonstrated that the isolated viruses were antigenically close to RSSE virus (12). They also showed that captured rodents had antibodies to TBE virus and that the viruses isolated from the rodents were also antigenically close to RSSE virus (13). These reports indicate that the TBE virus endemic in Hokkaido is closely related to RSSE viruses. Further studies are necessary for understanding the ecologic features of TBE virus in other islands of Japan.

Dr. Kurane is director of the Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan. His research interests include human T-cell responses to dengue viruses and pathogenesis of dengue hemorrhagic fever.

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Trends in Antimicrobial-Drug Resistance in Japan

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Multidrug resistance in gram-positive bacteria has become common worldwide. In Japan until recently, gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Serratia marcescens* were controlled by carbapenems, fluoroquinolones, and aminoglycosides. However, several of these microorganisms have recently developed resistance against many antimicrobial drugs.

In Europe and the United States, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) have been widely disseminated in many medical institutions; gram-negative rods, including *Klebsiella pneumoniae* and *Escherichia coli* producing extended-spectrum ß-lactamases (ESBLs), are also becoming a clinical threat. In Japan, many antimicrobial drugs, such as carbapenems, fluoroquinolones, and aminoglycosides, have been freely used as first-line drugs for more than 15 years. During that time, drug-resistant bacteria have been emerging and proliferating (1-3). Isolation of VRE and ESBL-producing gram-negative rods is still rare (4), but MRSA and penicillin-intermediate-resistant and penicillin-resistant *Streptococcus pneumoniae* are widely distributed in clinical settings (5), as in western countries. In addition, several carbapenem- and fluoroquinolone-resistant gram-negative rods have been isolated from geographically separate hospitals (6-8).

Development and Use of Antimicrobial Drugs

Many clinically effective antimicrobial drugs have been developed by Japanese pharmaceutical companies since the 1960s. These drugs are mainly broad-spectrum cephems, fluoroquinolones, macrolides, and aminoglycosides, which are quite effective against various pathogenic bacterial species. Moreover, the official prices of these antimicrobial drugs are set at relatively high levels compared with those of earlier drugs such as penicillins, older quinolones, and kanamycin. Therefore, newer drugs have been used preferentially as first-line drugs under the Japanese health insurance system, which allows medical institutions to obtain benefit from the difference between the official price (selling price) and the actual market cost. In Japan, three carbapenems (imipenem, panipenem, and meropenem) and at least nine fluoroquinolones (enoxacin, fleroxacin, norfloxacin, ofloxacin, ciprofloxacin, lomefloxacin, tosufloxacin, sparfloxacin, and levofloxacin) are used as first-line drugs in every clinical setting, although use of these drugs is restricted in many western countries, including the United States. Arbekacin, clarithromycin, and teicoplanin are also widely used for chemotherapy.

Drug-Resistant Bacteria

The following summary is based on a preliminary survey of VRE and MRSA and a report of surveillance for antimicrobial susceptibility in Japan conducted by the Medical Information System Development Center.

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Gram-Positive Cocci

VRE

Since vancomycin was approved for IV use in 1991 only for MRSA infections, isolation of VRE is still rare in Japan; however, VanA-type *Enterococcus faecium* was first isolated in Kyoto in 1996 (9). Fewer than 50 cases of *vanA*- or *vanB*-type VRE isolation have been reported throughout Japan (unpublished data). An increase in VRE isolation may be inevitable because of international travel and importation of chicken contaminated with VRE (10).

MRSA

The percentage of MRSA among nosocomial *S. aureus* strains in Japan is estimated to be 50% to 70%. Several deaths associated with MRSA infection have been reported (11,12), despite preventive measures against nosocomial infections. However, the actual number of deaths associated with MRSA infection, as well as the trend, is unknown.

Isolation of a vancomycin-homo-resistant *S. aureus* strain Mu50 from a clinical sample in Japan was reported in 1997 (13). This strain was selected from vancomycin-hetero-resistant *S. aureus* strain Mu3, which intrinsically contains a few Mu50. Identifying Mu50 by the antibiotic susceptibility testing method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (14) may be difficult. Therefore, clinicians were concerned that emergence of strains corresponding to Mu3 and Mu50 could become a serious clinical problem.

We conducted a nationwide survey for MRSA by collecting >6,600 clinical isolates from 245 medical settings. When $>10^5$ CFU of bacteria were inoculated, 3% to 4% of clinical isolates grew colonies on brain-heart-infusion agar (BHI agar) plates containing 4 mg/L of vancomycin, as recommended (13). However, the assay reproducibility was poor and we were unable to confirm a stable resistant phenotype. Several vancomycin-tolerant strains (MIC, 4 mg/L by NCCLS method) were selected after repeated in vitro selection with BHI agar plates containing sub-MIC concentrations of vancomycin. No strain corresponding to Mu50, which had an MIC for vancomycin of 8 mg/L by the standard NCCLS method, could be identified in 6,625 clinical isolates. If strains with vancomycin-resistance profiles similar to those of Mu3 or Mu50 were widely disseminated in Japan, vancomycin-homo-resistant strains such as Mu50 would be expected in clinical settings and would be isolated by the routine NCCLS antibiotic-susceptibility testing method (15). Since vancomycin has been in widespread use for nearly 9 years in Japan, several Mu50-like strains should have been selected and predominant. Possibly the Mu3 and Mu50 strains reported earlier are unusual strains that may have critical defects or mutations in the genes responsible for with synthesis or degradation of the peptidoglycan layer. The clinical importance and genetic background of these strains need to be elucidated. Clinicians should be alert for emergence of glycopeptide-resistant Staphylococci that have acquired endogenous gene mutations or exogenous genes responsible for vancomycin resistance (e.g., the *vanA* or *vanB* gene clusters found in VRE).

Glycopeptide-Resistant Coagulase-Negative Staphylococci

Sporadic isolation of teicoplanin-resistant coagulase-negative Staphylococci such as *Staphylococcus haemolyticus* has been reported to several medical, clinical, or microbiological societies in Japan; however, no report has been published in English.

Penicillin-Resistant Pneumococci

The overall isolation frequency of penicillin-intermediate *S. pneumoniae* and penicillin-resistant *S. pneumoniae* is estimated to be approximately 50%, according to reports from individual hospitals and health districts in Japan (16,17). Several clinically isolated pneumococci are resistant to cephems, new macrolides, and fluoroquinolones (16,18). Nosocomial penicillin-resistant *S. pneumoniae* isolates usually belong to serotypes 19, 6, and 23 (19), and substitution at Thr371 was associated with penicillin resistance in many such isolates (20).

Gram-Negative Rods

Pseudomonas aeruginosa and Related Microorganisms

P. aeruginosa is resistant to a wide range of antimicrobial drugs. Carbapenems, fluoroquinolones, and aminoglycosides such as amikacin are the last resort for treatment. However, several clinical isolates that have acquired resistance to

these drugs have been identified in Japan. Of special concern are highly carbapenem-resistant strains producing plasmid-dependent IMP-1-type metallo-ß-lactamase, which are proliferating rapidly (1). According to a preliminary survey of 2,533 *P. aeruginosa* isolates, 88 (1.3%) strains had the bla_{IMP} gene cassette responsible for IMP-1 production. Moreover, the bla_{IMP} gene cassette has been dispersing into various glucose-nonfermenting gram-negative bacteria such as *Pseudomonas putida*, *P. fluorescens*, and *Burkholderia cepacia* (7).

Furthermore, approximately 20% and 5% of clinically isolated *P. aeruginosa* have acquired resistance to fluoroquinolones and amikacin, respectively. *Alcaligenes* and *Acinetobacter* spp. have also acquired a wide range of drug resistance.

Enterobacteriaceae

Two nosocomial outbreaks of *Serratia marcescens* were recently reported in Japan. This bacterium is consistently resistant to penicillins and classic cephalosporins (e.g., cephalothin and cephaloridine), but not to carbapenems. However, IMP-1 producers have recently been isolated in geographically separate clinical settings, and they often show high-level resistance to ß-lactams, including cephamycins and carbapenems (21). According to a preliminary survey of 3,222 clinically isolated *S. marcescens* strains, at least 141 (4.4%) had IMP-1 productivity. Some of these strains also have acquired resistance to fluoroquinolones or amikacin.

E. coli and *K. pneumoniae* are still susceptible to oxy-imino-cephalosporins such as cefotaxime and ceftazidime. *K. pneumoniae* and *E. coli* strains that produce TEM- or SHV-derived extended-spectrum ß-lactamase and are resistant to these drugs are still rare in Japan, while those producing CTX-M-2 type ß-lactamase predominate (22,23). This disproportion may be related to the widespread use of cephamycins and carbapenems. However, as a result of recent restricted use of these broad-spectrum drugs, several *K. pneumoniae* and *E. coli* strains producing SHV-derived ESBLs, such as SHV-12, are emerging (4,24).

Several multidrug-resistant *Salmonella* spp. have been reported in Japan, and some of them were recently identified as *S.* Typhimurium DT104 (25). However, isolation frequency of DT104 is thought to be lower than in western countries.

Fluoroquinolone-resistant gonococci are also increasing in Japan, as in south Asian countries (26). *Haemophilus influenzae* clinical isolates that show ampicillin resistance, some of which are penicillinase nonproducers, have also been increasing (27). More than 90% of *Moraxella catarrhalis* have acquired penicillinase productivity.

Isolation frequency of multidrug-resistant *Mycobacterium tuberculosis* is estimated to be low (28) but may be increasing, as indicated by recent reports of several outbreaks (29).

Drug-Resistant Bacteria in the 21st Century

Multidrug-resistant gram-positive cocci such as MRSA and VRE have emerged and spread worldwide in the 20th century. Gram-negative rods such as *P. aeruginosa*, *K. pneumoniae*, and *S. marcescens* have until recently been controlled by carbapenems, fluoroquinolones, and aminoglycosides. However, several of these microorganisms have become resistant to many antimicrobial drugs. Extraordinary efforts will be needed to prevent global dissemination of multidrug-resistant gram-negative bacteria in the next millennium (30).

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Developing National Epidemiologic Capacity to Meet the Challenges of Emerging Infections in Germany

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In January 1996, the Robert Koch Institute, Germany's national public health institute, began strengthening its epidemiologic capacity to respond to emerging and other infectious diseases. Six integrated strategies were initiated: developing employee training, outbreak investigation, and epidemiologic research programs; strengthening surveillance systems; improving communications to program partners and constituents; and building international collaborations. By December 1999, five employees had completed a 2-year applied epidemiology training program, 186 health department personnel had completed a 2-week training course, 27 outbreak investigations had been completed, eight short-term research projects had been initiated, major surveillance and epidemiologic research efforts for foodborne and nosocomial infections had begun, and 16 scientific manuscripts had been published or were in press. The German experience indicates that, with a concerted effort, considerable progress in building a national applied infectious disease program can be achieved in a short time frame.

National and international strategies for detecting and preventing emerging infectious diseases have been created in both the civilian and military sectors (1-5). However, epidemiologic capacity at national, regional or state, and local levels is necessary to successfully implement many of these strategies. Health officials are conducting increasingly complex outbreak investigations, implementing and analyzing new surveillance systems, and conducting sophisticated applied epidemiologic research. However, the ability to carry out these tasks varies among countries and remains largely unevaluated. In 1996, Germany began strengthening its epidemiologic capacity to respond to emerging and other infectious diseases (6). We report the strategies used and their outcome through 1999.

Background

Germany is a highly industrialized country with 82 million inhabitants. It has 16 federal states, including five from the former German Democratic Republic. The principal responsibility for public health resides with the 16 state health ministries and approximately 420 local health departments. Although Robert Koch and his contemporaries built a strong tradition for infectious disease epidemiology in Germany in the late 19th and early 20th centuries, this tradition all but disappeared in the 1930s and 1940s. In the former West Germany, the national infectious disease institute (Robert Koch Institute [RKI]) was mainly focused on basic science research until the AIDS epidemic demanded a national public health response. In 1987, an independent AIDS Center was formed at the Federal Health Ministry. Reunification with the former East Germany and subsequent integration of the East German institutes for hygiene and microbiology gave additional incentives to improve infectious disease epidemiology. In 1994, a combined AIDS Center and Infectious Disease Epidemiology Division was created at RKI.

Substantial barriers hindered the further development of applied infectious disease

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epidemiology in Germany. Nearly all outbreak investigations were conducted at the local level; however, local health officials had little or no training in applied infectious disease epidemiology. No federal infrastructure existed to investigate outbreaks, nor was epidemiology recognized as a discipline distinct from microbiology.

In 1995, representatives of RKI, the Federal Ministry of Health and the Federal Ministry for Education and Research developed a concept for a network of collaborators whose goal would be intensifying epidemiologic research and improving infectious disease surveillance (7). As part of this concept, RKI was to develop a weekly epidemiologic bulletin, redefine national reference laboratories, form an infectious disease epidemiology commission, train epidemiologists in a 2-year applied training program similar to the Epidemic Intelligence Service at the Centers for Disease Control and Prevention (CDC) (8), and create networks of investigators capable of gathering existing public health data, identifying deficits, and collecting additional data as needed. A senior epidemiologist was seconded from CDC to help initiate this program, which began on January 1, 1996.

Program Goals, Strategies, and Outcomes

General Approach

The initial goal was to build sustainable national epidemiologic capacity over a 4- to 5-year period. Initial priorities were to develop capacity to identify and respond to epidemiologic emergencies, conduct applied epidemiologic research, and support state and local health departments in conducting these activities and in developing their own epidemiologic programs. Six integrated strategies were undertaken by RKI: developing employee training, outbreak investigation, and epidemiologic research programs; strengthening surveillance systems; improving communications between RKI and its partners and constituents; and building international collaborations (Figure 1).

Training and Manpower Development

The lack of personnel trained in applied infectious disease epidemiology made long- and short-term training programs a critical priority. The long-term program was to develop a cadre of epidemiologists (capable of performing outbreak investigations, epidemiologic research, and surveillance) who would later serve as trainers themselves. The short-term training program focused on providing health department personnel with practical skills and understanding to conduct and report on simple epidemiologic investigations and to conduct more complex epidemiologic research and surveillance activities in collaboration with national or state epidemiologists.

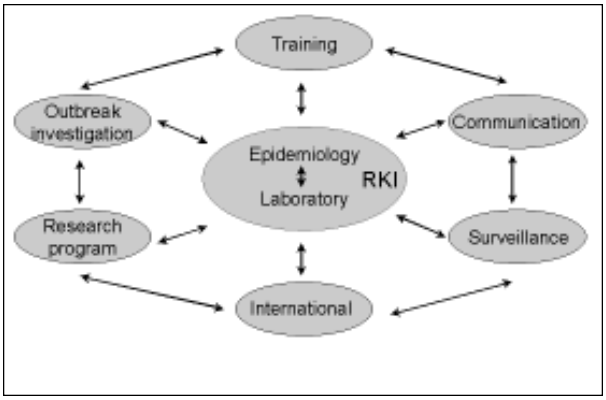

Figure 1. Integrated strategies used by the Robert Koch Institute (RKI) for the development of applied infectious disease epidemiologic capacity in Germany.

The 2-year training program, known as the German Field Epidemiology Training Program (FETP), began with two trainees in January 1996. Two additional cohorts of three trainees each began their training in 1998 and 1999, respectively. Seven of these eight trainees were physicians, and one was a veterinarian. Two of the physicians also had masters of public health degrees. All trainees were stationed at RKI. All were required to complete at least one outbreak investigation, surveillance project, and research project, as well as present their work at a scientific conference, participate as trainers in epidemiologic courses, and write at least one article each in a peer-reviewed scientific journal and in the national epidemiologic bulletin. Beginning with the second cohort, trainees also rotated for 2 weeks through RKI laboratories. This helped them understand the laboratory aspects of their epidemiologic investigations and further solidified the working relationships between the epidemiologists and laboratorians.

The German FETP started nearly simultaneously with the European Program for Intervention Epidemiology Training (EPIET), a Europe-wide, 2-year training program, also developed on an Epidemic Intelligence Service

model, in which a trainee from one European country trains in another. Four EPIET trainees from other countries have trained or are being trained at RKI, and three Germans have trained or are training in other countries as part of EPIET. The European program also provided a crucial training function for the German FETP by allowing each German FETP trainee to participate in EPIET's 3-week introductory training course and four 1-week training modules.

All five FETP graduates and three German EPIET graduates are employed. Two FETP graduates are now RKI epidemiologists, another is in charge of infectious diseases for the city and state of Hamburg, one works at a local health department with a focus on epidemiology, and one is a World Health Organization consultant. Of the three German EPIET graduates, one works in the EPIET program office, one is an RKI epidemiologist, and the third is an epidemiologist for the Health Ministry of North Rhine-Westphalia, the most populous German federal state.

The annual short-term training program consists of a 2-week applied epidemiology course for public health officials designed to impart practical skills. The first week involves lectures on basic epidemiologic study design, outbreak investigation methods, basic statistics, and three case studies. During the second week, students learn an epidemiologic data management and analysis computer program (EPI-Info) (9), collect data for a survey, enter the data in a computer, analyze data, and prepare a 10-minute scientific presentation, press release, and epidemiologic bulletin article based on the survey results. To date, 186 students from health departments have been trained, and the enrollment has increased from 29 in 1996 to 64 in 1999. Two short-term trainees later joined the German FETP. Seven outbreak investigations have been conducted jointly with RKI and the course graduates.

Outbreak Investigation

Benefits of developing the capacity to investigate outbreaks included filling a public health gap in the capacity to respond to epidemiologic emergencies; developing relationships between RKI and its partners, such as public health and research laboratories and health departments; forming hypotheses and bases for future research; providing training opportunities; and bringing recognition to public health, epidemiology, and RKI.

Twenty-seven outbreak investigations requiring travel to the field have been completed, with the number increasing from four in 1996 to nine in 1999 (Table). Each investigation was at the request and approval of the state health ministries, with the local health department or state ministry retaining overall control and responsibility. Eight of these outbreaks were foodborne or waterborne: Four were traditional common-source, foodborne outbreaks arising from single kitchens (Table 1, outbreaks 7,14,15,22), and four were from widely distributed commercial products (outbreaks 1,4,17,19). The other outbreaks were due to a diverse group of agents and modes of transmission and covered areas as large as Europe (Table).

Among the findings of these investigations were the recognition of *Escherichia coli* as a potentially common foodborne pathogen (outbreak 1), the recognition of Q fever as an emerging pathogen in Germany (partially due to urbanization in close proximity to sheep farms or grazing areas; outbreaks 2,18,23), and the role of Norwalk-like viruses in producing outbreaks in Germany (outbreaks 4,10,21). Germany's new epidemiologic capacity enabled it to participate in two multinational outbreaks among returnees from overseas travel (outbreaks 11,26) as well as in a World Health Organization investigation in Romania (outbreak 25).

Epidemiologic Research

The aim is to create a self-sustaining program of applied epidemiologic research at RKI focusing on foodborne and diarrheal diseases; AIDS and other sexually transmitted diseases, including hepatitis; vaccine-preventable diseases; respiratory diseases; travel-associated, vector-borne, and parasitic diseases; and nosocomial infections. The program has four developmental stages: outbreak investigation, initial targeted investigations, comprehensive line of research, and a fully realized research program.

Outbreak investigations provided an initial concrete focus of activity between RKI and collaboration partners in the field, such as health departments and laboratory scientists. In addition, outbreak investigations revealed health problems in need of further planned epidemiologic study. An example was the discovery from an outbreak investigation of the emergence of a sorbitol-fermenting enterohemorrhagic *E. coli*

Table. Outbreaks investigated by the Robert Koch Institute, 1996-1999

No.	Year	Setting	Syndrome	Pathogen	Cases (no.)	Comment (references)
1	1996	statewide	hemolytic-uremic syndrome	*Escherichia coli* O157	28	raw, spreadable sausage, new sorbitol-fermenting *E. coli* O157 strain (10)
2	1996	community	Q fever	*Coxiella burnetii*	45	community downwind from sheep farm. Climatic factors promoted airborne transmission (11-13)
3	1996	4 day-care centers	meningitis	echovirus 30	71	clinical illness increased risk of transmission to family members, high attack rate (14)
4	1996	vacation center for veterans	gastro-enteritis	Norwalk-like virus	86	bottled mineral water; national diagnostic capability for Norwalk-like viruses established as a result of outbreak
5	1997	ski group	upper respiratory syndrome, toxic shock syndrome	influenza A virus *Staphylococcus aureus*	38	followed foreign travel (15)
6	1997	ski group	upper respiratory syndrome	influenza A virus	39	followed foreign travel
7	1997	6 day-care centers	gastroenteritis	*Campylobacter jejuni*	186	common source from one kitchen
8	1997	community	meningitis	echovirus 30	353	high attack rate, person-to-person transmission (16)
9	1997	case investigation	hepatic failure, rhabdo-myalysis, bleeding	unknown	1	false-positive test for Lassa fever virus, 3 other persons of same ethnic origin identified with similar clinical syndrome (17)
10	1998	residence for elderly	gastroenteritis	Norwalk-like virus	189	person-to-person transmission
11	1998	nationwide	gastroenteritis	*Salmonella* livingstone	119	Europe-wide outbreak following foreign travel
12	1998	community	fever, sudden death	unknown	3	infants, no common risk factors identified
13	1998	community	meningitis, death	*Neisseria meningitidis*	9	associated with discotheque attendance in carnival season (18)
14	1998	company employees	gastroenteritis	*Salmonella* enteritidis	531	common source from a single kitchen
15	1998	community	gastroenteritis	*S.* enteritidis	103	common source from a single kitchen, alcohol protective
16	1998	hospital employees	myocarditis	unknown	40	pseudo-outbreak
17	1998	multiple states	gastroenteritis	*S.* blockley	12	imported eel smoked at multiple smokeries (19)
18	1998	community	Q fever	*Coxiella burnetii*	101	urban area in close proximity to sheep grazing and shearing
19	1999	10 commun-ities	trichinosis	*Trichinella spiralis*	52	investigation revealed two simultaneous outbreaks from different sources (20)
20	1999	hospital patients	sepsis, death	methicillin-resistant *S. aureus*	26	cardiac surgery intensive-care unit
21	1999	home for elderly	gastroenteritis	Norwalk-like virus	71	person-to-person transmission
22	1999	nationwide	gastroenteritis	*S.* enteritidis	48	national convention attendees, common source from one kitchen
23	1999	community	Q fever	*C. burnetii*	81	urban area in close proximity to sheep farm; uninvestigated outbreak from same sheep farm occurred 6 yrs. earlier
24	1999	case investigation	yellow fever	yellow fever virus	1	originally thought to be hemorrhagic fever (21)
25	1999	Romania	meningitis	echovirus	>5000	widespread outbreak involving multiple serotypes
26	1999	nationwide	gastroenteritis	*S.* paratyphi	43	Europe-wide outbreak from foreign travel
27	1999	hospital patients	sepsis, death	methicillin-resis-tant *S. aureus*	18	cardiac surgery intensive-care unit

(Table, outbreak 1). This investigation forged alliances between the RKI laboratories, a university laboratory, federal veterinarians, and a state health department. A working group was formed that developed a future research and surveillance program for enteric diseases.

The second stage of the program was to initiate targeted investigations involving single research projects to answer specific health problems. Eight short-term research projects were begun. These included a study of hepatitis B and C in Berlin dentists (22), risk factors for emergence of quinolone-resistant *E. coli* in a hospital, prevalence and risk factors for methicillin-resistant *Staphylococcus aureus* in Berlin nursing homes, three studies of influenza vaccine coverage (23,24), incidence and risk factors of borreliosis in an area where Lyme disease was highly endemic, and risk factors for echinococcus in Germany.

The third stage—a more comprehensive line of applied epidemiologic research conducted over a period of years in each of the six above-named fields—is being developed sequentially since it requires considerable personnel resources, financial commitment, and experience. In 1999, the Ministry for Education and Research began to fund the development of applied infectious disease research and surveillance networks. The selection criteria and proposal review panel were chosen to ensure that funded projects had an appropriate mixture of epidemiologic and laboratory science. RKI will receive DM 1,684,000 (approximately US $842,000) to develop a foodborne infections research network, which will include a German version of PulseNet (25) for the molecular characterization of Shiga toxin-producing *E. coli*, a national case-control study of sporadic enterohemorrhagic *E. coli* infections, and a national nosocomial infections network with integrated surveillance, research, and outbreak investigation components.

The last stage—a fully realized epidemiologic research program with field epidemiologists specialized in each of the six research areas and with integrated surveillance, laboratory, and prevention components—is a long-range goal. The program would have a steady research-funding stream for short- and long-term projects, whose priority would be determined by RKI and its collaboration partners based on immediate and long-term public health needs.

Strengthening Surveillance Systems

Federal law governs nationally notifiable disease surveillance. Reporting by local health departments to state and federal authorities is by aggregate number of cases only. The current law does not use case definitions, and many newly identified pathogens, such as hepatitis C, are not reported separately. There is no provision for laboratory-based reporting. Beginning in 1996, data obtained by the system have been published weekly in the newly formed epidemiology bulletin.

A new infectious disease law was passed by the German parliament and becomes effective in January 2001. In addition to allowing for single case reports using case definitions and specifying both laboratory- and health-care-provider-based reporting, the new law also gives a clear mandate for RKI to be the central federal authority for organizing and conducting surveillance and applied infectious disease epidemiologic research on a federal level and provides 30 additional staff.

The time it took to draft and gain passage of the new infectious disease law allowed RKI and new state epidemiologic programs to develop. As a result, 186 health department personnel have already been through short-term training, which will ease implementation of the new surveillance system in the field. A pilot study using components of a new computerized surveillance system is being set up in selected health departments. In addition, German FETP trainees have started six studies to evaluate existing surveillance systems or initiate sentinel surveillance. These studies include developing a national surveillance system for enterohemorrhagic *E. coli* and hemolytic uremic syndrome, evaluating a laboratory-based sentinel system for monitoring viral infections, and evaluating a system for monitoring foodborne outbreaks.

Improving Communications

Because the impact of emerging and reemerging infectious diseases on public health in Germany was grossly underestimated, a deliberate communications strategy was necessary to heighten public awareness. Also important was involving health department personnel in implementing new surveillance initiatives and conducting outbreak investigations by using analytic epidemiology. Finally, the collaboration of other scientific authorities, such as university research departments and professional associations, was needed.

Five communication strategies were used, and principal target groups were identified (Figure 2). Outbreak investigation was initially a high priority, in part to bring public recognition of infectious disease threats and the role of RKI and local public health authorities in responding to these threats. Subsequently developed public health guidelines also highlighted RKI's and health departments' roles in promoting science-based public health. Guidelines about diagnosing Shiga toxin-producing *E. coli* and investigating foodborne and nosocomial outbreaks are in preparation.

The new epidemiology bulletin reaches a wide audience, including the media, public health departments, and other researchers. It provides a weekly source of epidemiologic findings and surveillance data, giving RKI and participating health departments a regular, visible central role in infectious disease epidemiology. In addition, the bulletin publishes public health guidelines for one transmissible infectious disease per month. As of December 1999, guidelines for 11 infectious diseases (influenza, meningococcus, early summer tick-borne meningoencephalitis, Lyme borreliosis, hepatitis A, enterohemorrhagic *E. coli*, Q fever, campylobacter, rabies, measles, and Legionnaire's disease) have been published. The guidelines provide public health practitioners with an accessible resource for decision-making and guidance for obtaining national reference laboratory and epidemiologic assistance.

To gain credibility and support for the national epidemiologic program, 65 scientific presentations were given at conferences, and 19 scientific manuscripts based on original epidemiologic data were written; 16 of these are in press or have already been published in refereed journals or international bulletins (10-22, 26-28). The German FETP trainees and the EPIET trainees who have trained in Germany were first authors on 14 of these articles and wrote 18 articles in the German epidemiologic bulletin. Although publication in international medical journals was emphasized, publication in the epidemiology bulletin was vital because it was more widely read among public health practitioners.

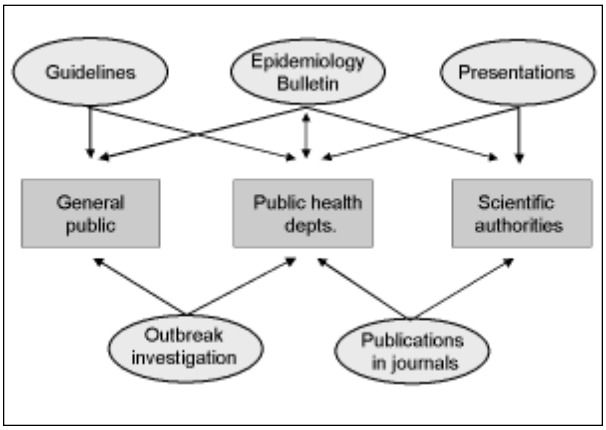

Figure 2. Program communication activities and principal target groups for the development of applied infectious disease epidemiology at the Robert Koch Institute.

Building International Collaborations

Although the principal focus of the national infectious disease epidemiology program was domestic, the integration of Germany into the European Union required the program to have an international presence. RKI's collaboration in training with EPIET and in investigating international outbreaks within Europe are two examples. The initial decision not to build a Europe-wide epidemiology center, but to approach Europe-wide surveillance through networks (29), requires many member states to take an active role in initiating and running one or more multinational surveillance systems. RKI has received funding for an exploratory study to determine the feasibility of a European campylobacter surveillance system.

Outside Europe, Germany has been extremely underrepresented in providing epidemiologic technical assistance for health projects or international assessment missions, largely because trained personnel were lacking. To help build this capacity, German FETP trainees have participated in 3-month assignments in Chad (28), Burkina Faso, and India as part of the World Health Organization's global polio eradication effort. In addition, RKI sent a team as part of a World Health Organization mission to support an outbreak investigation in Eastern Europe.

Funding

The Federal Ministry for Education and Research provided DM 1,144,000 (US $572,000) for the CDC consultant's salary, travel, and other costs related to outbreak investigations from 1996 through 1999, as well as short-term training courses for public health officials in 1997. The Federal Ministry of Health funded trainees' salaries (DM 752,000 [US $376,000]) and travel

to outbreak investigations, EPIET training modules, and scientific meetings (DM 98,000 [US $49,000]). The total cost of the program from 1996 through 1999 was approximately US $997,000. Additional funding was provided to enhance the nosocomial infections network and develop the foodborne disease network.

Future Plans

After the first 2 years of operation, the demands placed on the program greatly exceeded the available trained staff. A new nationwide surveillance system in 2001 will further tax the personnel resources. Although the new infectious disease law will provide 30 new federal positions for RKI, many of the new employees will need training. For this reason, FETP training will continue to be a high priority. States will likely continue to build up their epidemiologic capacity, further increasing demand for trained epidemiologists. The current demands on the program's resources do not permit expansion into other areas than infectious diseases. Another high priority will be building up the epidemiologic research program, financed in part through changes in funding for Germany's national reference laboratories. Laboratories participating in collaborative epidemiologic and surveillance projects will be eligible for discretionary funds beginning in 2000.

Conclusions

Several factors have contributed to the success of the new applied epidemiology program at RKI. The first was achieving a broad consensus about the program's scope and objectives among the program's future participants and customers. In Germany, this included the Federal Ministry of Health, the Ministry for Education and Research, the state health departments, and RKI. The second ingredient in the program's success was the initial emphasis on training, particularly for building a national epidemiologic program. The short-term training program quickly produced a growing network of collaborators in health departments. The long-term training programs (German and Europe-wide) produced technically competent investigators, who are now assuming key positions in infectious disease epidemiology. Because experiences in many countries have shown that at least 2 years is needed to train a field epidemiologist (8), achieving a critical mass of field epidemiologists at federal and state levels will take many years. Thus, the full effects of the long-term training program are not likely to be seen in the near future. This delay challenged the program: Many persons called for more rapid and less expensive alternatives (e.g., traditional classroom teaching or shortening the training period to 1 year) that would have severely compromised quality.

Because epidemiology is an applied science, the quality of the epidemiologic training and research depends on the experience of the program managers. Thus, an international consultant with considerable experience vastly improved the quality of training and research programs. Our experience suggests that an external consultant should remain in a country for 4 to 5 years for the program to become self-sustaining. A final key to the program's success was a stepwise and deliberate plan for developing a network of partners in the field and in the laboratory and for selling the program to consumers of the information. The simultaneous buildup of a Europe-wide epidemiology training program was crucial to developing the German one. The German trainees' participation in the EPIET training modules allowed them to be part of a larger regional network of trainees, thus simultaneously developing German and European networks of epidemiologists. The number and quality of EPIET's training modules could not have been achieved in Germany with the resources then available. Furthermore, EPIET provided a crucial external evaluation of the German FETP through periodic site visits. For example, one evaluation pointed out the need for better integration of the epidemiology and surveillance programs at RKI. As a result, the trainees' involvement with the analysis and interpretation of routine surveillance data was increased.

Our experiences in the program's first 4 years have shown that an organized applied epidemiology program with a high degree of technical expertise at the national level is necessary to effectively respond to emerging infections. For example, of the 27 outbreaks investigated to date, 4 were the traditional common-source, foodborne outbreaks from a single kitchen. Most of the others involved multinational or multi-state outbreaks, threats of imported exotic diseases, or community outbreaks of uncommon agents or bacterial or viral strains that were new, difficult to detect, or resistant to multiple drugs. The

traditional outbreak investigation approach that emphasizes a single health department's collection of samples from cases for microbiologic analysis would not have been adequate to determine the scope of nearly all of these outbreaks nor to identify their modes or vehicles of transmission.

RKI's activities thus far have formed the foundation for the continuing development of a national epidemiology program with integrated surveillance, research, and prevention components for most transmissible pathogens. Further development is critical, as the experiences to date suggest that epidemiologists, public health officials, and laboratory scientists are likely to become involved in increasingly complex scientific endeavors as they respond to emerging infections in Germany.

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Evidence Against Rapid Emergence of Praziquantel Resistance in *Schistosoma haematobium*, Kenya

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We examined the long-term efficacy of praziquantel against *Schistosoma haematobium*, the causative agent of urinary schistosomiasis, during a school-based treatment program in the Msambweni area of Coast Province, Kenya, where the disease is highly endemic. Our results, derived from treating 4,031 of 7,641 children from 1984 to 1993, indicate substantial year-to-year variation in drug efficacy. However, the pattern of this variation was not consistent with primary or progressive emergence of praziquantel resistance. Mathematical modeling indicated that, at current treatment rates, praziquantel resistance will likely take 10 or more years to emerge.

Schistosomiasis remains a public health problem in many regions, including Africa, the Middle East, Asia, and South America (1). For many of the *Schistosoma* species that infect humans, isoquinolin-4-one, praziquantel, is the only effective drug (2,3). Its minimal side effects and high degree of efficacy against both trematodes and cestodes have made it the drug of choice for many human and veterinary parasitic infections (2,4). However, praziquantel has been in use for more than 20 years (5), and concern is increasing that resistance has emerged, or will soon emerge, in human parasites (6,7).

Loss of praziquantel efficacy would set back helminth control efforts. Many community-based programs depend on praziquantel for treating patients with schistosomiasis, cysticercosis, echinococcosis, and tapeworm and other fluke infections (5,8-13). Concern over possible loss of efficacy prompted the European Commission to establish an International Initiative on Praziquantel Use, which met in February 1998 (14) and again in February 1999 (15). The group reviewed reports of low efficacy in clinical trials in Senegal and Egypt (16-19) and of laboratory isolation of schistosome strains resistant to standard and high doses of the drug (20-23). Although there was no definitive laboratory evidence of genetically transmissible and drug-selectable resistance (as had been demonstrated for the antischistosome drug hycanthone [24]), concern was raised over possible low-level resistance. The need was expressed for continued monitoring for resistant strains under the pressure of widespread praziquantel use (14).

We examined drug efficacy in the community and among schoolchildren given repeated praziquantel treatment for *S. haematobium* in Coast Province, Kenya. Year-to-year variation in treatment response was assessed, and the likely time-to-emergence of resistance was evaluated through the use of mathematical modeling of resistance-gene transmission in this obligately diecious parasite.

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Materials and Methods

Study Design

The overall goal of the community study was to treat urinary schistosomiasis in schoolchildren (initial N = 3,196) in a nine-village area in Kwale District, Coast Province, Kenya. After oral informed consent was obtained under a human investigations protocol approved by the institutional review boards of University Hospitals of

Cleveland and the Ministry of Health, Kenya, case-finding was performed by school-based and follow-up village surveys. Details of the protocols have been published (25-27). In 1984, the initial treatment year, *S. haematobium*-infected children were randomly assigned to groups for treatment with either praziquantel (Biltricide, Bayer, Leverkusen, Germany), 40 mg/kg once a year, or metrifonate (Bilarcil, Bayer), 10 mg/kg three times a year. In years 2 and 3, the initial treatment was repeated, independent of parasitologic findings. New entrants to the study were assigned randomly to either the praziquantel or metrifonate treatment groups, according to the original 1984 protocol. In 1987, half the 1984 cohort, treated either with praziquantel or with metrifonate, was randomized to receive a single dose of metrifonate, 10 mg/kg, as a "consolidation" treatment. After a 2-year hiatus, annual treatment was resumed in 1989 to 1991; during this period, only children in whose urine samples eggs were identified (egg-positive children) were treated with praziquantel alone.

Infection status was determined by Nuclepore (Whatman, Kent, UK) filtration of two 10-mL samples from stirred midday urine specimens. Infection-associated disease was determined by physical examination, dipstick urine examination for hematuria and proteinuria, and ultrasound examination of the kidneys and bladder (25). Clinical and parasitologic testing was performed each year. Results were coded and entered for analysis in databases at Case Western Reserve University and the Kenyan Ministry of Health.

Data Analysis

Results of annual treatments were scored as "cure" for patients whose status changed from egg-positive to egg-negative, "noncure" for those whose status remained egg-positive, and "infected or reinfected" for those whose status changed from egg-negative to egg-positive between yearly examinations. Because of the skewed distribution of egg counts in the infected population, the effects of treatment on average intensity of infection were assessed by determining the change in geometric mean egg count between examinations. Differences between outcome rates were assessed by the chi-square test with Yates' correction or Fisher's exact test.

Mathematical Modeling

The potential for development of praziquantel resistance in the study population was first estimated by the Hardy-Weinberg equilibrium analysis (28). We then used a deterministic, simultaneous differential equation model of helminth resistance (Appendix, 29). This more advanced model takes into account the skewed (negative binomial) distribution of number of worms in human populations, the obligate sexual reproduction of the parasites, and a possible decrease in fecundity as a result of parasite crowding in heavily infected humans. The model provides estimates of average level of infection in the study population, as well as the prevalence and density of resistant worms over time. Results are shown as three-dimensional graphs of mean numbers of worms over time (20 years), as a function of annual community drug use (p), and the reproductive fitness of resistant parasites. Treatment efficacy and aggregation constants for infection (k) were derived from our study area.

Results

Yearly Efficacy of Praziquantel

During 1984 to 1992, we observed substantial year-to-year variations in cure rates (conversion from egg-positive to egg-negative status on urine Nuclepore filtration examination) for both praziquantel and metrifonate (Table 1). The response to metrifonate treatment declined each year, from 79% in 1984 to 47% in 1987 ($p < 0.001$, Figure 1); in contrast, we observed no consistent downward trend in response to praziquantel treatment, despite repeated use of the drug in many patients (Figures 1 and 2). However, the response to praziquantel varied significantly from year to year ($p < 0.001$), from a cure rate of 96% in 1990 (year 7 of the project) to a cure rate of 65% in 1986 (year 3, $p < 0.001$). This level of efficacy was within the previous range of *S. haematobium* cure rates, both in Coast Province and elsewhere in Africa (Table 2). In suppression of infection intensity, the praziquantel-mediated reduction of mean *S. haematobium* egg counts was consistently $\geq$83% for all years of observation.

Impact of Repeated Treatment

In both the study periods during which repeated, annual praziquantel treatment was

used (1984-1987 and 1989-1993), the observed cure rate for egg-positive children increased in year 2 of treatment, then decreased in year 3 (Figure 1). The number of egg-positive children remaining to be treated in successive years was small, and this sample did not have sufficient statistical power to determine whether the year 3 decrease in praziquantel response was due to fluctuations in transmission (30), progressive selection of patients at high risk for exposure to *S. haematobium* (27,31), or gradual selection of praziquantel-resistant parasites.

Table 1. Effectiveness of praziquantel in eliminating *Schistosoma haematobium* infection, Msambweni, Coast Province, Kenya, 1984-1992

Patient's infection and treatment status	Prevalence of infection at 12 months after treatment (interval change in geometric mean of (egg count + 1) for group) Year treated with PZQ or metrifonate						
	1984	1985	1986	1987	1989	1990	1991
Egg-positive urine, Given PZQ, never previously treated	17% (97 to 1.5) n = 981	12% (33 to 1.5) n = 51	34% (29 to 3.7) n = 82	-	15% (18 to 1.5) n = 352	4% (22 to 1.1) n = 292	35% (24 to 4) n = 274
Egg-positive urine, Given PZQ, had ≥1 PZQ treatments	-	13% (10 to 1.5) n = 111	38% (16 to 2.6) n = 47	-	10% (19 to 1.1) n = 68	2% (14 to 1.0) n = 73	29% (13 to 2.8) n = 64
All egg-positive, PZQ-treated patients	17% (97 to 1.5) n = 981	13% (15 to 1.5) n = 162	35% (24 to 3.3) n = 129	-	14% (19 to 1.4) n = 420	4% (20 to 1.1) n = 365	34% (22 to 3.8) n = 338
Infection rate (egg - to egg + conversion)	15%	9%	11%	11%	13%	10%	21%
All patients, treated and untreated	18% n = 3,196	12% n =2,498	14% n = 2,372	19% n = 1,968	14% n = 1,398	13% n = 2,579	24% n = 1,938

PZQ = praziquantel.

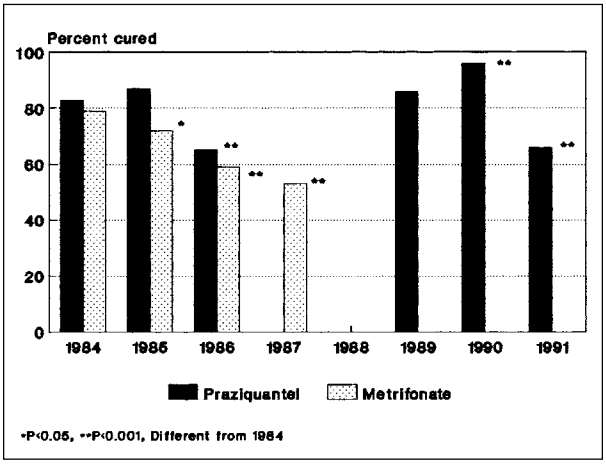

Figure 1. Yearly efficacy of drug therapy. Results of praziquantel treatment (solid bars) or metrifonate treatment (shaded bars) for *Schistosoma haematobium* infection in the Msambweni area during 1984 to 1992. Cure rates (conversion from egg-positive to egg-negative urine in annual follow-up testing) are shown for all egg-positive cases, by year of treatment. Only metrifonate therapy was given in 1987, and no treatment was given in 1988.

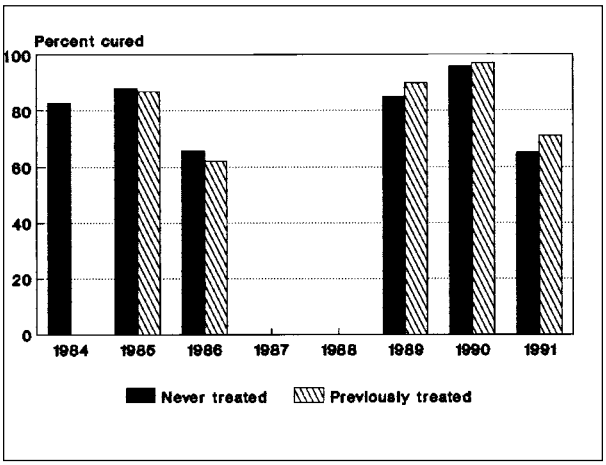

Figure 2. Efficacy of praziquantel therapy for *Schistosoma haematobium* according to prior treatment status. Solid bars indicate yearly cure rates for patients receiving their first praziquantel treatment. Hatched bars indicate cure rates for children with a history of prior praziquantel treatment. No significant differences in efficacy were noted in any of the years studied (1985-1991).

Table 2. Reported praziquantel (40 mg/kg) efficacy for *Schistosoma haematobium*, Coast Province, Kenya, and elsewhere in Africa

Site	Cure rate(s) observed (%)	Year(s)	Reference
Coast Province, Kenya			
Kajiwe, Kilifi district	62-65	1994	Olds et al., 45
Msambweni area, Kwale district	65-96	1984-91	present study
Southern Kwale district	84	1986	King et al., 46
Kwale district	87	1986	Stephenson et al., 47
Elsewhere			
Senegal	80-93	1995	Shaw et al., 48
Ghana	67	1994	Chan et al., 9
Zimbabwe	72	1986	Taylor et al, 49
Mauritania	90-95	1985-86	Etard et al., 50
Ghana	60	1984	Mott et al., 42
Egypt	59-62	1983	el Malatawy et al., 51
Gambia	57-93	1983	Wilkins et al., 52
Tanzania	66-83	1979	McMahon and Kolstrup, 53

Given the incomplete efficacy of praziquantel in eradicating infection (6) and the single annual follow-up, our study design could not distinguish reinfection from possible drug failure. Therefore, we used several indirect variables to estimate ongoing praziquantel efficacy: the level of persistent egg-positivity at 1 year after praziquantel treatment, compared with rates of new infection (egg-negative to egg-positive conversion) each year; the efficacy of the drug in children receiving a second, third, or fourth dose, compared with efficacy in children receiving a first dose; and the efficacy of the drug in children who remained egg-positive after their first dose compared with efficacy in children whose infections cleared but who subsequently became reinfected.

Persistent Infection vs. Reinfection

The yearly efficacy of praziquantel treatment and infection or reinfection rates (egg-negative to egg-positive conversion) were compiled for the study population (Table 1). Yearly infection or reinfection rates varied from 15% in 1984 to 1985 to a low of 9% in 1985 to 1986 and a high of 21% in 1991 to 1992 (average 13%). Infection rates for children initially treated with praziquantel who subsequently missed 1 year or more of treatment were 22% to 34% in subsequent surveys 2 to 4 years later. These rates suggested a time-dependent average accumulation of reinfection at an annual rate of 9% to 15%. The high transmission levels in 1991 to 1992 (21% new infection or reinfection) may explain the apparent decrease in praziquantel efficacy during this 12-month period. However, estimated transmission did not explain the decreased efficacy seen in 1986 to 1987, when the population egg-negative to -positive conversion rate was only 11%.

Efficacy of Retreatment with Praziquantel

To examine whether an increasing core of resistant infection might account for the higher post-treatment prevalence after year 2 of treatment, we compared the relative annual efficacy in egg-positive patients who received praziquantel for the first time with efficacy in patients receiving their second, third, or fourth treatments. We found that after the first cycle of treatment, i.e., from 1985 on, praziquantel-mediated cure rates did not differ for patients with first-time treatment and those with a history of praziquantel treatment (Figure 2). We further examined the response to second, third, and fourth treatments in patients who did not become egg-negative after their first dose (i.e., initial nonresponse, possibly resistant) (Figure 3). We compared these results with those of patients who tested negative after the first praziquantel dose, then became egg-positive in later years (those reinfected after cure, Figure 3). Children from these two groups, who were at greatest risk for infection with resistant parasites, eventually reverted to egg-negative status after treatment with one to three supplemental praziquantel doses. The two groups did not differ significantly in their response rates to the second, third, or fourth doses.

Discussion

Without clear evidence of emerging resistance in our treated population, we considered

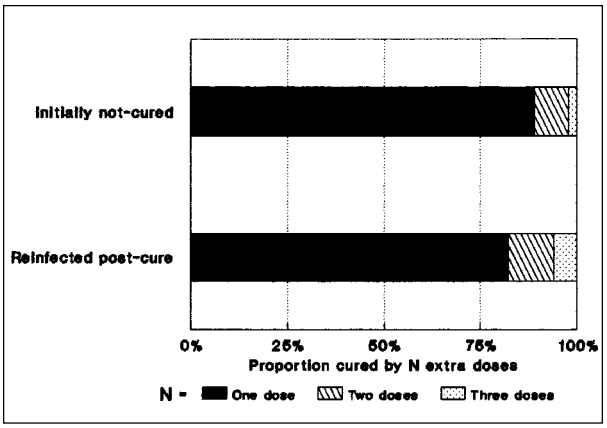

Figure 3. Extra doses needed to cure *Schistosoma haematobium* infection in 1984-entry cohort children who were not cured with a first dose of praziquantel (initial nonresponse, N=123, top bar) compared with doses needed to cure children reinfected after successful praziquantel treatment (reinfected post-cure, N= 36, bottom bar). The filled areas in each bar indicate percentage of each group requiring one (solid), two (hatched), or three (shaded) more doses of praziquantel to become egg negative. No significant differences were observed between the two groups in age, sex, or cure rates.

two related questions: Why was resistance not observed, and when might it be expected to emerge under the field conditions tested? To answer these questions, we turned to mathematical models of inheritance of drug resistance.

A number of factors may account for the lack of evidence of praziquantel resistance in the Msambweni project. Although *S. haematobium* resistance to praziquantel is likely to emerge at some future time, the interval required for detection of resistance may be much longer than our 8-year observation period. An effective praziquantel-resistance mutation may be so rare that the number of generations required for it to become the dominant phenotype has not yet occurred. In Egypt, for example, apparently resistant strains of *S. mansoni* are emerging >10 years after widespread availability of praziquantel treatment (32). For a single resistance gene mutation beginning at frequency of 10^{-6}, a Hardy-Weinberg equilibrium analysis (28) predicts it would take eight or more generations for the resistant phenotype to become clinically detectable (i.e., 25% to 50% of worms) at the community treatment rates used in this study, if the resistance gene heterozygotes are fully resistant to treatment (i.e., a dominant trait [Figure 4a]). In our study, we estimate that 25% to 50% of infections were treated, but because the targeted

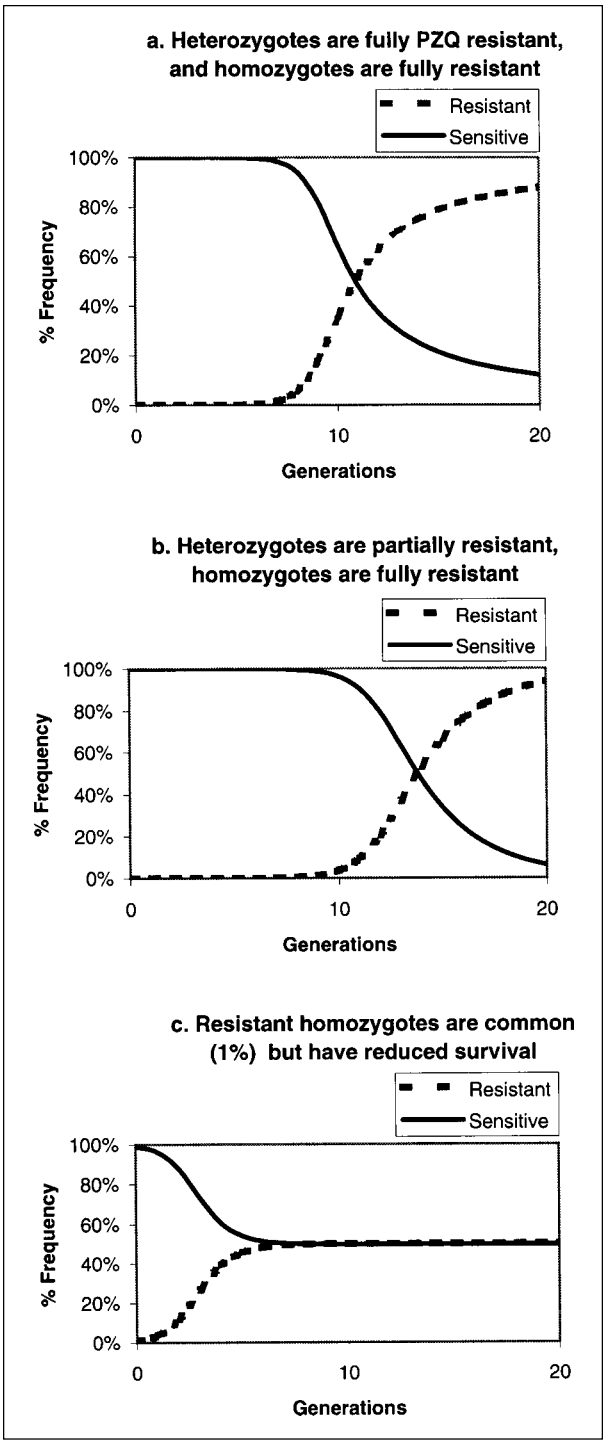

Figure 4. a. Hardy-Weinberg equilibrium analysis of the increase in resistance gene frequency in a parasite population where the initial R gene frequency is 10^{-6}, heterozygotes and R gene homozygotes are fully resistant, and 75% of susceptible worms are lost to treatment each generation. b. As in a, but 40% of heterozygotes are lost to treatment each generation. c. As in a, but 99% of resistant homozygotes do not survive to reproduce.

school-age groups have the highest numbers of worms, 50% to 75% of worms may have been exposed to praziquantel. Emergence times for the resistance phenotype are longer if heterozygotes remain fully or partially susceptible to the drug (11 generations for 25% heterozygote loss after treatment and 14 to 18 generations for 50% heterozygote losses) and only the homozygotes are fully resistant (Figure 4b). Given the seasonal nature of rainfall and water exposure, the requirement of two sexes for schistosome reproduction, and the uneven aggregation of worms in the human population, the effective generation time for *S. haematobium* is likely to be at least 6 to 12 months, so we observed no more than 8 to 16 generations; our observation period may have been too short to identify praziquantel resistance. Our annual follow-up was an insensitive means to detect drug failure due to resistance. However, under the pressure of continued praziquantel treatment in the community, if resistant worms are fully fit, rapid predominance of resistant strains would be expected (Figure 4).

Praziquantel failure on initial treatment in *S. mansoni*-endemic areas of Senegal suggests primary resistance to praziquantel (16,18) and a high prevalence of resistance genes in the local *S. mansoni* strain (21,33,34). More recent reports indicate, however, that in this area of Senegal, retreatment after 40 days adequately reduces infection levels and achieves better cure rates (19). The latter results suggest that immature schistosomes, which are known not to be susceptible to praziquantel, are typically present in Senegalese patients at the time of treatment. If the treated patient has been recently exposed to infection (≤6 weeks ago) in an area with continuous—rather than seasonal—transmission, apparent treatment failure may be observed when unaffected juvenile worms reach maturity and pass eggs several weeks after praziquantel treatment (14). Annual reinfection rates for *S. haematobium* may be high in some areas, such as Niger (35), and post-treatment infection detected after a single round of therapy should not be immediately interpreted as evidence of praziquantel resistance.

Nevertheless, animal studies of *S. mansoni* strains from Senegal indicate they are less sensitive to praziquantel than strains from other parts of the world (21,33,34). There is another explanation for an apparent low-level prevalence of drug resistance that fails to predominate in the parasite population: if a praziquantel-resistance mutation compromises reproductive fitness, the homozygous, fully resistant worm will never predominate. Instead, under the pressure of continued treatment, the resistance gene will achieve a stable-equilibrium share of the worm population, at a level dependent on its lower survival efficiency relative to that of the praziquantel-sensitive genotype (Figure 4c). This situation is analogous to the effect of the sickle cell-hemoglobin gene in human populations exposed to malaria.

Another possible factor likely to be slowing the emergence of praziquantel resistance in *Schistosoma* is the parasite's obligate diecious sexual reproduction. Unlike drug-resistant bacteria, praziquantel-resistant schistosomes must find a mate of the opposite sex to reproduce, which requires a sufficient density of human infection. In a disease-endemic area where most of the population has been treated, the initial heavy loss of susceptible worms (>80%) may actually reduce mean number of worms sufficiently (i.e., to fewer than one male and one female per host) to prevent most resistant worms from finding suitable mates (29). Worm distribution is highly aggregated in the human population, with most (75%) patients having light infections and a small proportion (approximately 5%) having heavy infections. Heavy infection can result in reduced worm fecundity, slowing the production of eggs and thus the transmission of genes from a resistant worm, even though the chances of mating are enhanced. After treatment, the reduced number of worms in a heavily infected human could increase fecundity, so the net effect of treatment on praziquantel-resistance gene transmission would be difficult to predict. In an age-targeted program such as ours, praziquantel-sensitive parasites would also persist in the untreated adult and infant human subpopulations, slowing the dominance of drug resistance gene(s). This would occur by allowing interbreeding of resistant and susceptible worms in host locations not having the environmental pressure (i.e., praziquantel treatment) that favors the resistance gene.

Using a dynamic model of parasite transmission that accounts for these reproductive features of macroparasite transmission (Appendix), we examined the impact of treatment coverage and duration of control programs on the emergence of

resistant infection, to estimate the time required for drug failure. We also examined the impact of varying reproductive fitness of the resistant worms on the time to recrudescence of infection. Figures 5 and 6 show the predicted recrudescence of infection over a 20-year period, as influenced by treatment coverage and by reproductive fitness of resistant worms. Both simulations indicate at least an 8- to 10-year delay in the return of infection to pretreatment levels. This delay is especially noted if treatment coverage is incomplete (Figure 5) and resistant parasites are less reproductively fit than susceptible ones (Figure 6). The model further indicates that under the conditions of our program (targeted mass treatment, with 25% to 75% of infections praziquantel-treated), resistance becomes clinically apparent (>50% resistant worms) only after a period years longer than that we were able to observe.

In our model, we assumed an effective spontaneous mutation rate for praziquantel resistance of 10^{-6}. Recent work by Moxon and colleagues (36-38) on the evolution of bacterial pathogens indicates that genetic mutation becomes accelerated (i.e., nonrandom) in a subset of organisms under environmental stress, such as drug pressure. The "hypermutable" phenotype prevails under these rapidly changing conditions because of its ability to adapt quickly to the new environment. Rapid genetic diversification is enhanced by simultaneous modular changes in DNA, which are facilitated by the presence of multiple copies of critical genes, transposons, and repeat regions or Z-DNA boundaries flanking key intron/exon boundaries (39). Higher organisms, such as schistosomes, are likely to use similar mechanisms in adaptation. Drake (40) has estimated that the effective spontaneous mutation rate per genome per sexual generation may be as high as 10^{-2} for the nematode *C. elegans* and 10^{-1} for the insect *Drosophila*. In sensitivity analysis of our differential model, reducing the mutation rate from 10^{-6} to 10^{-1} in our base case (50% coverage with 85% efficacy) reduced the expected time to emergence of resistance from 10 to 7 years. At higher levels of drug use (90%-100% coverage), the time to resistance was reduced from 7 to 5 years at the highest mutation rate (10^{-1}). This effect is not as dramatic as would be predicted by the simpler Hardy-Weinberg model

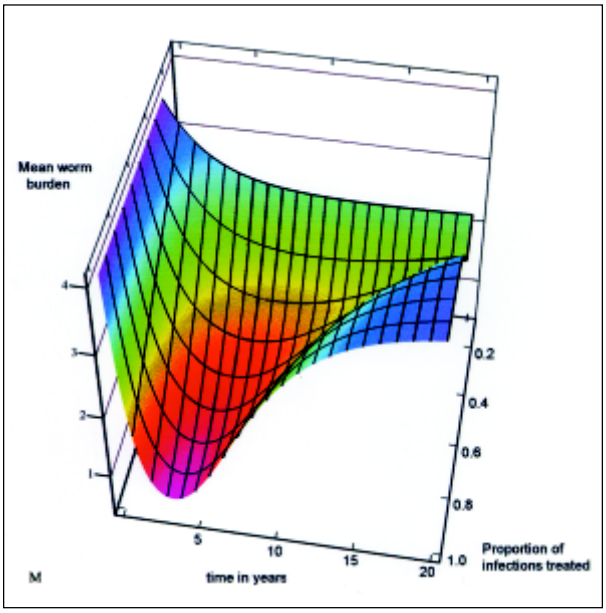

Figure 5. Three-dimensional graph of estimated mean community level of infection over time (20 years) as a function of the proportion of infections treated each year (20% to 100%). Results are obtained from the model described in the appendix. Resurgence of infection (due to emergence of resistance) was predicted to occur soonest (~7 years) when yearly treatment coverage was greatest (100%). Under the conditions of our study (25% to 75% coverage), detectable increases in community level of infection (due to resistance) were estimated to take a minimum of 10 to 15 years.

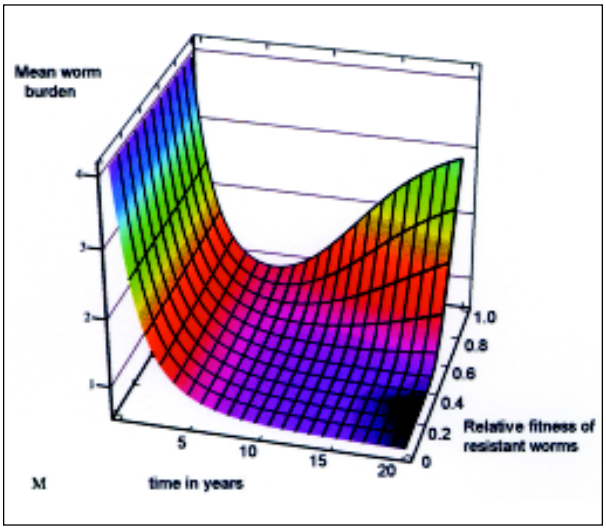

Figure 6. Three-dimensional graph of mean community level of infection, over time, as a function of the reproductive fitness of resistant parasites during an ongoing control program in which 50% of infected patients are treated each year. Limited fitness of resistant parasites (<0.6) delays or severely limits the reemergence of parasite infection intensity.

(i.e., a reduction from 14 to 3 generations for dominance of resistance), apparently because of the retarding effects of aggregation and mating on emergence of new genotypes.

In summary, our results indicate that significant resistance to praziquantel treatment had not emerged in *S. haematobium* in the Msambweni area as of 1991. As noted since its first use, praziquantel treatment was not 100% effective in eliminating infection (41). It was, however, reliably capable of suppressing intensity of infection in each of six rounds of treatment spaced over 8 years. These findings, together with our modeling analysis, support targeted use of chemotherapy for disease control in *S. haematobium*-endemic populations (1,26,42-44). In our program, praziquantel treatment was focused only on schoolchildren, the subpopulation having the highest numbers of worms (26,27). Higher treatment coverage increases evolutionary pressure on the schistosome population and hastens emergence of resistance. The limited coverage of a targeted (vs. mass-treatment) program may have reduced the tendency for resistance to emerge, compared with the experience reported for *S. mansoni* in Egypt (32).

For the treated Msambweni population, the benefits of treatment appeared to be maximal after 2 years of repeated yearly treatment (26,27). After this, a 2- to 3-year interval may be sufficient to maintain effective suppression of infection and disease (27). Because reduced exposure to the drug slows the emergence of drug resistance, the efficacy of longer intertreatment intervals in suppressing *S. haematobium*-related disease should be studied. Although efficacy should be maintained for a number of years, regular monitoring of drug efficacy is appropriate for all long-term praziquantel-based control programs (14). Emergence of praziquantel resistance should be anticipated within 10 to 20 years, and continued antischistosomal drug development should be pursued.

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Appendix

The following system of differential equations was used to model the level of schistosome infection and prevalence of resistant worms in the treated study population, where

σ is the number of sensitive schistosomes per host,

ρ is the number of resistant schistosomes per host, and

$\sigma + \rho = M$, the mean number of worms per host.

If p = the proportion of treated infections each year, and

h = the efficacy of one course of treatment, then

$c = -\ln(1 - ph)$ is the relative increase in the per capita death rate of sensitive worms due to treatment.

The basic death rate for sensitive and resistant parasites is an aggregate function of parasite and host death rates, represented in the equations as

$$\gamma_s = \frac{1}{\text{sensitive parasite lifespan}} + \frac{1}{\text{host lifespan}}$$

and

$$\gamma_r = \frac{1}{\text{resistant parasite lifespan}} + \frac{1}{\text{host lifespan}}$$

R_0 represents the basic reproductive rate of the susceptible worm. If ϕ = the relative loss of reproductive fitness of the resistant phenotype (with value from 0 to 1), then $R_{0r} = R_0 (1 - \phi)$. X represents the spontaneous mutation rate of resistant worms. To reflect the impact of obligate sexual mating and unequal distribution of worms among hosts, F is a fecundity expression where

Ψ = the mating probability,

z = the density-dependent effect on worm fecundity, and

k = the aggregation constant of the negative binomial distribution of worms,

such that

$$F(\sigma + \rho) = \Psi \left[1 + (\sigma + \rho)\frac{(1-z)}{k}\right]^{-(k+1)}$$

Then, along the lines of equation 17.5 of Anderson and May (29) for repeated treatment,

$$\frac{d\sigma}{dt} = \gamma_s \cdot \sigma \cdot \left[R_0 \cdot F(\sigma + \rho) - 1 - \frac{c}{\gamma_s}\right]$$

and assuming resistant parasites to be completely unaffected by treatment,

$$\frac{d\rho}{dt} = \gamma_r \cdot \rho \cdot (R_{0r} \cdot F(\sigma + \rho) - 1) + \chi \cdot p \cdot \sigma$$

For the numerical analysis of these equations, we used the ODE solver of MathCAD (MathSoft, Inc., Cambridge MA) based on the Runge-Kutta estimation with 1,000 steps for a model time span of 20 years.

Initial variable estimates were taken from our field data (treatment coverage among total infected population, p = 0.25 to 0.75; treatment efficacy, h = .65 to .95; aggregation constant, k = 0.067; host lifespan = 50 years) or from previously published estimates for *S. haematobium* biology (R_0 = 2 to 4; parasite lifespan = 3 to 5 years; density effect on fecundity, z = 0.96 to 0.99) (29).

Investigating Disease Outbreaks under a Protocol to the Biological and Toxin Weapons Convention

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The Biological and Toxin Weapons Convention prohibits the development, production, and stockpiling of biological weapons agents or delivery devices for anything other than peaceful purposes. A protocol currently in the final stages of negotiation adds verification measures to the convention. One of these measures will be international investigation of disease outbreaks that suggest a violation of the convention, i.e., outbreaks that may be caused by use of biological weapons or release of harmful agents from a facility conducting prohibited work. Adding verification measures to the current Biological and Toxin Weapons Convention will affect the international public health and epidemiology communities; therefore, active involvement of these communities in planning the implementation details of the protocol will be important.

The Biological and Toxin Weapons Convention (1) prohibits the development, production, stockpiling, or transfer of biological weapons agents (microbial pathogens and toxins) for other than peaceful purposes and any devices used to deliver these agents.[1] The convention was the first treaty to outlaw the development and possession of an entire category of weapons, and the first to outlaw any weapon of mass destruction. There are now 143 states parties to the convention and an additional 18 signatories. Even though the convention's renunciation of biological and toxin weapons was categorical, it was not accompanied by effective provisions for verification. Article VI of the convention provides that states parties that suspect another state party of noncompliance may submit a complaint to the U.N. Security Council, and all states parties are obliged to cooperate fully with any investigation that the security council may initiate. However, this mechanism has proved inadequate. Despite several allegations of noncompliance in the 25 years the treaty has been in force, Article VI has never been invoked, probably in recognition of the intensely political nature of security council decisions, and the crippling effect of the veto power of the five permanent members (2,3).

Recent information about the covert biological weapons programs of the former Soviet Union (4) and Iraq (5) gave added momentum to verification efforts begun in 1991. A draft text for a protocol to the Biological and Toxin Weapons Convention is now in the final stages of development and could be opened for signature and ratification within a year. One of the provisions of this >200-page protocol will be a mechanism for investigating certain outbreaks of disease to resolve suspicions about compliance with the convention (6). There are several reasons for having such a mechanism (7). An outbreak of disease might be the result of biological attack on troops, civilians, crop plants, or domestic animals. Alternatively, an outbreak could be the result of accidental escape of harmful agents from a secret biological weapons facility. A mechanism for the prompt investigation of such outbreaks by the international community would address and resolve such concerns; it would also deter the use of unsubstantiated accusations (e.g., the 1952 allegation of U.S. propaganda use of biological

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[1]Although the Biological and Toxin Weapons Convention does not explicitly prohibit the *use* of biological weapons, it does so implicitly because any use presupposes prior production and stockpiling.

agents in Korea and Manchuria [8,9]) and would reduce the credibility of fraudulent investigations controlled by the accuser. Finally, the political costs of being identified as a biological aggressor could deter covert biological weapons programs (7,10).

What Outbreaks Will Be Investigated?

Thousands of outbreaks of disease occur annually among humans, domestic animals, crop plants, and wild animals and plants. The only outbreaks relevant to the weapons convention and its verification protocol are "suspicious" outbreaks, which have features suggesting an unnatural cause. The draft protocol calls for requests to investigate outbreaks to include "detailed evidence, and other information, and analysis that such an outbreak(s) of disease is not naturally occurring and is directly related to activities prohibited by the Convention" (6). Thus, very few outbreaks would likely become issues of treaty compliance.

An outbreak might be suspicious because epidemiologic features suggest an unnatural origin. For example, in the 1979 anthrax outbreak in Sverdlovsk, former Soviet Union, the distribution of both human and animal cases in a narrow corridor downwind from a military microbiology facility was a strong indication of unnatural origin (11,12). Also, the etiologic agent may differ from agents naturally found in the environment, as would be the case if the agent were genetically engineered; in such an event, the unusual phenotype of the agent would signal something anomalous. Detailed molecular study, including DNA sequencing, should reveal the recombinant nature of the organism.

Even in the absence of genetic engineering, agents used as biological weapons may differ in recognizable ways from those causing natural cases of the same disease. This would be the case if the weapons agent had been maintained in laboratory culture for some time before use. Because of natural selection and genetic drift, each population of an organism continually diverges genetically from others of the same organism. It is thus possible in principle (and in practice for many agents) to determine the geographic origin of an outbreak with the tools of molecular epidemiology, by determining to which local population its etiologic agent belongs. An outbreak caused by a strain last seen many years ago could be suspicious (7,10).

Other features of the agent can also be suspicious. For instance, in the Sverdlovsk outbreak, retrospective molecular analysis of retained pathology samples showed that patients appeared to have been simultaneously infected with several strains of the anthrax agent (13); multiple infections are not normally encountered in natural outbreaks.

In addition to unusual epidemiologic features, devices used to disseminate the agent and intelligence information can help identify a suspicious outbreak. For example, the 1993 outbreak of intestinal illness among the insurgent Karen of Burma (Myanmar) (14-16) was suspicious largely because a number of putative delivery devices (balloons attached to meteorologic radiosondes and parachutes) were recovered.

Labeling an outbreak suspicious reflects a judgment that the evidence suggests unnatural causes. Different analysts can come to different conclusions, and political or ideological factors can affect the judgment. Thus, compiling a list of features that would automatically render an outbreak suspicious is not possible. Probably, most or all suspicious outbreaks will turn out to be natural occurrences.

Types of Suspicious Outbreaks

Suspicious outbreaks can be grouped into four main categories, depending on the nature of the suspicions they provoke. They may be thought to be the result of covert biological attack by another nation, criminal or terrorist attack, covert attack by a nation on a subnational group within its borders, or escape of a biological agent from a facility developing prohibited weapons.

If a nation suspects it has been biologically attacked by another nation, it would most likely be the one to request an investigation and could be expected to cooperate fully. However, despite the cooperative attitude of the host nation, its vested interest in the outcome of the investigation requires the investigating team to be alert to the possibility that information provided by official sources may be biased, incomplete, or even fraudulent.

Normally an outbreak suspected to be the result of criminal or terrorist attack would be investigated as a police matter by the country on whose territory it took place. However, in some instances, assistance under the protocol would be requested, e.g., if the affected country did not

have sufficient resources to mount an investigation, or if state-sponsored terrorism were suspected. Such investigations would also be conducted in a cooperative climate.

Outbreaks thought to be the result of attack by a nation on one of its own subnational groups or the result of an accidental release of harmful agents could lead to a request by one state party for an investigation on the territory of another. If the suspected nation is innocent of the allegations, it might cooperate fully with the investigating team. However, even for an innocent party, considerations of national sovereignty or concerns about revealing sensitive information about internal matters might incline the national government to oppose investigation under a convention protocol. Even the simple desire not to complicate an ongoing public health investigation could lead to reluctance; for example, the 1993 outbreak of hantavirus pulmonary syndrome in the United States had several highly suspicious features (7). Had the protocol been in force at that time, a request for an international on-site investigation, in parallel with the ongoing investigation of the Centers for Disease Control and Prevention, would have been likely. The United States would probably not have welcomed such a complication.

If the international community were to proceed with an unwelcome investigation, it would do so under decidedly uncooperative conditions. This could complicate and even compromise the investigation. Failure to cooperate with the investigation would constitute a violation of the protocol and could be interpreted as confirming guilt, an incentive to give at least the appearance of cooperation. The great difficulties that the U.N. Special Commission had performing facility inspections in Iraq with an uncooperative government remind us that actually implementing unwelcome investigations requires a daunting amount of political will and persistence (17-19). However, the success of the U.N. Special Commission in unearthing details of Iraqi weapons programs, despite failure of the Iraqi government to cooperate, shows that such investigations can be worthwhile.

Who Decides If an Outbreak Is Suspicious?

The negotiated protocol is expected to establish the Organization for the Prohibition of Biological Weapons, which will administer certain aspects of the protocol, including investigations. An executive council composed of selected states parties to the protocol will authorize all investigations. Only states parties to the protocol will have standing to request an investigation.

This restricted standing could present a dilemma to public health professionals who might conclude from their investigation that an outbreak was deliberately instigated. For them, a professional approach would be to convey their suspicions and evidence to the organization sponsoring the investigation (typically the World Health Organization [WHO], a national or subnational health organization, or a nongovernmental organization). However, if the responsible organization is not willing to convey the suspicions to the government or to the Organization for the Prohibition of Biological Weapons, the public health experts may be in an awkward position, fraught with conflicting professional, political, and ethical responsibilities.

How a request from a state party for an outbreak investigation would be handled by the executive council is not yet clear. Existing precedents in arms control treaties are either of the "strong red light" or "strong green light" types. Under a strong red light mechanism, once a request is formally made, a substantial majority of the executive council must vote to oppose an investigation in order to stop it; otherwise, the investigation goes forward. Under a strong green light mechanism, once a request is made formally, a substantial majority of the executive council must vote in favor of an investigation in order for it to proceed. Both models raise concerns: the former because it may not provide sufficient protection from nuisance requests, and the latter because it may make investigating truly suspicious outbreaks very difficult if they are controversial.

The evidence brought to the executive council to support a request for an investigation is likely to be somewhat ambiguous, given the prevalence of natural outbreaks of disease. Its evaluation is thus not a trivial matter, and serious attention to this issue is warranted; two levels of analysis might be useful. First, the Organization for the Prohibition of Biological Weapons might assist the executive council by providing a technical analysis of the evidence. The organization is expected to have a staff of impartial experts in epidemiology and weapons control issues,

qualified to evaluate technical evidence. Second, each state party on the executive council should have a mechanism in place for consulting with its own experts on very short notice, since the executive council vote is expected to be mandated within a day of the request.

Sources of Information about Suspicious Outbreaks

Investigation of most suspicious outbreaks will be initiated by national or international public health agencies. In many cases the results of this initial public health investigation will implicate the outbreak as suspicious and provide the basis for a request for an investigation. The evidence should not be expected to be conclusive; however, it should provide reasonable grounds for suspicion that the outbreak may involve intentional or accidental release of prohibited materials. The investigation itself, once approved, will have the role of gathering sufficient additional evidence to decide if the suspicions are correct.

This evidence is likely to include one or more of the following: intelligence gathered by the state party requesting the investigation, epidemiologic data gathered by the state party requesting the investigation, or epidemiologic evidence gathered by an international organization (e.g., WHO). Other evidence could include recovered delivery devices and information gathered by nongovernmental organizations.

Intelligence information can be relevant when prohibited activities in a particular nation are suspected of causing an outbreak, and the intelligence directly addresses those activities. However, intelligence commonly requires substantial redaction to protect sources and capabilities, which can seriously compromise its persuasiveness. Thus, intelligence is unlikely to be a useful source of information for the executive council, except under unusual circumstances.

Commonly, relevant information suggesting that the outbreak is suspicious will be epidemiologic and will be obtained by a national or international health organization. If the source is a national health organization, the information can be expected to be freely available if the requesting party is the one that performed the investigation; if, however, a second party is requesting an investigation in a country that is unreceptive to it, the available information is likely to be limited and unreliable. Even when freely shared, epidemiologic information from an interested party may not be complete and accurate.

Different problems are associated with epidemiologic information gathered by an international organization. Currently, for example, WHO's procedures prevent official release of information not approved by the host country. While permission to release information to the executive council would be expected when the host country and requesting country are the same, such permission would be unlikely if the host country opposed the investigation; nevertheless, the outlines of the results would likely be widely known, given the rapid expansion of epidemiologic information on the Internet (e.g., through ProMED Mail). However, the unavailability of official information could be a serious problem within the executive council, as it provides a credible rationale for questioning technical information.

As procedures are developed to share information among health organizations and the Organization for the Prohibition of Biological Weapons, the implications for public health need to be considered. The effectiveness of health organizations could be compromised if nations fear that a natural outbreak might be mistakenly judged suspicious. International health organizations will thus need to operate with great tact and caution when they encounter a potentially suspicious outbreak.

Finally, no matter how important the resolution of suspicions of convention noncompliance is, protecting ongoing public health response to an outbreak is essential. Containment of the outbreak and prompt treatment of patients cannot be compromised by a simultaneous arms control investigation.

Features of an Outbreak Investigation under a Biological and Toxin Weapons Convention Protocol

Most scientific aspects of an outbreak investigation in a Biological and Toxin Weapons Convention context are identical to those of a comparable investigation in a public health context. However, some striking differences occur because of the international scope and the quasi-forensic aspects of the investigation. While it is probably possible under most circumstances to discriminate between a natural (but unusual) outbreak, and one that results from accident or from intentional use (7), the acceptability of such

conclusions in a political context requires a high level of credibility.

The investigation's team leader will need to be a full-time member of the Organization for the Prohibition of Biological Weapons, with experience in supervising epidemiologic investigations and in handling the diplomatic issues of weapons control compliance. This leader will have to ensure scientific rigor, proper handling of evidence, impartiality, and tactful handling of publicity. Other members of the team will be chosen for their relevant expertise from the organization staff or a pre-approved list of experts employed outside the organization.

The possibility of interference from the host state makes it important that the team be as independent as possible. This is particularly critical for interpretative and translation services, but applies as well to such areas as communications, transportation, diagnostic reagents, standards, analytic equipment, and microbiologic media. Failure to ensure such independence may seriously compromise the effectiveness of investigations. However, the current draft protocol does not ensure independence of communications and transportation.

Unlike more routine investigations where sample tampering is not normally considered a risk, investigations of suspicious outbreaks will require strict documentation of the provenance and chain-of-custody of all samples. Sample analysis will require certifiably calibrated analytic equipment and standardized reagents. Such analysis should be done on-site or in approved diagnostic laboratories. Internal standards, replicate analysis, and blind testing should be used for the highest possible credibility of results.

Most investigations can be expected to overlap with ongoing public health investigations. There could thus be substantial difficulties in coordinating the two, with their very different goals and divergent requirements for sample handling and analysis. Information sharing will also be an issue, since both investigations need access to all relevant information about the outbreak, yet they must be independent.

Conclusions

For the public health community and their colleagues in plant and animal epidemiology, negotiating a protocol to the Biological and Toxin Weapons Convention will establish formal procedures for the investigation of certain outbreaks. These procedures will have certain elements, such as forensic standards for evidence handling, that are generally not familiar to field epidemiologists. However, the very formal procedures will help insulate scientists from the political dimensions of such investigations. Nevertheless, several aspects of the ongoing negotiations deserve the careful consideration of public health organizations and practitioners, for example, ways in which epidemiologic information gathered during a public health investigation can be used to justify a weapons-control investigation or to support or rebut the conclusions of such an investigation. Additionally, further consideration is needed about the implications of public health investigations' sharing personnel with highly politicized arms control investigations, a consequence of the small numbers of professionals with expertise in relevant disciplines.

The negotiated weapons convention protocol will improve security against biological attack. However, the costs to routine public health measures should be minimized to the greatest extent possible. This will require continued attention from both the arms control and the public health communities. Negotiations on the protocol are nearing the final stages in Geneva, and there is no longer opportunity for technical consideration to have much influence on the final language. However, advice from public health professionals will be needed as the detailed operating procedures are developed to implement the necessarily general diplomatic language of the new protocol. Such advice will be important at both the international level, as the Organization for the Prohibition of Biological Weapons develops detailed operating procedures, and at the national level, as each state party implements legislation and domestic operating procedures.[2]

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[2]To learn more about the protocol and how to have input into the process, interested scientists can contact their professional organizations or nongovernmental organizations, such as the Federation of American Scientists (www.fas.org/bwc/), the University of Bradford Department of Peace Studies (www.brad.ac.uk/acad/sbtwc/), or the Monterey Institute of International Studies (http://cns.miis.edu/research/cbw/). All the relevant documents, including copies of the current rolling text of the Biological and Toxin Weapons Convention protocol, can be downloaded from these sites.

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Hemophagocytic Syndromes and Infection

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Hemophagocytic lymphohistiocytosis (HLH) is an unusual syndrome characterized by fever, splenomegaly, jaundice, and the pathologic finding of hemophagocytosis (phagocytosis by macrophages of erythrocytes, leukocytes, platelets, and their precursors) in bone marrow and other tissues. HLH may be diagnosed in association with malignant, genetic, or autoimmune diseases but is also prominently linked with Epstein-Barr (EBV) virus infection. Hyperproduction of cytokines, including interferon-γ and tumor necrosis factor-α, by EBV-infected T lymphocytes may play a role in the pathogenesis of HLH. EBV-associated HLH may mimic T-cell lymphoma and is treated with cytotoxic chemotherapy, while hemophagocytic syndromes associated with nonviral pathogens often respond to treatment of the underlying infection.

The term hemophagocytosis describes the pathologic finding of activated macrophages, engulfing erythrocytes, leukocytes, platelets, and their precursor cells (Figure 1) (1). This phenomenon is an important finding in patients with hemophagocytic syndrome, more properly referred to as hemophagocytic lymphohistiocytosis (HLH) (2). HLH is a distinct clinical entity characterized by fever, pancytopenia, splenomegaly, and hemophagocytosis in bone marrow, liver, or lymph nodes. The syndrome, which has also been referred to as histiocytic medullary reticulosis, was first described in 1939 (3). HLH was initially thought to be a sporadic disease caused by neoplastic proliferation of histiocytes. Subsequently, a familial form of the disease (4) (now referred to as familial hemophagocytic lymphohistiocytosis [5]) was described. However, the nearly simultaneous development of fatal HLH by a father and son in 1965 indicated that infection might play a role (6).

HLH has since been associated with a variety of viral, bacterial, fungal, and parasitic infections, as well as collagen-vascular diseases (7-11) and malignancies, particularly T-cell lymphomas (12-14). This diversity has prompted the suggestion that HLH secondary to an underlying medical illness should be designated reactive hemophagocytic syndrome. The association between HLH and infection is important because

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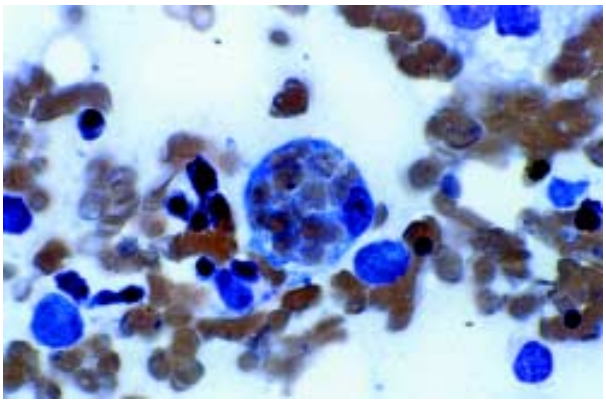

Figure 1. Hemophagocytosis in the bone marrow of an 18-year-old woman with Epstein-Barr virus (EBV)-associated hemophagocytic lymphohistiocytosis. The patient visited her physician in September 1997 with pharyngitis and an elevated heterophile agglutinin titer. She was diagnosed with infectious mononucleosis, and her symptoms resolved in 2 weeks. Approximately 2 months later, she had persistent, spiking fevers and became jaundiced; her immunoglobulin (Ig) M to EBV capsid antigen was positive; and EBV capsid IgG and nuclear IgG were negative. She had pancytopenia and was hospitalized. Bone marrow evaluation revealed a hypocellular marrow, with active hemophagocytosis. The macrophage in the center of this image appears "stuffed" with phagocytosed erythrocytes. Phagocytosis of platelets and leukocytes by macrophages was also seen (not shown). The patient was treated with intravenous immunoglobulin, steroids, and cyclosporine A, but not etoposide. Her condition worsened; she had respiratory, renal, and hepatic failure; and she died of an intracerebral hemorrhage on hospital day 34, 3 1/2 months after her initial illness.
Original photomicrograph 100X magnification with oil immersion, courtesy of Frank Evangelista, Beth Israel Deaconess Medical Center. Photomicrograph published in Blood 1999;63:1991 and reproduced by permission of the publisher.

1) both sporadic and familial cases of HLH are often precipitated by acute infections; 2) HLH may mimic infectious illnesses, such as overwhelming bacterial sepsis and leptospirosis (15); 3) HLH may obscure the diagnosis of a precipitating, treatable infectious illness (as reported for visceral leishmaniasis [16]); and 4) a better understanding of the pathophysiology of HLH may clarify the interactions between the immune system and infectious agents.

This article describes the clinical features and epidemiology of HLH and summarizes its association with infection; reviews evidence that this syndrome results from disordered cellular immunity; outlines options for treatment of patients with infection-associated HLH; and discusses issues related to hemophagocytosis in genetic, malignant, and autoimmune disease. The genetic basis of hemophagocytic syndromes has been reviewed in detail (17).

Clinical Features

Clinical criteria for the diagnosis of HLH, proposed by the Histiocyte Society (2), include clinical, laboratory, and histopathologic features (Table). Fever and splenomegaly are the most common clinical signs, but hepatomegaly, lymphadenopathy, jaundice, and rash are also seen. The rash is commonly described as maculopapular, but nodular eruptions have also been described (22). Of central nervous system manifestations, encephalopathy, meningismus, and seizures are the most commonly reported (23,24). These clinical findings may suggest an acute viral infection, such as Epstein-Barr virus (EBV), cytomegalovirus (CMV) infection, viral hepatitis, or acute HIV seroconversion, a situation complicated by the association of these infections with HLH.

Table. Clinical signs and laboratory abnormalities associated with hemophagocytic lymphohistiocytosis

Clinical sign	% of patients affected	Reference
Fever*	60-100	5,18-21
Splenomegaly*	35-100	5,19-21
Hepatomegaly	39-97	5,19-21
Lymphadenopathy	17-52	5,18-21
Rash	3-65	5,18-21
Neurologic signs	7-47	19,21
Laboratory abnormality		
Anemia*	89-100	5,19,20
Thrombocytopenia*	82-100	5,19,20
Neutropenia*	58-87	5,19,21
Hypertriglyceridemia*	59-100	5,19,21
Hypofibrinogenemia*	19-85	5,19,21
Hyperbilirubinemia	74	19

*Proposed diagnostic criterion for HLH (2)

The most prominent laboratory abnormalities noted are cytopenias, which may be profound. Serum chemistry findings may suggest hemolysis, with hyperbilirubinemia and elevation of lactate dehydrogenase. Most patients have hypertriglyceridemia and marked elevation of ferritin (25,26). Serum fibrinogen is typically low, and there may be disseminated intravascular coagulation (18). Elevated circulating fibrin degradation products and serum ferritin in patients with HLH appear to be associated with increased risk for death (27).

Histopathologically, hemophagocytosis is seen in bone marrow, spleen, and lymph nodes (1,28) and occasionally the central nervous system (23,29) and skin (22). Activated macrophages may engulf erythrocytes, leukocytes, and platelets, their precursors, and cellular fragments. These cells appear "stuffed" with other blood cells. Hemophagocytosis may be present in the liver, but infiltration of the hepatic portal tracts with lymphocytes is also common (1,28).

Epidemiology

HLH appears to affect all ages, although the hereditary and sporadic cases are reported primarily in children (30); a crude annual incidence of 1.2 cases of familial hemophagocytic lymphohistiocytosis per million children has been reported in Sweden (19). Large series of HLH cases have been reported in Hong Kong (18,31) and Taiwan (12,32), but whether the incidence of HLH is higher in Asia than in Europe or North America is not known. A seasonal pattern has been suggested in which cases may occur more often in the summer (32).

The familial form of HLH occurs in young children as a genetic disorder with autosomal recessive inheritance; possible loci for a responsible gene or genes have recently been mapped to the long arms of chromosomes 9 (33) and 10 (34). HLH may also occur as a complication of Chediak-Higashi syndrome (35) or after EBV infection in patients with X-linked lymphoproliferative syndrome (36). In these patients, fatal infectious mononucleosis may be pathologically indistinguishable from HLH (37).

In 1979, HLH was described in a cohort of patients who had serologic evidence of recent

viral infections (38), and virus-associated hemophagocytic syndrome was proposed as a distinct clinical entity. Subsequently, HLH has been reported in association with a variety of infections, and the term reactive hemophagocytic syndrome has been suggested to distinguish HLH associated with an identifiable infectious or noninfectious etiology from its hereditary forms. However, the reactive and hereditary forms of the disease are difficult to distinguish; for example, patients with familial forms of HLH may have hemophagocytic syndrome after a documented viral infection (39).

HLH and Infection

Case reports and case series on the association of infections and HLH are summarized at URL:http://www.cdc.gov/ncidod/eid/vol6no6/fisman_refs.htm.

Disseminated infection with an unusual organism in a patient with HLH may represent secondary infection in an immunocompromised host; however, the resolution of HLH following treatment of infection suggests that, in many cases, HLH is secondary to the underlying infection.

A diagnosis that takes into account all the underlying diseases associated with HLH would not be practical, and formal guidelines for evaluating patients with suspected infection-associated HLH have not been established. Nevertheless, all patients meeting the criteria for HLH should undergo initial diagnostic tests that include routine cultures of blood and urine and chest radiography to screen for such infections as miliary tuberculosis. Attempts should be made to screen for infection with EBV, CMV, and parvovirus B19, either through serologic testing or polymerase chain reaction, in-situ hybridization, or (in the case of CMV) immunofluorescent antigen testing. Serologic testing for HIV and human herpesvirus-6 infection should also be considered, and throat and rectal swabs should be taken for viral culture. Because of the association between HLH and fungal infections, lysis-centrifugation blood cultures and fungal antigen testing should be considered for all patients with HLH. Even if an infection known to be associated with HLH has been confirmed, cell marker and T-cell receptor gene rearrangement tests should be performed on bone marrow or other tissue specimens to determine whether an underlying T-cell lymphoma is present.

Extensive testing for underlying infecting organisms should be guided by epidemiologic data and the patient's medical history. For example, in a patient with underlying HIV infection, HLH has been associated with infections that commonly affect patients with AIDS (e.g., pneumococcal disease, pneumocystosis, histoplasmosis, and infection with *Penicillium marneffei*) and with T-cell lymphoma. Patients with a history of travel or animal exposure should be screened for such infections as leishmaniasis, brucellosis, rickettsioses, and malaria. In bone marrow transplant patients, attempts should be made to isolate adenovirus from urine, nasopharyngeal and rectal swabs, and tissue specimens.

Because so many immunologic, neoplastic, genetic, and infectious disorders may be associated with HLH, clinicians should work closely with pathologists and microbiologists to clearly define precipitating or underlying illnesses.

Pathophysiology

Phagocytosis of blood cells and their precursors is a hallmark of hemophagocytic syndromes. Hemophagocytosis is achieved mostly by monocytes and macrophages, and nitro-blue tetrazolium reduction by monocytes from patients with HLH is approximately six times that of control monocytes (40). Splenic macrophages from patients with HLH exhibit an activated phenotype with increased expression of MHC class I and II molecules and increased M-CSF receptor expression (41). Phagocytosis of platelets in HLH may be enhanced by increases in antiplatelet immunoglobulin (Ig) G, which has been reported in parvovirus B19-associated HLH (42).

Excessive activation of monocytes in HLH may be due to stimulation by high levels of activating cytokines. High levels of interferon-γ (IFN-γ) (43-45), soluble interleukin-2 receptor (43,46), tumor necrosis factor-α (TNF-α) (44,47,48) interleukin-1 (49), and interleukin-6 (43) have been demonstrated, suggesting that elaboration of activating cytokines by T-helper cells promotes activation of macrophages in this disease (Figure 2). Higher levels of IFN-γ and TNF-α correlate with poor clinical outcome in children with virus-associated HLH (45,49).

Recently, oversecretion of interleukin-18 by monocytes in patients with HLH has been described (50); interleukin-18 production may further enhance TNF-α and IFN-γ production by

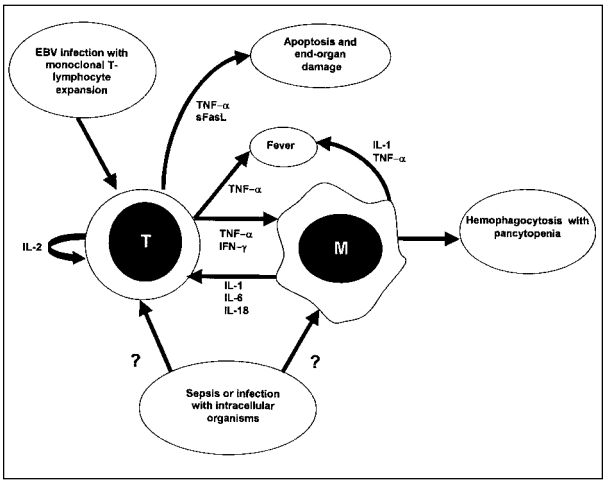

Figure 2. Schematic representation of possible immunopathologic mechanisms in infection-associated hemophagocytic lymphohistiocytosis (HLH). In Epstein-Barr virus (EBV)-associated HLH, infection of T lymphocytes results in clonal proliferation, with production of high levels of activating cytokines. Elaboration of TNF-α and other cytokines causes fever and systemic illness. TNF-α and IFN-γ production contributes to macrophage activation with resulting hemophagocytosis, as demonstrated by the ability of anti-TNF-α and anti-IFN-γ antibodies to attenuate hemophagocytosis. The immunopathology of infection with nonviral pathogens is less well understood, but may be related to exaggerated production of TNF-α and IFN-γ in response to infection. TNF-α = tumor necrosis factor-α; IFN-γ = interferon-gamma; IL-1 = interleukin-1; IL-2 = interleukin-2; IL-6 = interleukin-6; IL-18 = interleukin-18; sFasL = soluble Fas ligand; T = T-lymphocyte; M = macrophage; EBV = Epstein-Barr virus.

T-lymphocytes and NK cells, as well as induce Fas ligand expression on lymphocytes, which enhances their cytotoxic effect. Serum levels of soluble Fas ligand, which can trigger apoptosis in such Fas-expressing tissues as the kidney, liver, and heart, also appear to be increased in HLH (51).

The exact mechanisms by which abnormal cytokine elaboration by T lymphocytes results in HLH remain unclear. However, data from patients with EBV-associated HLH, as well as HLH associated with EBV-positive T-cell lymphomas, may be instructive. Although T lymphocytes lack the putative EBV receptor CD21, the presence of episomal EBV genome in T-cell lymphomas (52,53) and T lymphocytes from patients with virus-associated HLH is well described (54,55). EBV-positive T-cell lymphomas appear to elaborate TNF-α more frequently than either EBV-positive B-cell lymphomas or EBV-negative T-cell lymphomas (53).

Lay and colleagues induced the expression of CD21 in T-lymphoma cell lines and subsequently infected these cells with EBV. High levels of TNF-α , IFN-γ , and IL-1α were secreted by these cells after EBV infection; when the lymphocytes were co-cultured with monocytes, enhanced phagocytosis by monocytes was observed. The enhanced phagocytosis was eliminated by the addition of antibodies against TNF-α and IFN-γ (53).

The clonal expansion of EBV-infected T lymphocytes has been demonstrated in both EBV-associated HLH (55-57) and EBV-positive T-cell lymphoma (53) by the presence of homogeneous viral terminal repetitive sequences. EBV-infected cells stain positive for such T-lymphocyte markers as CD45RO and T-cell receptor-ß (54,55). Clonality of infected T lymphocytes is further suggested by the finding of monoclonal rearrangements of the T-cell receptor-ß gene in EBV-associated HLH (58).

The distinction between the monoclonal proliferation of T lymphocytes seen in EBV-associated HLH and EBV-positive T-cell lymphomas may describe extremes of a spectrum of disordered T-lymphocyte proliferation and cytokine elaboration following EBV infection of T lymphocytes. Elaboration of such viral proteins as LMP1, essential to the immortalization of EBV-infected B-lymphocytes, may affect EBV infection of T lymphocytes, although studies suggest otherwise (59). It is also unclear whether clonal proliferation of T lymphocytes occurs in HLH associated with pathogens other than EBV. The fact that these syndromes seem more likely to resolve with control of the underlying infection suggests that this may not be the case. The apparent utility of cyclosporin A in HLH (60-62) and the morphologic similarity of the liver disease seen in HLH to acute graft rejection in transplant patients (1) lend further credence to the role of lymphocytes as central to the pathogenesis of HLH.

The pathophysiology of infection-associated HLH following infection with nonviral pathogens may also be related to production of high levels of activating cytokines by host lymphocytes and monocytes. The relative frequency of association between infecting organisms (e.g., *Mycobacterium tuberculosis, Salmonella* Typhi, and *Leishmania* sp.) that trigger a T_H1 immune response and reactive hemophagocytic syndromes might suggest that the syndromes result from a poorly

regulated or inappropriate T_H1 response to intracellular pathogens. However, Tsuda and colleagues found no evidence of a marked shift towards a T_H1 cytokine profile in patients with HLH associated with nonviral infections (63).

Prognosis and Therapy

Because these disorders are rare, no controlled clinical trials of therapy have been performed. For patients with reactive HLH associated with pathogens other than EBV, supportive care and treatment of the underlying infection is associated with recovery in 60%-70% (20,64). Among adults with HLH, age >30 years appears to be associated with an increased risk for death (27).

Epstein-Barr virus-associated HLH is almost universally fatal if untreated, with death usually resulting from hemorrhage, infection, or multiorgan failure (64,65). The poor prognosis of this syndrome suggests that patients should be treated initially with combination chemotherapy and immunotherapy, regardless of whether they are thought to have familial HLH. Chemotherapy with etoposide (which is toxic to macrophages) and dexamethasone is recommended, with the use of intrathecal methotrexate in patients with neurologic symptoms or persistent cerebrospinal fluid abnormalities (66-69). In a group of children with EBV-associated HLH, investigators induced complete remission (median 15 months) in 15 of 17 patients (68). The increasing recognition of the important role of T lymphocytes in HLH has led to the recommendation that chemotherapy be combined with cyclosporin A immunotherapy (60-62,67). Antithymocyte globulin may also have a role in therapy (60).

HLH associated with viral infection may be difficult to distinguish from familial HLH triggered by a viral infection (39), although familial HLH should be considered more likely in infants even in the absence of a positive family history (19). The distinction is important, as allogeneic bone marrow transplantation is the therapy of choice in patients with familial HLH who attain remission (67,70). In patients without a clear diagnosis of familial HLH, bone marrow transplantation should be considered if remission is not attained by 8 weeks of chemotherapy and immunotherapy. Patients in remission without a clear diagnosis of familial HLH should be monitored closely for signs of relapse (67).

The role of intravenous immunoglobulin in the treatment of HLH is unclear. Remission after such therapy has been reported in adults and older children with underlying immune dysfunction (71,72). However, Chen and colleagues noted remission in only two of nine children with virus-associated HLH treated with intravenous immunoglobulin alone (65).

Acyclovir does not appear to be useful in the treatment of EBV-associated HLH. However, resolution of HLH associated with other viral pathogens has been reported after antiviral chemotherapy. For example, adenovirus-associated HLH in a bone marrow transplant patient was reported to resolve with vidarabine (73), while human herpesvirus-8-associated HLH in a patient with HIV infection appeared to improve with the use of foscarnet (74).

Conclusions

HLH and related hemophagocytic syndromes are uncommon but severe illnesses associated with a variety of infectious agents, as well as genetic, neoplastic, and autoimmune diseases. HLH in the context of infection is best described as part of a spectrum of EBV-associated illness resulting in clonal proliferation of T-lymphocytes, with excessive activation of macrophages. This syndrome may be difficult to distinguish from T-cell lymphoma and should be treated aggressively with etoposide-based chemotherapeutic regimens.

Hemophagocytic syndromes associated with other infectious illnesses, including sepsis, typhoid fever, tuberculosis, and leishmaniasis, may resolve with treatment of the underlying infection, and their recognition is important as they may mimic malignant disease. Further study of these reactive hemophagocytic syndromes may yield important insights into the biology of macrophage activation.

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Predominance of HIV-1 Subtype A and D Infections in Uganda

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To better characterize the virus isolates associated with the HIV-1 epidemic in Uganda, 100 specimens from HIV-1-infected persons were randomly selected from each of two periods from late 1994 to late 1997. The 200 specimens were classified into HIV-1 subtypes by sequence-based phylogenetic analysis of the envelope (*env*) gp41 region; 98 (49%) were classified as *env* subtype A, 96 (48%) as D, 5 (2.5%) as C, and 1 was not classified as a known *env* subtype. Demographic characteristics of persons infected with the two principal HIV-1 subtypes, A and D, were very similar, and the proportion of either subtype did not differ significantly between early and later periods. Our systematic characterization of the HIV-1 epidemic in Uganda over an almost 3-year period documented that the distribution and degree of genetic diversity of the HIV subtypes A and D are very similar and did not change appreciably over that time.

HIV strain characterization and phylogenetic analysis have played a key role in elucidating epidemiologic and historical aspects of HIV transmission worldwide (1). In Thailand, for example, molecular analyses of virus isolates documented the independent introduction and spread of two different HIV-1 subtypes, B and E, as well as the increasing proportion of subtype E relative to B in injection drug users over time (2-8). Information from these studies clarified the dynamics of the Thai epidemic and influenced the decision to introduce a bivalent subtype B/E vaccine in the first HIV-1 vaccine efficacy trials recently initiated there (6). However, few other studies have described HIV-1 strains or the phylogenetic relationships of sequences from infections sampled in a systematic manner. This is especially true in central Africa, where the HIV epidemic has been present for a relatively long time (1-3,6,7,9-12).

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Uganda, where the prevalence of HIV-1 is one of the highest in the world, has been a focus of research and intervention efforts, including vaccine development for the first vaccine trial in Africa. Previous studies documented HIV-1 subtypes A and D as important strains, with a limited distribution of other variants (13-19). The primary objective of our study was to characterize the recent virus isolates of the HIV-1 epidemic in Uganda by describing the distribution and genetic diversity of the principal HIV-1 subtypes.

Methods

In an ongoing collaborative effort between the Uganda Virus Research Institute and the Centers for Disease Control and Prevention to study HIV-1 genetic diversity, isolates from a number of different clinical sites and counseling and testing centers in Uganda were collected and characterized (19). To obtain uniform and consistent sampling over time from the Kampala and Mpigi districts of central Uganda, where most specimens were collected, we selected two sites in Kampala (Mulago Hospital, Nsambya

Mobile Unit) and one site in Entebbe (Uganda Virus Research Institute Clinic). From more than 1,800 specimens collected from these sites from late 1994 through 1997, we chose all seropositive specimens from the earliest period (the last quarter of 1994 through the first quarter of 1995, n=393, and the last two quarters of 1997, n=135). After determining that the major demographic factors, such as mean age (p=0.76), age group (p=0.20), sex (p=0.12), and marital status (p=0.14), were not significantly different between persons seen in either period, we selected 100 specimens at random from each period for further analysis. Because local resources were limited, clinical (World Health Organization stage) and immunologic (CD4 and CD8 counts) data were available from only a subset of patients. Although limited by the number of specimens available and by laboratory capacity, our sample size could detect a minimum absolute change of more than 20% in either subtype A or D with a power of 80% and with 95% confidence.

Data collection, specimen processing, viral isolation, and polymerase chain reaction (PCR) amplification of the *env* gp41 fragment (460 bp) used methods previously described (19-21). After amplification, DNA from the nested PCR was cycle-sequenced (60 ng DNA per sequencing reaction) with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA), according to manufacturer's protocol, by using the PCR nested primers gp46F2 and gp47R2 (21).

Results

Of all 200 specimens, 98 (49%) were classified as *env* subtype A, 96 (48%) as D, 5 (2.5%) as C, and 1 unclassified into a known *env* subtype category. The demographic characteristics of persons infected with the two principal HIV-1 subtypes, A and D, were very similar, and the proportion of either subtype did not differ significantly between early and later periods (Table 1). In addition, there were three subtype C isolates and one isolate of undetermined subtype in the early period and two subtype C isolates in the later period.

The Figure illustrates the phylogenetic trees for all HIV-1 *env* subtype A and D isolates. The mean pairwise intrasubtype nucleotide divergence for *env* gp41 was 7.8% (3.6%-22.3%) for subtype A specimens and 7.2% (2.8%-12.5%) for subtype D specimens. The translated amino acid regions of gp41 revealed only minor substitutions in subtypes A and D (data not shown). When specimens were further stratified by period and clinic, intrasubtype divergence of the two subtypes was similar, with the percentage of divergence slightly higher in the later period (Table 2). There was no clear segregation or clustering by period or locale (Figure).

Table 1. Demographic characteristics of persons infected with HIV-1 subtype A or D

Characteristics	Subtype A n=98	Subtype D n=96	
Period			
Early (1994/95)	n=51	n=45	p=0.47
Late (1997)	n=47	n=51	
Age (yrs)			
Median	30.0	28.0	
Mean	31.2	30.8	p=0.39
Sex			
Female	66%	73%	p=0.26
Male	34%	27%	
Marital status			
Married	37%	40%	p=0.66
Single, divorced, widowed	63%	60%	
Clinic (district)			
UVRI[a] (Mpigi)	37%	33%	p=0.81
Mulago (Kampala)	23%	24%	
Nsambya (Kampala)	40%	43%	

[a]UVRI = Uganda Virus Research Institute.

In the subset of persons with clinical or immunologic data, the clinical characteristics of persons infected with *env* subtype A or D were very similar; any differences were not significant (Table 3).

Discussion

Our detailed and systematic characterization of the HIV-1 epidemic in Uganda over an almost 3-year period has shown that the distribution and degree of genetic diversity of the two predominant *env* subtypes, A and D, are very similar and have not changed appreciably over that time. However, there are two caveats when interpreting our results. First, our sample was collected cross-sectionally without known dates of infection. Second, our available sample size was not large enough to document small changes in the proportion of either A or D subtypes. Nevertheless, the proportion of those two subtypes observed in specimens from the two periods were virtually identical.

Table 2. Mean (range) percentage of pairwise nucleotide divergence stratified by time period, subtype, and clinic (district)

Subtype by clinic (district)	% Mean (range) 1994-95	1997	Overall
Subtype A[a]			
UVRI[b] (Mpigi)	5.0 (3.6- 9.5)	7.7 (3.9-12.6)	6.4 (3.6-12.6)
Mulago (Kampala)	7.3 (3.9-10.4)	8.3 (4.2-14.0)	7.8 (3.9-14.0)
Nsambya (Kampala)	7.3 (3.9-11.7)	10.8(3.9-22.3)	9.1 (3.9-22.3)
Subtype D			
UVRI (Mpigi)	5.9 (3.1- 8.8)	8.0 (2.8-12.4)	6.9 (2.8-12.4)
Mulago (Kampala)	7.9 (3.6-12.4)	6.7 (3.1- 9.8%)	7.2 (3.1-12.4)
Nsambya (Kampala)	6.4 (3.3- 9.5)	8.5 (3.9-12.5)	7.4 (3.3-12.5)

[a]Since two isolates from 1994-1995 were almost identical and may represent persons who were epidemiologically linked, these were treated as one sequence for overall calculations.
[b]UVRI = Uganda Virus Research Institute.

Table 3. Clinical characteristics among a subset of persons infected with HIV-1 subtype A or D[a]

Characteristic WHO[a] clinical	Subtype A n=37	Subtype D n=46	p value
Stage			
1 or 2	8%	9%	p=0.97
3	57%	54%	
4 (AIDS)	35%	37%	
CD4	n=29	n=26	
Mean	253	187	p=0.24
Range	19-1,061	2-713	
CD8	n=29	n=26	
Mean	812	734	p=0.45
Range	288-2,000	139-1,458	

[a]WHO = World Health Organization.

Our results are consistent with those of previous studies indicating that both subtypes A and D have been present as major strains in the Ugandan epidemic since the mid-1980s (13-17). The similar mean and range of intrasubtype variation for subtypes A and D in the *env* gp41 region in this report, as well as in the *env* C2-V3 we reported earlier (19), also suggest that the two predominant HIV-1 subtypes have both been present in Uganda for a relatively long period.

The lack of significant clustering by period or clinic in the phylogenetic analyses suggests that infections from both subtypes are transmitted in a broad, heterogeneous manner in central Uganda. Similarly, our examination of a subset of patients with clinical or immunologic data, in combination with similar overall demographic characteristics, suggests that persons with subtype A or D had similar levels of immune suppression. Since this information was independently collected at three sites before genetic characterization of infecting HIV-1 strains, a systematic selection bias with respect to subtype appears unlikely.

Although we did not document any major recent changes in the proportion of subtypes A or D, our ongoing surveys may show future changes in subtype distribution. In contrast to the large proportion of subtypes A and D, the proportion of subtype C appeared to be small in both periods studied and in our earlier study (19). The minor, nonexpanding, role of subtype C in central Uganda contrasts with the higher proportions of C in southeastern Africa. Recently, we reported that subtype C isolates from Uganda formed a distinct subcluster, unlike subtype C isolates from other locales (24).

The presence or absence of significant changes in subtype distribution in different populations is ecologic and by itself does not constitute definitive evidence for or against potential subtype-specific differences in transmissibility, pathogenesis, or clinical symptoms (25). In Uganda, where subtypes A and D are found in a large proportion of the population, current and future prospective studies will help to address these crucial research questions.

Although few countries, especially those with limited resources, have the necessary infrastructure to systematically monitor the HIV-1 epidemic, periodic molecular analyses such as this one in Uganda are needed, particularly if trials of subtype-specific vaccines are being considered. Since the HIV-1 pandemic is becoming increasingly complex, with greater heterogeneity and the presence of recombinant viruses (26), a major challenge will be to develop cost-effective technologies to characterize viruses without sequencing (1). While more rapid typing methods are available to determine the subtype distribution (19), we needed the labor-intensive sequencing and phylogenetic analysis in this study to describe the genetic diversity in detail.

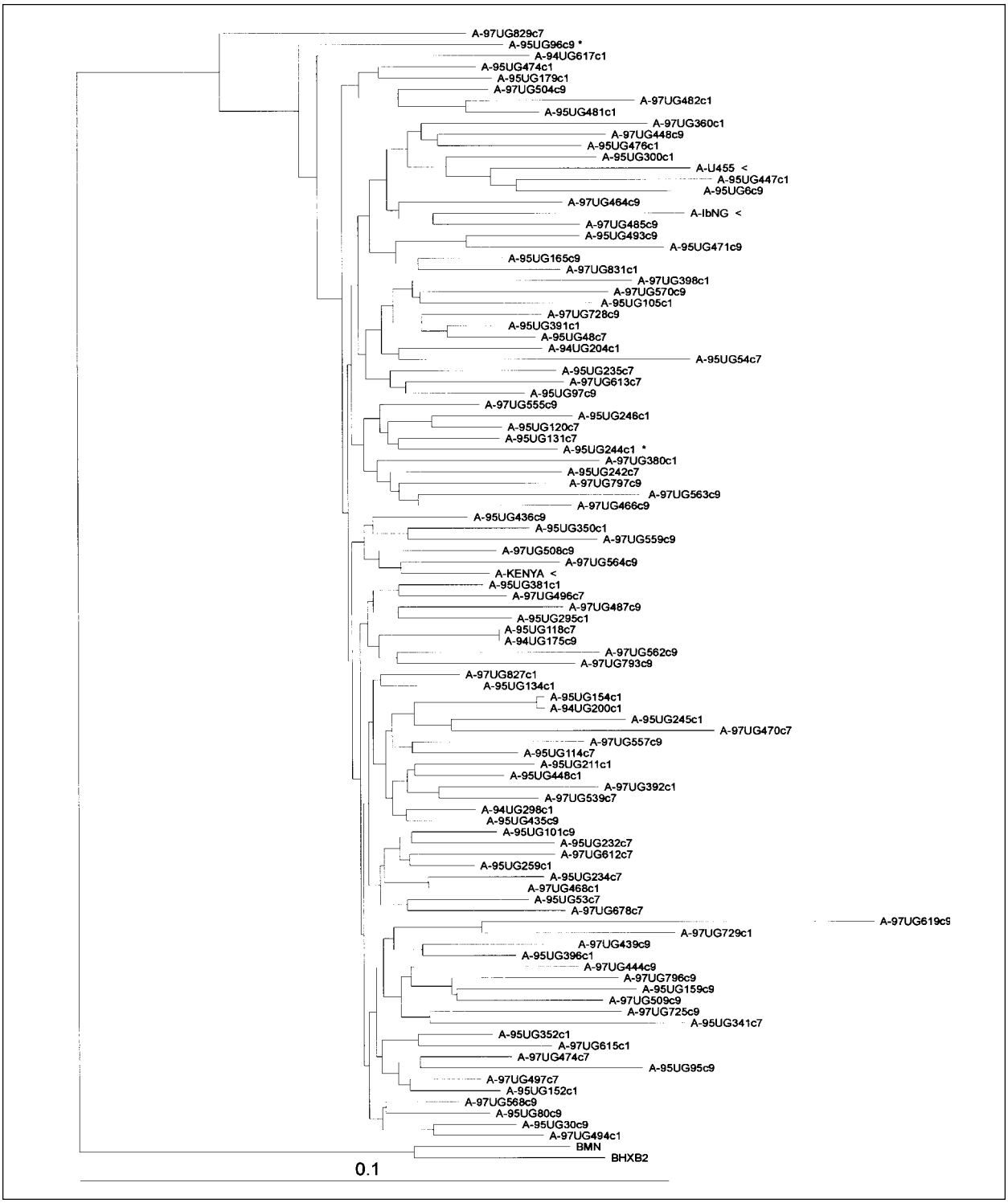
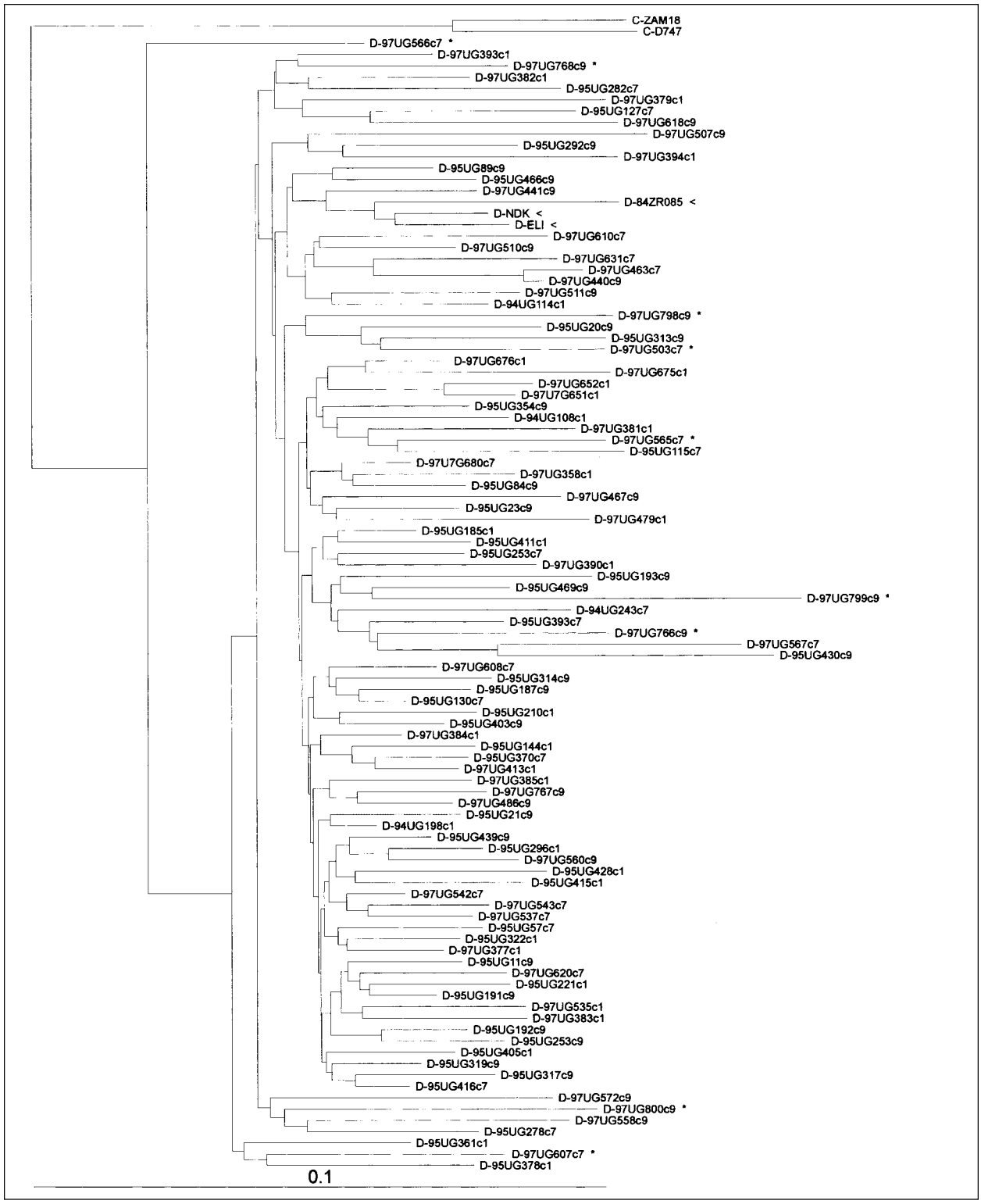


Figure. Phylogenetic classification of *env* gp41 HIV-1 sequences from Ugandan (UG) patients (GenBank accession numbers for subtypes A and D are pending). Numbers before the abbreviation UG indicate the year of specimen collection; c1, c7, and c9 denote the UVRI, Mulago, and Nsambya clinics, respectively. The trees were constructed on the basis of 354-bp DNA sequences by the neighbor-joining method with nucleotide distance datum sets calculated by Kimura's two-parameter approach and rerooted by using SIV-cpz as the outgroup. Arrows indicate reference subtype A and D sequences; asterisks indicate sequences, which decrease the bootstrap value from 90% to 73% in subtype A and from 85% to 55% in subtype D sequences. The scale bar indicates the evolutionary distance of 0.10 nucleotides per position in the sequence. Vertical distances are for clarity only. An automated DNA sequencer (Applied Biosystems Model 373, Foster City, CA) was used to generate sequence data for alignment



with the CLUSTAL version V multiple sequence alignment program and subsequent phylogenetic analysis. Phylogenetic relationship of sequences was analyzed by the neighbor-joining method (PHYLIP package version 3.5c with and without bootstrapping), and the maximum-likelihood method (fastDNA program, version 1.0.8, which uses randomized data input and global rearrangement). The stability of tree topology was tested by pruning, which consisted of removing one species from the alignment and rerunning the phylogenetic analysis. Accurate subtype determination using *env* gp41 has been shown to be similar to that based on *env* C2V3 sequences (22). The gp41 DNA sequences Environment package, and immunodominant regions were analyzed (23). The reference sequences for subtypes A-J, groups O and N, and SIVcpz were retrieved from the 1997 HIV-1 Molecular Immunology Database (Los Alamos National Laboratory, Los Alamos, NM).

Furthermore, given the time and resources necessary to develop vaccine candidates, confirmation from this and previous studies that subtypes A and D continue to be the predominant subtypes with consistently minor contributions from other strains may have important implications for vaccine research.

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Hantavirus Pulmonary Syndrome Associated with Monongahela Virus, Pennsylvania

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The first two recognized cases of rapidly fatal hantavirus pulmonary syndrome in Pennsylvania occurred within an 8-month period in 1997. Illness in the two patients was confirmed by immunohistochemical techniques on autopsy material. Reverse transcription-polymerase chain reaction analysis of tissue from one patient and environmentally associated *Peromyscus leucopus* (white-footed mouse) identified the Monongahela virus variant. Physicians should be vigilant for such Monongahela virus-associated cases in the eastern United States and Canada, particularly in the Appalachian region.

Sin Nombre virus (SNV) was isolated and characterized as the cause of the 1993 cluster of 17 cases of hantavirus pulmonary syndrome (HPS) in the southwestern United States (1,2). HPS cases in other states were predicted because of the widespread distribution of the rodent hosts of hantavirus (2). Subsequent reports described human infection with and rodent carriage of novel genomic variants of hantavirus in New York (3), Florida (4), and Louisiana (5). Each of the four initially characterized strains of hantavirus causing HPS in North America is carried by a primary rodent host species, although spillover to other rodents in the area can occur (6-9).

We describe the first two recognized cases of HPS acquired in Pennsylvania; both were fatal and at least one was caused by infection with the newly characterized Monongahela variant of hantavirus (10). This variant can be present in *Peromyscus leucopus* mice, in addition to *P. maniculatus nubiterrae* with which they often share microhabitat.

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Case Reports

Case 1. A 40-year-old, previously healthy crossbow hunter was taken by ambulance to a Lehigh County, Pennsylvania, hospital in November 1997, with complaints of back muscle pain, dizziness, diarrhea, fever, and abdominal pain of 3 days' duration. He had been taking oral penicillin and ibuprofen for several weeks for a chronically infected tooth, and initially his symptoms were attributed to severe dental infection or antibiotic-associated diarrhea. A chest X-ray at the time of the first emergency room visit was normal. The patient was given intravenous fluids and was referred for dental extraction, but he was hospitalized the next day for progressively severe respiratory and generalized systemic distress. He was transferred to the intensive care unit, where he was placed on mechanical ventilation 11 hours after hospital admission because of increasing respiratory failure. Sputum, blood, and urine cultures and Gram stains were negative or nondiagnostic; these tests included respiratory viral cultures for respiratory syncytial virus; adenovirus; parainfluenza virus types 1, 2, 3; and influenza virus types A and B (Table 1). Chest X-rays over a 3-day period showed pleural effusions and progressive,

Table 1. Laboratory data

Complete blood count	Hantavirus pulmonary syndrome Patient 1				Hantavirus pulmonary syndrome Patient 2				
Date	3/10/97	3/12/97	3/13/97		11/13/97	11/14/97	11/15/97	11/16/97	
Time	2057	1443	0700	1100	1356	1856	0450	0400	0925
White blood cells	4,300	4,800	12,100	10,900	4,400	5,100	8,000	11,100	15,700
Polymorpho nuclear cells (percent)	58	60	45	53	67	62	81	58	73
Bands (percent)	3	23	34	27	0	9	N/D	11	N/D
Immunoblasts	4	1	3	5	2	N/D	N/D	N/D	N/D
Platelets	100,000	27,000	13,000	2,000	128,000	74,000	59,000		
Hematocrit	45.9	47.0	55.5	44.0	47.4	46.4	48.2		
Creatinine (mg/dL)	0.8	0.8	1.9		1.2	1.1	1.0		

refractory bilateral pulmonary infiltrates, initially interstitial then alveolar. Intravenous therapies included fluids, vasopressors, and inotropic agents, including dobutamine, dopamine, and norepinephrine. Antimicrobial drugs administered during the hospital stay included azithromycin, ceftriaxone, doxycycline, clindamycin, levofloxacin, and trimethoprim/sulfamethoxazole. The patient's fever persisted throughout the 3-day hospital stay, his pulmonary and cardiac status deteriorated, and he died 5 days after onset of illness, despite intensive critical care support.

Hantavirus infection was confirmed by both immunohistochemical study of lung and kidney tissue, using a cross-reactive monoclonal antibody (GB04-BF07) as described (11). Enzyme-linked immunosorbent assay for serum antibodies showed an immunoglobulin (Ig) G titer of 1:400 and an IgM titer of 1:1600 for Sin Nombre antibody.

Patient 1 lived in a rural area in Upper Macungie Township near Allentown, Pennsylvania (Figure 1). In the 8 weeks before his death, he hunted deer almost every day and was potentially exposed to mouse habitats in four Pennsylvania counties. The longest exposure was a 3-day, 2-night stay in a small cabin near Germania in Potter County, Pennsylvania (Figure 1), 3 weeks before onset of illness. The patient slept on a mattress in a loft. During an investigation of the cabin in December 1997, rodent droppings were found on the loft floor around the mattress. A dead *Peromyscus leucopus* (white-footed mouse) was found in a trap on the cabin floor. When unoccupied, the cabin was closed and the downstairs windows were boarded shut. On January 6, 1998, 70 live traps were set in and around the cabin. Eleven small mammals were captured: seven *P. leucopus*, two *P. maniculatus* (deer mouse), one *Clethrionomys gapperi* (red-backed vole), and one *Blarina brevicauda* (short-tailed shrew). The animals were anesthetized and bled through a capillary tube from the periorbital sinus. Blood was placed on Nobuto blood collection strips, air-dried, and analyzed for hantavirus antibodies. Lungs were collected from the mammals, placed on ice, and kept frozen at -70°C until virus isolation.

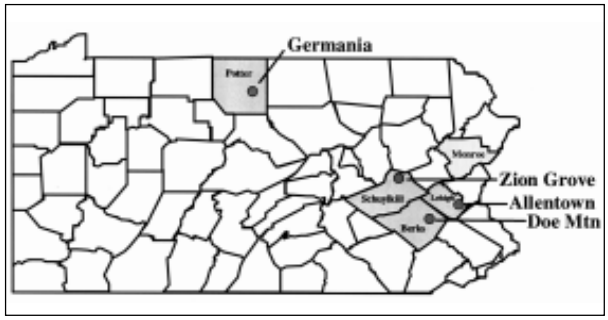

Figure 1. Pennsylvania county map highlighting the four counties involved (Potter, Schuylkill, Lehigh, and Berks) in the environmental investigations of Patient 1, and the single county of residence (Monroe) and rodent exposure of Patient 2.

Patient 1 had also cleaned a small trailer near Zion Grove in Schuylkill County, Pennsylvania, approximately 6 weeks before onset of illness, and had noted mouse droppings there. He hunted avidly with a crossbow, often from shelter blinds on Doe Mountain in Berks County, Pennsylvania, in the 8 weeks before his illness.

Case 2. A 39-year-old machine shop worker and mother of two children visited a Monroe County, Pennsylvania, hospital emergency department in March 1997. She had fever, myalgias, profound weakness, cough, and shortness of breath, and had not urinated for 3 days. She received intravenous fluids, and

laboratory testing was performed (Table 1). Because of acteriuria, an initial diagnosis of urinary tract infection was made. Treatment with clarithromycin and cefuroxime oral antimicrobial drugs was initiated, and the patient was discharged; 48 hours later, she returned to the hospital, complaining of severe backache, dizziness, abdominal pain, and shortness of breath. She was admitted to the hospital and 12 hours later was placed on mechanical ventilation because of respiratory distress. She rapidly became progressively hypotensive despite fluid and inotropic therapy, and she died on hospital day 2, day 5 after onset of illness. Antimicrobial drugs administered included erythromycin, ceftazidime, and cefotaxime. Blood cultures remained sterile, and sputum cultures grew oral flora. A urine culture taken at admission yielded >100,000 *Escherichia coli*/mL. A year after the patient's death, immunohistochemical testing of archival tissue from an initially nondiagnostic autopsy confirmed the diagnosis of HPS. No serum was available for testing.

Patient 2 lived in a mobile home in a rural, wooded area near Cresco, Barrett Township, Monroe County, Pennsylvania (Figure 1). The machine shop where she worked was also in an isolated area. Family members stated that she had not left the area for several months before her death. During the environmental studies begun 14 months after the patient's death, rodent droppings were found under the kitchen sink of her home. That evening, 70 rodent live traps were placed in and around the mobile home, and six *P. leucopus* mice were captured. The rodents were bled, and lung tissue was collected. On June 2, 1998, the machine shop was examined, but no evidence of rodent activity was noted. Seventy traps were set in and around the shop. Six *P. leucopus,* one *Tamias striatus* (eastern chipmunk), and one *B. brevicauda* were captured in the surrounding wooded area; no rodents were captured inside the building.

Materials and Methods

RNA Extraction, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and Genetic Analysis

RNA was extracted from Patient 1's lung tissue, and RT-PCR and nucleotide sequence analysis were carried out as described (12). RNA was also extracted from six seropositive rodents, and RT-PCR and nucleotide sequence analysis were performed (13).

Results

Genetic Analysis of Virus from Lung Tissue

The nucleotide sequences of the fragments S, M-G1, and M-G2 were compared with the equivalent regions of other previously characterized *Peromyscus*-associated hantavirus. The hantavirus in Patient 1's lung was clearly identified as Monongahela virus, which was first detected in *P. maniculatus nubiterrae* (Cloudland deer mice) captured in West Virginia in 1985 (10). Virus S segment fragment showed 95.7% nucleotide identity and 100% deduced amino acid compared with the Monongahela-1 virus strain. A shorter, overlapping fragment (246 nucleotide) was available to compare with the Monongahela-3 virus strain. Again, a 96.7% nucleotide identity and 100% amino acid identity were found. Other *Peromyscus*-borne hantaviruses were more distantly related. Comparison of this patient's hantavirus sequence with those of New York virus (strains NY-1 and RI-1) and SNV (strains NMH10, CC74 and CC107) identified a nucleotide difference of 14.8% to 19.6% and an amino acid difference of 2.3% to 3.1% (14-16).

Results were similar, with 95.1% nucleotide and 100% amino acid identity, when the virus M-G2 fragment sequence from Patient 1 was compared with the Monongahela-2 virus strain. Nucleotide and amino acid differences of 15.6% to 21.5% and 1.5% to 7.4%, respectively, were seen when this fragment was compared with the more distantly related *Peromyscus*-borne hantaviruses, including Blue River virus (strains Indiana and Oklahoma), New York virus (strains RI-1, NY-1, and NY-2), and SNV (strains NM H10, CC74, and CC107) (15-19). No comparable piece of Monongahela virus sequence was available to align with the virus M-G1 fragment from this patient. However, sequence identity differences of 15.1% to 18.1% and 3.5% to 7.0% were seen at the nucleotide and amino acid levels, respectively, when the patient M-G1 sequence was compared with those of the other *Peromyscus*-associated hantaviruses.

Analysis of Rodent Samples

Three of seven *P. leucopus* mice (PA1, PA, and PA9) trapped at the suspected exposure site

for Patient 1 in Potter County and three of six *P. leucopus* mice (MC-3, MC-4, and MC-6) trapped at the Monroe County location (suspected exposure site for Patient 2) contained hantavirus-specific antibodies. With the exception of MC-3, Monongahela virus-specific RNA was detected in each of the seropositive rodents by RT-PCR. *P. leucopus* MC-3 was a juvenile mouse, suggesting that maternal antibody may have been detected. The virus nucleotide sequences obtained from the Potter County mice closely matched those obtained in Case 1, with the M-G1 PCR fragment sequences being identical. The virus nucleotide sequence fragments obtained from the Monroe County mice differed from those from Patient 1 and the Potter County rodent samples by approximately 10%.

A detailed phylogenetic analysis of the M-G2 fragment sequences of these and other published hantavirus sequences was carried out. A 50% majority rule consensus tree was generated by bootstrap analysis with 1,000 replications (Figure 2). Hantaan and Seoul hantavirus sequences were used as an outgroup. This analysis showed that the viruses detected in Patient 1 (human 584) and rodents (PA1, Potter County; and MC-4, Monroe County) were clustered in the Monongahela virus lineage.

Pathology

Examination of lung tissue from Patient 1 by microscopy showed a mild to moderate interstitial pneumonitis, with mononuclear infiltration and congestion, and edema typical of HPS. Typical immunoblasts were seen within the red pulp in periarteriolar sheaths of the spleen. Immunohistochemical examination showed widespread staining of hantavirus antigens within

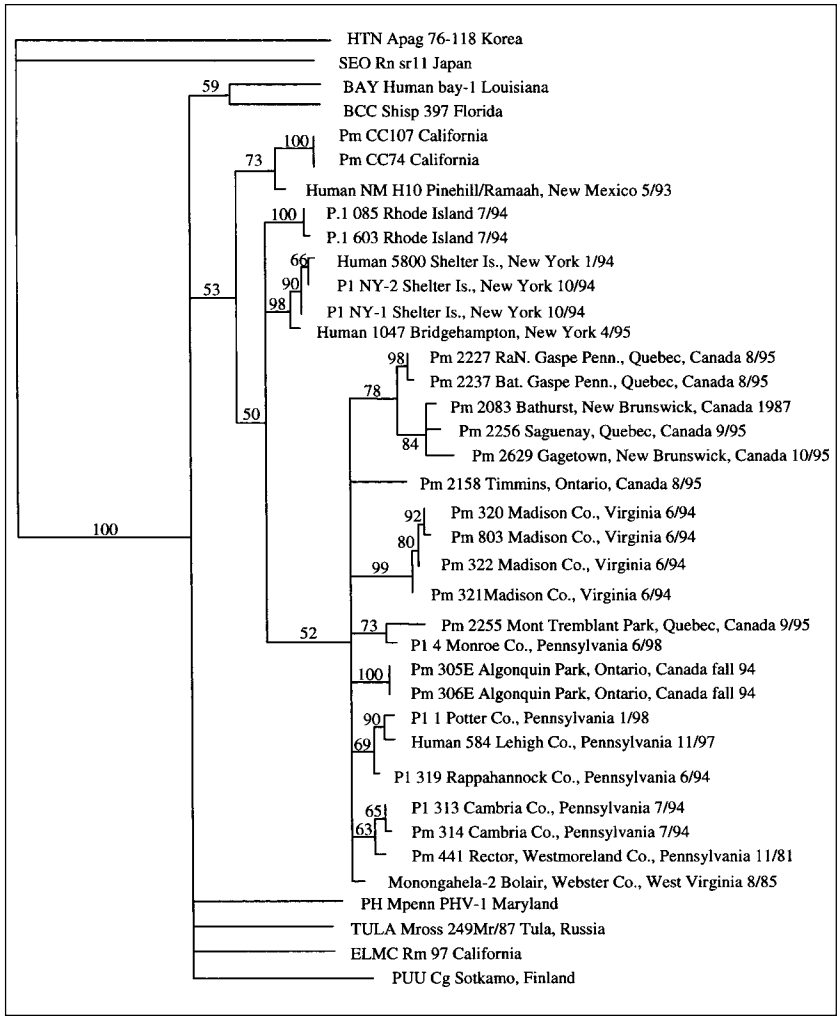

Figure 2. Phylogram of the 139-bp fragments of the G2 coding region.[1] The number above the branch is the bootstrap probability (shown as percentages). HTN Apag - Hantaan virus strain 76-118, *Apodemus agrarius*; SEO Rn sr11 - Seoul virus strain SR-11, *Rattus norvegicus*; BAY - Bayou virus; BCC Shisp - Black creek canal virus, *Sigmodon hispidus*; Pm - *Peromyscus maniculatus*; NM - New Mexico; Pl - *Peromyscus leucopus*; PH Mpenn - Prospect Hill, *Microtus pennsylvanicus*; TULA Mross - Tula, *Microtus rossiaemeridionalis*; ELMC Rm 97 - El Moro Canyon virus, *Reithrodontomys megalotis*; PUU Cg - Puumala virus, *Clethrionomys glareolus*.
[1]Three fragments of the virus genome were amplified by nested polymerase chain reaction to allow generation of nucleotide (nt) sequences 393 nt in length for an S segment region encoding the N protein, 259 nt in length for an M segment region (M-G1) encoding the G1 protein, and 205 nt in length for an M segment region (M-G2) encoding the G2 protein (12). Sequence data were analyzed by using the Wisconsin GCG Package (version 9.1) software. Initial multiple sequence alignment was done with the GCG Pileup program, and phylogenetic analysis was performed by using the Phylogenetic Analysis Using Parsimony (*and other methods) program (version 4.0) (20).

endothelial and follicular dendritic cells, similar to the pattern seen in previous cases of HPS. However, instead of the typical granular staining, the lung immunostaining was more linear and curvilinear, similar to staining seen in infections with Hantaan virus. The immunostaining in the kidney was also more abundant than with typical HPS cases.

Conclusions

We describe two Pennsylvania cases that add incrementally to knowledge about North American hantavirus. Rodent hosts carrying hantavirus in an asymptomatic but communicable form are found in most areas of North America (21). Clinicians should be vigilant for possible cases of HPS, even in areas where no cases have been previously recognized.

These Pennsylvania cases share with earlier (2) and more recently described cases (22) the clinical picture of a nonspecific and often nonpulmonary flulike prodromal phase. The surprising rapidity with which noncardiogenic pulmonary edema affects often previously healthy victims separates HPS from many other common febrile diseases seen in North America. Some reports have suggested that patients be transferred to facilities with extracorporeal membrane oxygenation capability for patients who do not respond to respiratory and inotropic support (23). However, data may be insufficient to establish the precise role of extracorporeal membrane oxygenation support in the management of these patients (24).

These Pennsylvania cases also reflect the first report of human infection with the Monongahela variant of hantavirus. The initial description of this strain was from archival tissue of *P. maniculatus nubiterrae* captured in the Monongahela National Forest in West Virginia. Retrospective serologic diagnoses of HPS have been made in nonfatal human cases from Virginia and West Virginia, but none of these cases, to our knowledge, have had RT-PCR confirmation of the Monongahela variant. Recent analysis of hantavirus RNA and rodent mitochondrial DNA has shown genetically distinct clades of virus and rodents useful in understanding suspected cospeciation of virus types within host rodent subspecies (25-27). Monongahela hantavirus appears to be closer in evolutionary distance to the New York/Rhode Island strains than to SNV strains found in the midwestern and southwestern United States (16). Finding Monongahela variant of hantavirus in both *P. leucopus* and *P. maniculatus* can be explained by spillover between the two mouse species, given their frequent sympatric and synchronistic existence (16). Allopatric migrations and other biogeographic influences may explain the existence of hantavirus variants in more than one species or subspecies of host rodent (16).

Given the complexity of the genetic relationships between the *Peromycine*-borne hantaviruses, it is unclear whether the Monongahela virus lineage will be considered a distinct hantavirus species or a subspecies of SNV (18). However, the pathologic findings in Monongahela-associated HPS, together with its primary association with a distinct *P. maniculatus* subspecies, suggest biologic differences between Monongahela virus and classic SNV lineages.

Clinical differences have been noted for HPS cases due to different hantavirus types in North and South America. Renal disease and myositis are more evident in patients infected with hantavirus carried by *Sigmodon* sp. and *Oryzomys* sp. than by *Peromyscus* sp.(4). The unusual linear immunohistochemical immunostaining and heavy renal staining seen in autopsy material from the two Pennsylvania HPS cases may also have clinical importance. The ultimate goals of effective treatment and prevention of HPS will require continued close cooperation between clinicians, laboratorians, virologists, mammalogists, and epidemiologists.

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Risk Factors for Otitis Media and Carriage of Multiple Strains of *Haemophilus influenzae* and *Streptococcus pneumoniae*

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We studied genetic diversity in *Streptococcus pneumoniae* and *Haemophilus influenzae* in throat culture isolates from 38 children attending two day-care centers in Michigan. Culture specimens were collected weekly; 184 *S. pneumoniae* and 418 *H. influenzae* were isolated from the cultures. Pulsed-field gel electrophoresis identified 29 patterns among the *S. pneumoniae* isolates and 87 among the *H. influenzae* isolates. Of the cultures, 5% contained multiple genetic types of *S. pneumoniae*, and 43% contained multiple types of *H. influenzae*. Carriage of multiple *H. influenzae* isolates, which was associated with exposure to smoking, history of allergies, and age 36 to 47 months, may increase risk for otitis media in children.

Acute otitis media is the most frequently diagnosed bacterial illness in young children in the United States, resulting in an annual economic cost of approximately $3.8 billion per year (1). *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* are the bacterial species most frequently associated with otitis media in young children, and nasopharyngeal colonization with these bacteria is associated with its development (2-6).

Simultaneous carriage of more than one genetically distinct isolate of *H. influenzae* has been observed in Australian Aboriginal children (7) and in children attending day-care centers (8), two groups at increased risk for otitis media (4,7,9-12). Carriage of multiple genetic types of *H. influenzae* may result from inadequate clearance of colonizing bacteria or from exposure to multiple circulating strains (4,7). Concurrent colonization with more than one serotype of *S. pneumoniae* has also been reported (13), but the degree of colonization with multiple genetic types of *S. pneumoniae* has not been investigated. In addition, the association between risk factors for otitis media and colonization with multiple types of *H. influenzae* and *S. pneumoniae* isolates has not been examined.

We examined genetic diversity among *S. pneumoniae* and *H. influenzae* isolates colonizing 38 children attending two Michigan day-care centers. We also investigated the degree to which the bacteria were shared among children attending these centers and the relationship between risk factors for otitis media and carriage of multiple isolates.

Methods

Study Population

Thirty-eight children from two Michigan day-care centers participated in the study. One hundred sixty children attended day-care center 1 full time, divided into nine classrooms by age and developmental level. Fifty-five children attended day-care center 2 full time, divided into six classrooms. A total of 12% of the children from day-care center 1 and 33% from day-care center 2

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participated in the study. At least one child from each classroom participated in the study, with the exception of the classroom for children ≥5 years of age in day-care center 1.

Study Protocol

Participants were recruited from day-care center 1 in February 1998 and from day-care center 2 in June 1998. Parents signed consent forms allowing four throat cultures to be collected over a 3-week period. Parents were also asked to complete a short questionnaire assessing history of and risk factors for otitis media, antibiotic use, and demographic characteristics. The study was approved by the University of Michigan Health Sciences Institutional Review Board.

Throat cultures were collected once a week for 3 weeks from each child, for a maximum of four cultures per child. At the time of each culture, the child's teacher was asked if the child had been ill in the preceding week, and if so, with what symptoms. The parents of children who had been ill were asked to describe the child's symptoms and whether an antibiotic was given.

Bacteriologic Methods

Throat swabs were collected with rayon-tipped swabs (VWR Scientific, Westchester, PA), were placed in 500 µL Brain Heart Infusion (BHI) medium, and were transported on ice. Swabs were vortexed vigorously in the medium and then discarded. Five hundred microliters of sterile skim milk were added to each sample, and cultures were frozen at -80°C.

Samples were thawed at room temperature, vortexed briefly, and 100 µL of sample was streaked on each of two chocolate agar plates supplemented with 300 mg bacitracin (BBL, VWR Scientific) for *H. influenzae* selection and two 5% sheep blood agar plates supplemented with 2.5 µg gentamicin (BBL) for *S. pneumoniae.* Plates were incubated at 37°C with 5% CO_2 for 24 hours to 48 hours. Five colonies were selected from each plate for a maximum of 10 colonies per bacterial species; colonies with differing morphologic features were selected. *H. influenzae* isolates were characterized by X- and V-factor dependence (BBL): 34% of the picked colonies were *H. influenzae*, 49% were *H. parainfluenzae,* and 17% were other species. Only *H. influenzae* isolates were characterized further. *S. pneumoniae* isolates were identified by optochin disc sensitivity (BBL).

DNA Isolation from *H. influenzae* and *S. pneumoniae*

H. influenzae and *S. pneumoniae* isolates were streaked from frozen stocks on chocolate agar plates and blood agar plates, respectively, and grown overnight at 37°C with 5% CO_2. Bacterial growth was diluted to an optical density of 1.0 (610 nm) (SP 830 spectrophotometer, Turner, Barnstead/Thermodyne, Dubuque, IA) in 3 mL PETT IV buffer (10 mM Tris-Cl, pH 7.6, 1 M NaCl). The bacterial suspension (350 µL for *H. influenzae* or 1 mL for *S. pneumoniae*) was centrifuged at 13,000 rpm for 30 seconds. Bacterial pellets were resuspended in 100 mL PETT IV buffer, 100 µL 0.8% InCert agarose (FMC Bioproducts, Rockland, ME) was added to each, and 20-mL plugs were pipetted on a weigh boat. Plugs were cooled at 4°C for 30 min and then incubated with 1 mL EC lysis buffer (6 mM Tris-Cl, pH 7.6; 1 M NaCl; 100 mM EDTA, pH 8.0; 0.5% Brij-58; 0.2% deoxycholate; 0.5% N-lauroylsarcosine; 1 mg/mL lysozyme; and 20 µg/mL RNAse) for 4 hrs at 37°C (*H. influenzae*) or 50°C (*S. pneumoniae*). The buffer was removed, and plugs were incubated with 200 µL ESP buffer (0.5 M EDTA, pH 8.0; 1% N-lauroylsarcosine; and 1 mg/mL Proteinase K) for 12 hrs to 24 hrs at 50°C. ESP buffer was removed, and plugs were incubated with 1 mL TE-phenylmethylsulphonyl fluoride (1 mM) for 2 hrs at 37°C. Plugs were then washed twice with 1 mL TE buffer and stored at 4°C in 1 mL fresh TE buffer.

DNA Digestion and Pulsed-Field Gel Electrophoresis

Single plugs were incubated 30 min in 100 µL 1X enzyme reaction buffer (Gibco-BRL, Rockville, MD) at 37°C. Buffer was removed, and plugs were incubated 4 to 6 hrs with 69 µL 1X reaction buffer plus 1 µL *SmaI* (Gibco-BRL). Following digestion, restriction buffer was removed, and plugs were incubated 15 min in 0.5X TBE buffer at room temperature. Plugs were melted briefly at 80°C and loaded on 1% SeaKem HGT agarose gels (FMC Bioproducts). DNA electrophoresis was performed for 16 hrs with 4- to 16-second switch times at 120 volts and 14°C, with the Bio-Rad CHEF-DR III System (Hercules, CA). Three of the *H. influenzae* isolates did not digest well with *SmaI* and were excluded from the analysis. Twenty plugs from each species were also digested with *ApaI.* Bacterial isolates whose restriction patterns appeared identical or nonidentical with

SmaI digestion also appeared to be identical or nonidentical, respectively, with *ApaI* digestion.

Gels were incubated 1 to 4 hrs with 20 µg/µL ethidium bromide, visualized under ultraviolet light, and photographed. The banding patterns were identified as either identical or different by the criteria of Tenover et al. (14). Identical isolates differed by no more than one band; closely related isolates by two or three bands; possibly related isolates by four to six bands; and different isolates by seven or more bands. All isolates of the same species collected from the same child were run on the same gel, so genetic fingerprints could be compared for all 3 weeks. For example, all *H. influenzae* isolates from child no. 32 were run on a single gel (Figure 1), while all *S. pneumoniae* isolates from child no. 37 were run on a single gel (Figure 2).

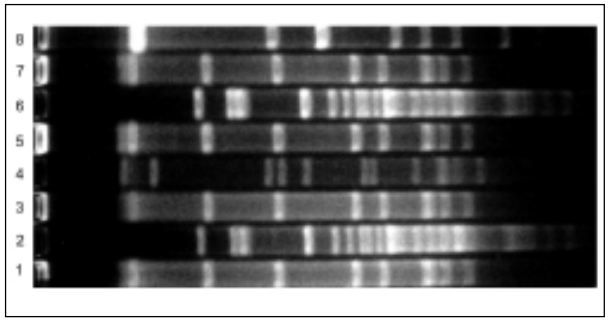

Figure 1. Four distinct *Haemophilus influenzae* isolates from one child during a sampling period. Lanes 1-8: *H. influenzae* isolates collected from child no. 32 at the third sampling period.

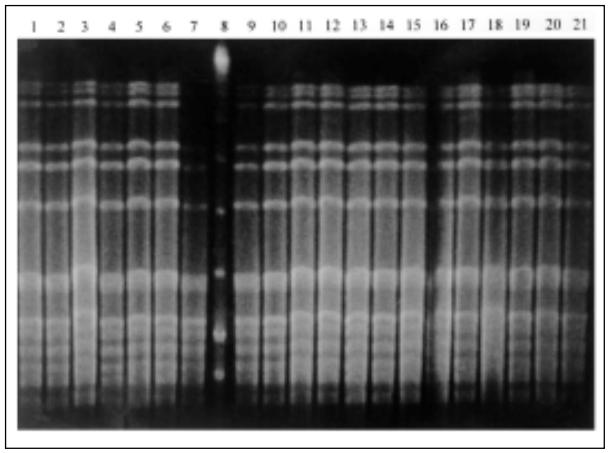

Figure 2. Example of *Streptococcus pneumoniae* isolates collected from child no. 37 during four sampling periods. Lane 1: Isolate collected at week 1; lanes 2-3: isolates collected at week 2; lanes 4-7, 9-13: isolates collected at week 3; lane 8: low molecular weight lambda ladder; lanes 14-21: isolates collected at week 4.

Analysis of Bacterial Sharing

Single isolates representing all observed pulsed-field gel electrophoresis (PFGE) patterns on the gels from individual children were run on a second gel to determine if particular patterns were shared among children in the day-care centers. Since not all unique *H. influenzae* patterns could be run on a single gel, individual *H. influenzae* gels were scanned into Adobe Photoshop (15) to permit comparison across separate gels. Any isolates that appeared identical in Adobe Photoshop were run together on a third gel for confirmation.

All statistics were performed with SAS (16). Fisher's exact test (two-tailed) was used to determine significant differences in the number of genetic types in day-care center 1 compared with day-care center 2. Univariate generalized estimating equation (GEE) logistic regression models were created to determine the risk factors for otitis media associated with carriage of multiple genetic types of *H. influenzae* or *S. pneumoniae*. The GEE SAS macro was used to control for correlation between multiple throat samples from the same child. Variables strongly associated with outcomes in univariate models were included in multivariate GEE models.

Results

Colonization with *S. pneumoniae* and *H. influenzae*

We obtained 126 throat cultures from 38 children at two day-care centers. One hundred two (81%) of the cultures were positive for at least one *H. influenzae* isolate and 60 (48%) for at least one *S. pneumoniae* isolate. We obtained 418 *H. influenzae* isolates (average of 4.1/positive culture) and 184 *S. pneumoniae* isolates (average of 3.0/positive culture) from the 126 throat cultures.

PFGE analysis identified 87 patterns among the *H. influenzae* isolates and 29 patterns among the *S. pneumoniae* isolates. Two of the *H. influenzae* isolates appeared to be closely related, as their PFGE patterns differed by only two bands. Four other pairs of *H. influenzae* isolates were possibly related, as their banding patterns differed by four to six bands. All other *H. influenzae* isolates differed by seven or more bands. All nonidentical *S. pneumoniae* isolates differed by seven or more bands.

Fifty-six distinct *H. influenzae* patterns were observed in day-care center 1. Eight (14%) of

those patterns were observed in more than one child during the study period. Thirty-one distinct *H. influenzae* patterns were observed in day-care center 2. Four (13%) patterns were observed in more than one child (Table 1). Thirteen distinct *S. pneumoniae* genetic patterns were observed in day-care center 1. Four (31%) patterns were observed in more than one child during the 3-week study period. Sixteen genetic patterns were observed in day-care center 2. Three (19%) of those patterns were observed in more than one child (Table 2).

Sharing of specific isolates varied from week to week and room to room within the day-care

Table 1. Genetic patterns of *Haemophilus influenzae* in children attending two day-care centers, Michigan

Child ID No.	Week 1	Week 2	Week 3	Week 4	No. of patterns[a]
			Day-care center 1		
20	3	—	0	—	1
21	6	—	Didn't cut[b]	9,10,11	4
22	1,2,12	1,2,13,14	15,16	8	8
23	17,18	4,19	18	4,18,19 20,21	6
24	—	5	5	5,22	2
25	0	0	—	0	0
26	6,23	4,7	4,24, 25,26	4,26, 27	8
27	—	7,28,29	28	28	3
28	1,30	—	31	1	3
29	0	0	32	0	1
30	—	2	—	—	1
31	39	0	40,41	5,42, 43,44	7
32	7,45	3,6,45	6,7,46, 47	48	7
33	1	—	—	—	1
34	0	7	7,33	Didn't cut[b]	2
35	49,50	49,51	51,52, 53	53	5
37	54,55	54,55	54,56	54	3
38	7,34,35	0	8	0	4
39	36	0	0	37,38	3
40	7	4,6	6	6	3
			Day-care center 2		
51	3	3	6	6	2
53	11	11	11	11,12	2
54	0	0	31	0	1
55	—	2,3	0	2,3	2
56	Didn't cut[b]	13,14	—	—	2
57	7,8	7	0	0	2
58	2,15	13	—	—	3
59	16,17	—	16,18	16	3
60	—	—	9	9,10	2
61	5,19	2,5,19, 20	2,5	2,5	4
62	2	2,27	2,27	2	2
63	—	1,28	28,29	28,29	3
64	—	1,21	0	21	2
65	4,30	4,30	30	4,30	2
66	0	0	2	0	1
67	22	23	23,24	23,24	3
68	25	—	—	25,26	2
69	4	—	1,4	0	2

[a]0 indicates that no *H. influenzae* were isolated. — indicates that no culture was collected from the child at that time.
[b]Isolate did not digest with *SmaI*.

Table 2. Genetic types of *Streptococcus pneumoniae* in children attending day-care centers 1 and 2, Michigan

Child ID no.	Week 1[a]	Week 2	Week 3	Week 4	No of patterns
			Day care 1		
20	0	—	1,2	—	2
21	0	—	0	3	1
22	2	2	0	0	1
23	0	4	0	0	1
24	—	0	0	0	0
25	0	0	—	0	0
26	0	5	5	0	1
27	—	0	5	5	1
28	0	—	0	2	1
29	0	6	0	6	1
30	—	6	—	—	1
31	7	0	0	7,8	2
32	0	8	9	0	2
33	0	—	—	—	0
34	0	8	8	0	1
35	0	10	0	0	1
37	11	11	11	11	1
38	0	0	0	0	0
39	0	0	0	12	1
40	13	0	0	0	1
			Day care 2		
51	0	0	0	0	0
53	0	14	0	14	1
54	0	0	0	0	0
55	—	1	1	1	1
56	2	3	—	—	2
57	4,5	0	0	5	2
58	0	0	—	—	0
59	6	—	6	6	1
60	—	—	5	5	1
61	7	7	7	7	1
62	0	0	8	8	1
63	—	0	9,10	9,10	2
64	—	11	11	0	1
65	11	11	11	12,15	3
66	12	12	12	12	1
67	0	0	0	0	0
68	0	—	—	0	0
69	0	—	13	16	2

[a]0 indicates that no *S. pneumoniae* were isolated. — indicates that no culture was collected from the child at that time.

centers. Most shared isolates (68%) appeared in no more than two children during the study period. For example, in day-care center 1, the *H. influenzae* fingerprint pattern #7 appeared in children from five classrooms on three sampling occasions (Table 1).

Genetic Diversity of Isolates Carried by Individual Children

Fifty-four (43%) throat cultures contained two or more genetically distinct *H. influenzae* isolates, and children in day-care center 1 carried more genetically distinct isolates at a single time than children in day-care center 2 ($p = 0.028$). The children carried an average of 1.4 genetically distinct *H. influenzae* isolates per throat culture (range 0-5; Table 1) and 2.9 (range 0-8) genetically distinct isolates during the study period.

Six (5%) throat cultures contained two genetically distinct *S. pneumoniae* isolates, and none contained more than two (Table 2). On average, children carried fewer genetically distinct *S. pneumoniae* isolates per culture than the average number of *H. influenzae* isolates per culture (0.5 *S. pneumoniae* isolates vs. 1.4 *H. influenzae* isolates per culture); this difference was statistically significant ($p < 0.001$, t test). One child carried three genetically distinct *S. pneumoniae* isolates during the study period; no other child carried more than two isolates during the study period. Five of the children who carried more than one genetically distinct *S. pneumoniae* isolate simultaneously carried more than one genetically distinct *H. influenzae* isolate. Overall, 31 children (82%) carried multiple isolates at some point during the study.

Association with Risk Factors for Otitis Media

Children who were 36 to 47 months old, had allergies, or were exposed to smoking were more than twice as likely to carry two or more genetically distinct *H. influenzae* isolates at a time (Table 3). Children who had a history of frequent otitis media episodes were half as likely to carry two or more genetically distinct *H. influenzae* isolates. Children who used a pacifier were one-fifth as likely to carry two or more genetically distinct *H. influenzae* isolates as children who did not use a pacifier (Table 3). No

Table 3. Univariate logistic regression odds ratios and 95% confidence intervals[a] predicting carriage of two or more genetically distinct *Haemophilus influenzae* or *Streptococcus pneumoniae* isolates during a sampling period.

Risk Factor	Carriage of two or more *H. influenzae* genetic types (n=55) OR (95% CI)	Carriage of two or more *S. pneumoniae* genetic types (n=6) OR (95% CI)
Male	1.11 (0.49-2.53)	0.63 (0.07-5.68)
Race (nonwhite)	0.93 (0.39-2.19)	0.92 (0.16-5.47)
Age (≤23 months, referent)		
24-35 months	0.41 (0.11-1.55)	—
36-47 months	2.49 (0.92-6.74)	—
≥48 months	1.43 (0.43-4.78)	—
Allergies	2.26 (0.87-5.87)	1.37 (0.20-9.56)
Exposure to smoking	3.73 (1.42-9.82)	—
Pacifier use	0.23 (0.07-0.79)	3.01 (0.29-31.55)
Otitis prone (>3 infections/year)	0.39 (0.15-1.02)	1.64 (0.22-12.44)
Presence of tubes	0.96 (0.26-3.49)	1.14 (0.11-11.80)
Having siblings	1.42 (0.60-3.39)	0.96 (0.10-9.38)
High parental education level (>4 years of college)	1.42 (0.60-3.39)	1.21 (0.16-9.24)
Parental income (<$10,000-$40,000 baseline)		
$41,000-$80,000/year	1.50 (0.39-5.72)	1.51 (0.14-16.00)
$81,000->$100,000/year	1.01 (0.26-3.89)	0.43 (0.03-6.07)
Day care site 1	1.06 (0.46-2.43)	2.26 (0.36-14.01)
Antibiotic use	0.25 (0.32-1.57)	
Respiratory illness	0.71 (0.32-1.57)	0.72 (0.11-4.56)
Otitis media episode	0.46 (0.11-1.84)	—

[a]Logistic regressions performed with the generalized estimating equations (GEE) SAS macro to control for correlation between multiple samples taken from the same child.
OR = odds ratio; CI = 95% confidence intervals.

exposure variables were associated with carriage of multiple distinct *S. pneumoniae* isolates.

In a multivariate GEE logistic regression model, exposure to smoking, having allergies, and being 36 to 47 months old were significantly associated with at least a twofold increase in carriage of two or more genetically distinct *H. influenzae* at a time (Table 4). Using a pacifier and having a history of frequent otitis media episodes were highly correlated with antibiotic use; therefore, only antibiotic use was included in the multivariate model. Even after the data were adjusted, children who had taken an antibiotic during the study were almost four times less likely to carry two or more genetically distinct *H. influenzae* isolates than children who had not taken an antibiotic (Table 4).

Table 4. Multivariate logistic regression odds ratios and 95% confidence intervals[a] predicting carriage of two or more genetically distinct *Haemophilus influenzae* isolates during a sampling period

Risk Factor	Carriage of two or more *H. influenzae* genetic types (N=54) OR (95% CI)
Exposed to smoking	3.89 (1.83-8.28)
Allergies	2.48 (1.03-5.95)
Age	
24-35 months	0.60[b] (0.28-1.27)
36-47 months	3.43[b] (1.35-8.71)
48+ months	1.28[b] (0.49-3.32)
Antibiotic use	0.26 (0.07-1.01)

[a]Logistic regression performed with the generalized estimating equations (GEE) SAS macro to control for correlation between multiple samples taken from the same children. All risk factors were included in a single model.

[b]Odds ratios compared with ≤23-month age group.

Conclusions

Multiple *H. influenzae* isolates are frequently present in the upper respiratory tracts of adults with cystic fibrosis (17) and chronic obstructive pulmonary disease (18). Multiple genetic types were identified in 44% of the throat cultures in our study, a rate similar to that observed in cultures obtained from three Australian Aboriginal children (7), but considerably higher than that recorded in previous studies (8,19,20).

The high genetic diversity we observed in individual children may be associated with use of throat culture specimens, which may contain a more diverse population of *H. influenzae* than nasopharyngeal cultures (8). Alternatively, these findings could be associated with our bacterial culture and genetic typing techniques. We used selective media for the initial bacterial isolation, chose 10 colonies from the plates for further examination, and used PFGE to type all isolates. Use of PFGE, the most sensitive method available for genetically typing bacteria, may have resulted in detection of more genetic differences than other commonly used typing methods, such as multilocus enzyme electrophoresis (8).

To address the possibility that colonization with multiple genetic types was due to colonization with a single isolate that was genetically evolving during the study period, we defined strain identity according to the criteria of Tenover et al. (14). Few nonidentical PFGE patterns in our study appeared related; most had seven or more restriction fragment differences, which implies at least three genetic differences. Although the evolutionary dynamics that have resulted in a fairly high diversity among several surface exposed proteins of *H. influenzae* (21) have not been well defined, the large differences we observed in the PFGE patterns are unlikely to have occurred by evolutionary changes over the 1-week sampling intervals.

The association between risk factors for otitis media and colonization with multiple genetic types of bacteria had not been examined before this study. We found that children who were exposed to smoking were more likely to carry multiple *H. influenzae* isolates than children who were not. Smoking has been described as a risk factor for otitis media (9,11,12,22,23), but the exact mechanism by which it contributes to otitis media had not been described. Mucociliary clearance is damaged in adult smokers (24), and *H. influenzae* is more likely to adhere to pharyngeal cells of middle-aged smokers than nonsmokers with bronchitis (25). Etzel et al. (22) suggested that secondhand smoke may also damage the upper respiratory environment in young children, leading to increased bacterial colonization. Increased carriage of diverse *H. influenzae* types may partially account for the increased episodes of otitis media observed in children exposed to smoking. However, the association of smoking with both increased carriage of *H. influenzae* and episodes of otitis media does not establish a causal relationship.

Children with allergies were more likely to carry multiple *H. influenzae* isolates than

children without allergies. Allergies in young children have also been described as a risk factor for otitis media (12,26-28) because upper respiratory mucosal swelling during an allergic episode may cause eustachian tube dysfunction, similar to that observed during an upper respiratory viral infection (26,28). Alternatively, allergic responses may result in impaired mucociliary activity (29,30), which may permit increased bacterial colonization of the upper respiratory tract. As with smoking, while allergies are associated with both increased carriage of *H. influenzae* and increased episodes of otitis media, increased carriage of *H. influenzae* does not necessarily increase episodes of otitis media.

Children 36 to 47 months of age were more likely to carry multiple types of *H. influenzae* than children ≥23 months of age. The reason for this association is unclear. Children in the 36- to 47-month age group were not significantly more likely to have specific otitis media risk factors than younger children. However, 36- to 47-month-old children may have more contacts than younger children, which could contribute to increased exposure to multiple genetic types of *H. influenzae*.

Children who were given an antibiotic during the study period were less likely to carry multiple types of *H. influenzae* than children who were not. As antibiotic use reduces or eliminates bacterial carriage, this factor may explain why children who took an antibiotic were less likely to carry multiple *H. influenzae* isolates.

No associations were found between risk factors for otitis media and colonization with multiple genetic types of *S. pneumoniae*. Only six of the throat culture specimens contained multiple *S. pneumoniae* isolates, and such a small sample size would probably not be sufficient to detect any associations; five of the six cultures also contained multiple genetic types of *H. influenzae*. Therefore, some children appear more likely to carry multiple types of both bacterial species.

Many parents were reluctant to allow their children to participate in our study (49% participation overall); thus, our study population may not accurately reflect the entire day-care population. Parents whose children had a history of frequent otitis media may have been more willing to participate. These children might also have been more likely to be exposed to smoking or have allergies and may have also carried more types of *H. influenzae*. However, we believe this bias is unlikely, as we found no association between carriage of multiple *H. influenzae* and history of frequent ear infections. Alternatively, parents who had other otitis media-prone children may have been more concerned about otitis media in general and more likely to allow their other children to participate. The participating children might therefore have been exposed to the same risk factors as their otitis-prone siblings and have been more likely to carry multiple *H. influenzae* isolates. This is also unlikely, as children with siblings were no more likely to carry multiple isolates than children without siblings. Therefore, the associations between specific risk factors for otitis media and colonization with multiple bacterial strains observed may be a real phenomenon in day-care populations.

We may also have been limited in our ability to detect colonizing *S. pneumoniae*. Pharyngeal swabs are less effective for isolation of *S. pneumoniae* than nasopharyngeal swabs (31,32); we may have missed approximately 13% of colonizing *S. pneumoniae* isolates. In addition, all isolates that were typed by optochin disc sensitivity had zones of inhibition at least 17 mm. (A zone of inhibition at least 14 mm is necessary for presumptive identification of *S. pneumoniae*.) We did not type (by PFGE) any potential isolates with a smaller zone of inhibition; therefore, we may have missed some isolates that would have yielded a questionable inhibition zone. These limitations in our sampling and culture techniques could have resulted in an underestimate of the genetic diversity of the *S. pneumoniae* isolates carried by this group of children.

In summary, this study describes high genetic diversity among *S. pneumoniae* and *H. influenzae* isolates colonizing individual children attending two day-care centers. Carriage of multiple genetic types of *H. influenzae* was associated with child's age, antibiotic use, exposure to smoking, and history of allergies. Carriage of multiple genetic types of *H. influenzae* may be an intermediate step in the biologic pathway leading to otitis media and may explain the increased episodes of otitis media observed in children with allergies or children exposed to smoking. Future studies will determine 1) if the high rate of carriage of multiple *H. influenzae* is specific to children attending day-care centers or if all children commonly carry multiple *H. influenzae* pathogens in their upper respiratory

tracts and 2) if children carrying multiple pathogens are also at increased risk for otitis media.

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Molecular Evidence of Clonal *Vibrio parahaemolyticus* Pandemic Strains

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The upsurge in worldwide incidence of *Vibrio parahaemolyticus* infection in the last 5 years has been attributed to the recent appearance of three serotypes with pandemic potential: O3:K6, O4:K68, and O1:K untypeable (KUT). Thirty-five strains of these serotypes, isolated from different countries over 4 years, were characterized by ribotyping and pulsed-field gel electrophoresis to determine their origin. The ribotypes of the strains of these serotypes were indistinguishable, except for a Japanese *tdh*-negative O3:K6 strain and a U.S. clinical O3:K6 isolate, which had slightly different profiles. The migration patterns of the *Not*I-digest of the total DNA of the strains were similar, and only slight variations were observed between the serotypes. By contrast, the O3:K6 and O1:KUT strains isolated before 1995 and strains of other serotypes had markedly different profiles. The O4:K68 and O1:KUT strains most likely originated from the pandemic O3:K6 clone.

Infections caused by *Vibrio parahaemolyticus*, a halophilic member of the genus Vibrio, have increased globally in the last 5 years. *V. parahaemolyticus* diarrhea results from eating raw or undercooked seafoods, although other routes of transmission have been documented (1). Studies implicate the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH), encoded by the *tdh* and *trh* gene, respectively, as the major virulence factors of this organism (2-5). Therefore, the presence of the *tdh* gene marked by a ß-type hemolysis on Wagatsuma agar (2,3), the *trh* gene correlated to a positive urease test (6), or both serve as markers for pathogenic strains. Recently, three major serotypes—O3:K6, O4:K68, and O1:K untypeable (KUT), listed in chronological order of appearance—have caused a pandemic of *V. parahaemolyticus* infection (7-10). Strains of these serotypes have been responsible for gastroenteritis in India, other Southeast Asian countries, and the United States (9-12). In Calcutta, strains of the O3:K6 serotype have been responsible for the high incidence of *V. parahaemolyticus*–mediated gastroenteritis since February 1996 (7). Likewise, this serotype was isolated in other Asian countries (including Laos, Taiwan, and Japan) and the United States.

This alarming rise of a serotype previously associated with only sporadic cases of gastroenteritis was monitored closely. The O3:K6 strains isolated before 1995 (henceforth referred to as old O3:K6) and those isolated since 1995 (referred to as new O3:K6) were analyzed for variation in nucleotide sequence of the *toxRS* region and differed invariably in seven bases within the 1,364-bp *toxRS* region (10). Two of the seven unique bases were exploited to develop the group-specific polymerase chain reaction (GS-PCR) that distinguished between the new and the old O3:K6 isolates. Examination of the non-O3:K6 isolates by GS-PCR showed that the *toxRS* sequences of the recent isolates of O4:K68 and O1:KUT serotypes were identical to those of the new

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O3:K6 isolates (10). All the strains of the new clone (regardless of serotype or place of isolation) carried the *tdh* gene but not the *trh* gene. These strains also exhibited a unique arbitrarily primed PCR profile distinct from the strains of other serotypes, supporting the hypothesis that the O4:K68 and O1:KUT strains evolved from the newly emerged O3:K6 clone. Using ribotyping and pulsed-field gel electrophoresis (PFGE), we examined whether a single clone expressed as three different serotypes of *V. parahaemolyticus* is causing the pandemic.

Materials and Methods

Bacterial Strains

A total of 35 pandemic strains of *V. parahaemolyticus* (21 of O3:K6, 10 of O4:K68, and 4 of O1:KUT) isolated from 1996 to 1999 from widely separated geographic regions were examined by ribotyping and PFGE (Table 1). Thirteen nonpandemic strains of O3:K6, O1:KUT, and other serotypes isolated before February 1996 were included as external controls (Table 2).

Table 1. Pandemic *Vibrio parahaemolyticus* strains used in this study

Serotype	Strain no.	Year of isolation	Origin
O3:K6	VP47	1996	Calcutta
	KX-V224	1996	Traveler at Kansai Airport from Thailand
	KX-V225	1996	Traveler at Kansai Airport from Thailand
	KX-V226	1996	Traveler at Kansai Airport from Singapore
	KX-V231	1996	Traveler at Kansai Airport from Thailand
	KX-V138	1995	Traveler at Kansai Airport from Indonesia
	AM-6383	1996	ICDDR, B
	146	1997	Wakayama, Japan
	2	1997	Laos
	AN-13938	1998	ICDDR, B
	OP411	1997	Osaka, Japan
	OP419	1998	Osaka, Japan
	1(1926)	1998	Kyoto, Japan
	FIHES98VI-32-4	1998	Fukuoka, Japan
	VP26	1998	Thailand
	22/3	1998	Thailand (an environmental strain)
	AN-8373	1998	ICDDR, B
	VP81	1996	Calcutta
	DOH958 15	1997	Taiwan
	VP2	1998	Korea
	BE98-2062	1998	United States
O1:KUT	VP185	1997	Calcutta
	AN-16000	1998	ICDDR, B
	VP250	1998	Calcutta
	KX-V737	1999	Traveler at Kansai Airport from Thailand
O4:K68	AN-5034	1998	ICDDR, B
	KX-V483	1997	Traveler at Kansai Airport from Thailand
	KX-V508	1997	Traveler at Kansai Airport from Nepal
	KX-V522	1997	Traveler at Kansai Airport from Singapore
	KX-V532	1997	Traveler at Kansai Airport from Thailand
	KX-V563	1998	Traveler at Kansai Airport from Thailand
	KX-V568	1998	Traveler at Kansai Airport from Vietnam
	VP232	1998	Calcutta
	OP-424	1998	Osaka, Japan
	KX-V740	1999	Traveler at Kansai Airport from Thailand

Table 2. Nonpandemic *Vibrio parahaemolyticus* strains used in this study

No.	Serotype	Strain no.	Year of isolation	Origin
1	O3:K6	AQ3810	1983	Singapore
2	O3:K6	AQ4093	1986	Maldive Islands
3	O3:K6	AQ4133	1986	Hong Kong
4	O4:K63	VP32	1995	India
5	O4:K8	VP221	1997	India
6	O4:K10	VP254	1998	India
7	O1:KUT	91A1437	1991	United States
8	O1:KUT	AQ4764	1992	Thailand
9	O2:K3	VP191	1997	India
10	O4:K13	VP206	1997	India
11	O3:K29	VP263	1998	India
12	O1:K25	TdP10	1998	Thailand
13	O4:K9	TdP16	1998	Thailand

Purification of Genomic DNA

A modification of the method of Murray and Thompson (13) was used to extract chromosomal DNA. Briefly, cells from 18-hour culture in Luria-Bertani (LB) broth, Miller (Difco Laboratories, Detroit, MI) with 3% NaCl, were harvested by centrifugation at 6,000 x g for 5 minutes. The pelleted cells were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), treated with 10% sodium dodecyl sulfate (SDS) and proteinase K and incubated for 1 hour at 37°C. After incubation, CTAB/NaCl (10% cetyl trimethyl ammonium bromide in 0.7 M NaCl) was added and incubated at 65°C for 10 minutes. The aqueous phase was then treated with phenol-chloroform, and the DNA was pelleted and washed with 70% ethanol. Purified DNA was suspended in TE buffer and treated with RNase at 37°C for 30 minutes.

Ribotyping

Restriction enzyme *Bgl*I (Boehringer GmbH, Mannheim, Germany) was used for ribotyping of the *V. parahaemolyticus* strains. The genomic fragments were electrophoresed on a 1% SeaKem agarose gel (FMC Bioproducts, Rockland, ME) using Tris-acetate (TAE) buffer (0.04 M TAE, 0.001 M EDTA [pH 8.0]). For Southern blotting, the gel was treated successively in 0.25 N HCl for 10 minutes to allow partial depurination and cleavage of large fragments, in denaturation solution composed of 0.5 M NaOH, 1.5 M NaCl for 30 minutes and in 0.5 M Tris-HCl (pH 7.4) for 30 minutes. DNA was then transferred to Hybond N^+ membrane (Amersham International PLC, Buckinghamshire, England), using 20X SSC (3 M sodium chloride, 0.3 M sodium citrate by vacuum blotter (Pharmacia, Sweden). The membrane was then washed with 20X SSC and dried at room temperature followed by fixation in a UV cross-linker (Bio-Rad Laboratories, Richmond, CA). A 7.5-kb *Bam*HI fragment of the recombinant plasmid pKK3535 containing an rRNA operon of *Escherichia coli* (14) was used as the *rrn* gene probe for ribotyping. Labeling of the probes, hybridization, and detection of bands were carried out according to the instructions of the manufacturer of the ECL detection system (Amersham Life Science, UK).

DNA Extraction and Digestion for PFGE

The test strains grown on LB agar, Miller (Difco) with 3% NaCl were transferred to 3 mL LB broth, Miller (Difco) with 3% NaCl and cultured overnight at 37°C with shaking at 100 rpm. One hundred microliters of the overnight culture was transferred to 8 mL of LB broth, Miller and incubated at 37°C with shaking at 100 rpm until the culture attained an optical density of 0.9 at 600 nm. Bacterial cells were harvested from 1 mL of the culture by centrifugation and resuspended in 0.5 mL cell lysis buffer (10 mM Tris-HCl [pH7.2], 20 mM NaCl, 50 mM EDTA). Agarose plugs were prepared by mixing equal volumes of bacterial suspension with 2% low-melting agarose. The bacterial cells in the agarose plugs were lysed by treatment with lysis solution (1 mg of lysozyme per mL, 0.4% N-sodium lauryl sarcosine, 0.2% Na-deoxycholate, 10 mM Tris [pH 7.2], 50 mM NaCl) at 37°C overnight and treated with proteinase K at 50°C overnight. The plugs were washed with washing buffer containing 20 mM Tris HCl (pH 8.0) and 50 mM EDTA. Agarose plugs containing genomic DNA were equilibrated in enzyme buffer for 1 hour at room temperature and were cleaved in 600 µL of enzyme buffer H (500 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl) containing 50 U of *Not*I enzyme at 37°C overnight.

PFGE

PFGE of *Not*I-digested inserts was performed on 1% agarose (Bio-Rad) by the contour-clamped homogenous electric field method on a CHEF Mapper system (Bio-Rad) in 0.5X TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA, pH 8.0) for 40 hours, 24 minutes. A DNA size standard (Bacteriophage λ-ladder,

Bio-Rad) was used as the molecular mass standard, and a minichiller (Bio-Rad) was used to maintain the temperature of the buffer at 14°C. Run conditions were generated by the autoalgorithm mode of the CHEF Mapper PFGE system by using a 20- to 300-kb size range. After electrophoresis, the gel was stained in ethidium bromide (1 µg/mL) for 30 minutes and destained in water for 15 minutes twice. The DNA bands were visualized and photographed with the Gel Doc 2000 (Bio-Rad) (8).

Results

The organization of the *rrn* genes in the chromosomes of *V. parahaemolyticus* was examined by Southern hybridization of *Bgl*I-digested total DNA with rRNA-specific DNA probe. The ribotyping patterns observed with the strains of the three different serotypes are shown in Figure 1. The DNA probe specific to *rrn* genes hybridized with 11 fragments, 23.0 kb to 4.0 kb. Strains of the new O3:K6, O4:K68, and O1:KUT serotypes exhibited the R4 ribosomal banding pattern, which was the major pattern exhibited by 76% of the O3:K6 isolates in India from February to August 1996 (8). However, the O3:K6 strain, FIHES98VI-32-4, isolated in Japan, which was devoid of the *tdh* gene and was included in this study because it was positive by GS-PCR (10), differed in the 23.1-kb region. Another O3:K6 strain, BE98-2062, isolated from the United States, differed from the other strains by a single band near the 6.0-kb region. Representative nonpandemic strains—including the old O3:K6, O1:KUT, and other unrelated serotypes—were similarly analyzed for the organization of the *rrn* genes (Figure 1D). The nonpandemic strains showed heterogenous ribotype profiles, each pattern markedly different from the other and from that exhibited by the pandemic strains.

The ribotyping data were substantiated by studying the electrophoretic migration pattern of *Not*I-digested fragments of the chromosomal DNA of each of the 35 pandemic strains of *V. parahaemolyticus* obtained by PFGE. The PFGE data for the O3:K6, O4:K68, and O1:KUT strains are shown in Figure 2. Except for the *tdh*-negative O3:K6 strain from Japan (FIHES98VI-32-4), all the O3:K6 strains show almost identical RFLP patterns. The O3:K6

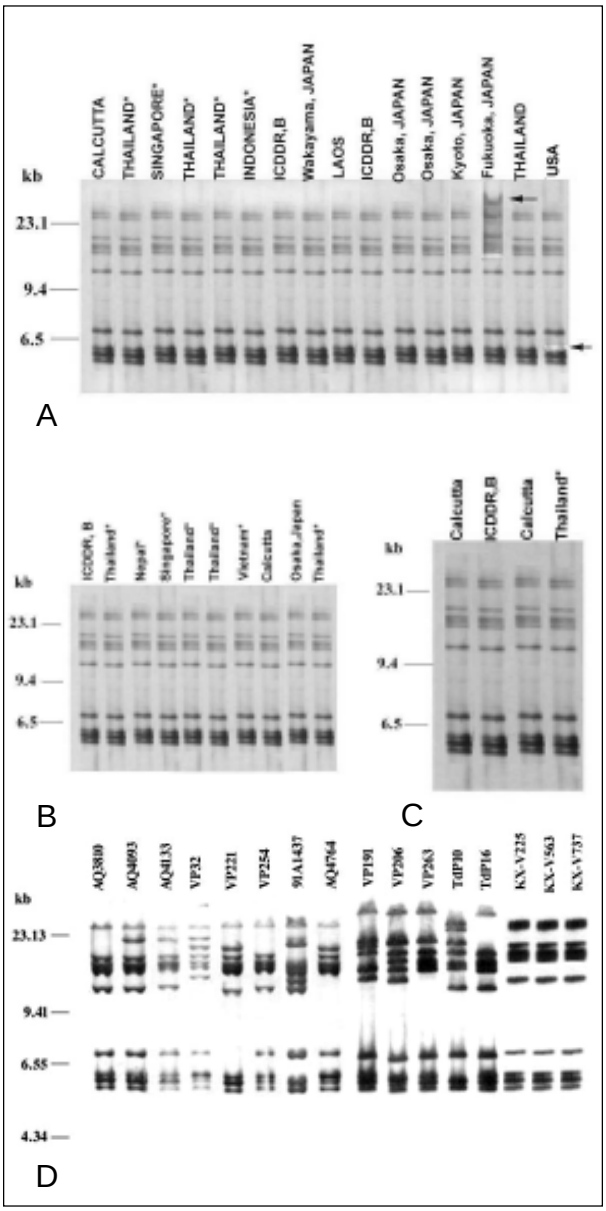

Figure l. Southern blot hybridization of *Bgl*I-digested *Vibrio parahaemolyticus* chromosomal DNA with rRNA probe. 1A-1C, ribotype patterns of the O3:K6, O4:K68, and O1:K untypeable (KUT) strains, respectively, isolated from different countries. 1D, ribotype pattern of the nonpandemic strains isolated from different countries and belonging to various serotypes (Table 2). The last three lanes indicate the pattern of the representative O3:K6(KX-V225), O4:K68(KX-V563), and O1:KUT(KX-V737) strains, respectively. Positions of λ-*Hin*dIII molecular-size markers run on the same gel are indicated on the left margin. *=Strains isolated from travelers to the Kansai Airport from the respective places.

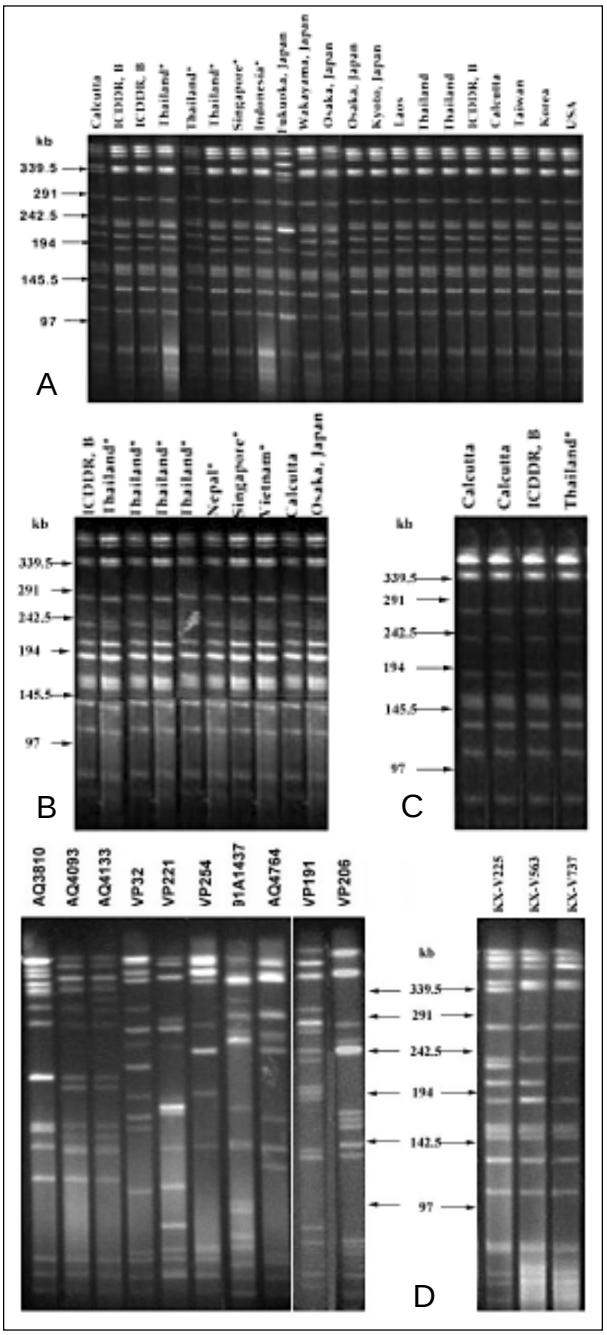

Figure 2. Electrophoretic migration pattern of the *Not*I-digested *Vibrio parahaemolyticus* genomic DNA obtained by pulsed-field gel electrophoresis (PFGE). 2A-2C, PFGE patterns of the O3:K6, O4:K68, and O1:K untypeable (KUT) strains, respectively, isolated from different countries. 2D, PFGE pattern of the nonpandemic strains isolated from different countries and belonging to various serotypes (Table 2). The lanes on right side of marker indicate the pattern of the representative O3:K6 (KX-V225), O4:K68(KX-V563), and O1:KUT(KX-V737) strains, respectively. Positions of molecular-size standards are also indicated. *=Strains isolated from travelers to the Kansai Airport from respective places.

strain, BE98-2062, isolated in the United States, showed minor variation from the other strains near the 58.5-kb region. The PFGE patterns of all O4:K68 strains isolated from different geographic locations were identical and nearly similar to the pattern obtained with the O3:K6 strains (Figure 2B). Strains of this serotype, however, differ from the O3:K6 strains by one band near the 240-kb region and by the higher intensity of a band at the 194-kb region, which could indicate the comigration of more than one band. Similarly, although all four O1:KUT strains were identical to each other, they differed from the O4:K68 serotype by the absence of a single band in the 200-kb region and from the O3:K6 serotype by the absence of bands at the 240-kb and 200-kb regions (Figure 2C).

Since all isolates of the three different serotypes, except FIHES98VI-32-4, varied by one or two bands, all appear to have originated from a common ancestor. The variation may be due to the difference in the genetic organization of the O and K antigen biosynthesis gene clusters in the strains. The banding pattern of the *Not*I-digested DNA fragments, obtained by PFGE of the nonpandemic strains, is shown in Figure 2D. Considerable polymorphism was observed between the pandemic and nonpandemic strains and between the nonpandemic strains of various serotypes.

Conclusion

Until recently, *V. parahaemolyticus* caused sporadic and localized diarrhea and—unlike toxigenic *V. cholerae* O1 and O139—was never associated with a pandemic. However, with the advent of the new O3:K6 strains in 1996, the epidemiology of this organism abruptly changed. The dominant and continued occurrence of this serotype was reported from eight countries. The extent and rapidity of spread of the new O3:K6 strains signaled the beginning of the first pandemic of *V. parahaemolyticus.* We have shown that the recent O3:K6 isolates from eight countries were identical in the RFLP of the rRNA genes and showed similar PFGE profiles. We have also shown that strains of two other serotypes, O4:K68 and O1:KUT, isolated since 1995 possessed ribotype and PFGE patterns similar to those of the new O3:K6 strains. Variations between the three pandemic serotypes are minor when compared to the differences seen with the nonpandemic strains.

Hence, from the molecular analysis and chronology of appearance of these strains, the O4:K68 and O1:KUT isolates appear to have originated from the existing O3:K6 clone. The ribotype and PFGE patterns displayed by the pandemic clone are unique. Therefore, a single clone may be responsible for the emergence of pandemic serotypes that have different somatic and capsular antigens. This study suggests that the epidemiologically related strains may also be genetically related. The abrupt origin of this pandemic clone and, more importantly, the sudden acquisition of pandemic properties by three different serotypes of *V. parahaemolyticus*, almost 5 decades after its discovery, require further scrutiny.

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Mass Die-Off of Caspian Seals Caused by Canine Distemper Virus

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Thousands of Caspian seals (*Phoca caspica*) died in the Caspian Sea from April to August 2000. Lesions characteristic of morbillivirus infection were found in tissue specimens from dead seals. Canine distemper virus infection was identified by serologic examination, reverse transcriptase-polymerase chain reaction, and sequencing of selected P gene fragments. These results implicate canine distemper virus infection as the primary cause of death.

During the spring of 2000, high death rates were reported in Caspian seals (*Phoca caspica*) (1), which live only in the Caspian Sea and are listed as a vulnerable species by the International Union for the Conservation of Nature (2). The die-off was first reported near the mouth of the Ural River, Kazakhstan, in late April; it subsequently spread south to the Mangistau region (Figure 1). More than 10,000 seals are estimated to have died during April and May along the Kazakhstan coast. High death rates were also reported in May and June along the Apsheron peninsula of Azerbaijan and the Turkmenistan coast. We present evidence that canine distemper virus infection was the primary cause of these deaths.

Clinical signs in infected seals included debilitation, muscle spasms, ocular and nasal exudation, and sneezing. In necropsies performed in June on eight seals from Azerbaijan (Table), no consistent gross lesions were found. However, microscopic lesions, including bronchointerstitial pneumonia, encephalitis, pancreatitis, and lymphocytic depletion in lymphoid tissues, were seen in these and four seals found in Kazakhstan in May. Multiple intracytoplasmic

Figure 1. Map of the Caspian Sea region. Seal samples were collected from Kazakhstan, Turkmenistan, and the Apsheron peninsula, Azerbaijan.

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Table 1. Caspian seals tested for canine distemper virus infection

Animal No.	Location	Date	Test			
			RT-PCR	IgM	IgG	IHC
1	KA	May 3	+	+	+	ND
2	KA	May 3	+	+	+	ND
3	KA	May 4	ND	ND	ND	+
4	KA	May 4	ND	+	+	+
5	KA	May 4	ND	+	+	+
6	KA	May 4	ND	+	+	+
7	AZ	May 15	+	ND	ND	ND
8	AZ	June 2	+	-	-	-
9	AZ	June 2	+	+	+	+
10	AZ	June 4	-	-	+	-
11	AZ	June 5	+	-	+	-
12	AZ	June 5	-	-	+	-
13	AZ	June 5	+	+	+	+
14	AZ	June 5	+	+	+	+
15	AZ	June 7	-	-	+	-
16	TU	June 10	+	ND	ND	ND

RT-PCR, reverse transcriptase-polymerase chain reaction; IHC, immunohistochemical labeling; KA, Kazakhstan; AZ, Azerbaijan; TU, Turkmenistan; +, positive; -, negative; ND, not determined because sample lacking.

and rare intranuclear acidophilic inclusions, characteristic of morbillivirus infection (3), were observed in many epithelial tissue specimens (Figure 2A). Paraffin-embedded tissue sections were examined for morbillivirus antigen by an immunohistochemical technique (4). A monoclonal antibody against the nucleoprotein of phocine distemper virus, known to cross-react with canine distemper virus and cetacean morbilliviruses, was used as primary antibody. Morbillivirus antigen was detected in multiple tissues, including lung, lymph nodes (Figure 2B), spleen, brain, pancreas, liver, and epithelial tissue of the reproductive, urinary, and gastrointestinal tracts. These multisystemic tissue lesions are characteristic of distemper in terrestrial and aquatic mammals (3).

Tissues from 12 seal carcasses found on the coasts of Kazakhstan, Azerbaijan, and Turkmenistan (Table) were examined for morbillivirus nucleic acid by reverse-transcriptase polymerase chain reaction (RT-PCR). One set of universal morbillivirus primers, based on conserved sequences in the phosphoprotein (P) gene, and a second set specific for the canine distemper virus fusion (F) gene, were used in this technique (5). Tissues from nine seals were positive with both P and F primers, yielding the expected products of 429 bp and 372 bp, respectively. Selected P gene fragments were sequenced for phylogenetic

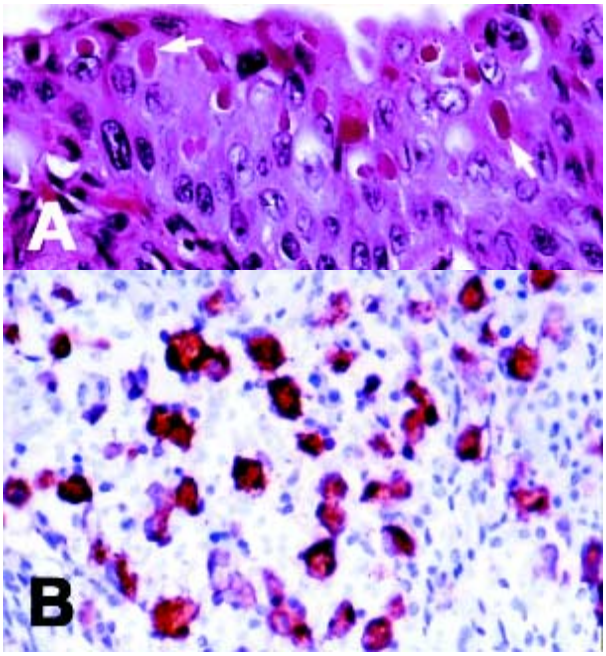

Figure 2. Tissue lesions from a Caspian seal with distemper. (A) Multiple intracytoplasmic, acidophilic viral inclusions in transitional epithelium of urinary bladder (arrows). Hematoxylin and eosin. (B) Immunohistochemical labeling of morbilliviral antigen in lymphoid cells in a lymph node. Avidin-biotin-peroxidase technique with hematoxylin counterstain.

comparison (Figure 3). The resulting sequences matched those of canine distemper virus and were clearly distinct from those of other members of the genus *Morbillivirus*, including phocine distemper virus. Except for one nucleotide change in the P gene fragment from seal 14, the sequences from Kazakhstan and Azerbaijan were identical, indicating that seals from widely separated regions of the Caspian Sea were infected by the same virus. This finding establishes spatial and temporal links between the seal deaths in these regions.

These sequences were identical to that of canine distemper virus detected in 1997 in brain tissue from a single Caspian seal in which no evidence of morbillivirus lesions was found (6). These results suggest either persistence of canine distemper virus in the Caspian seal population over a period of several years or repeated spillover from the same terrestrial reservoir.

Serum specimens from 13 seals (Table) were tested for canine distemper virus-specific immunoglobulin (Ig)M and IgG antibodies by an antibody-capture enzyme-linked immunosorbent assay (ELISA) and an indirect ELISA,

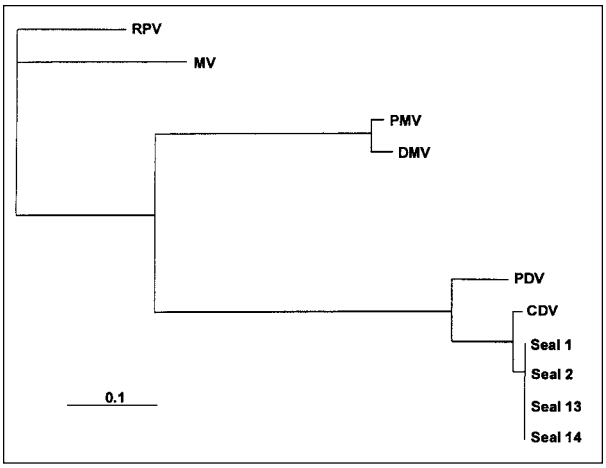

Figure 3. Phylogenetic analysis of P gene fragments from Caspian seals and representative members of the genus *Morbillivirus*. Sequences of samples from Caspian seals were generated in this study. Other sequences were obtained from GenBank. RPV, rinderpest virus strain RBOK (#X68311); MV, measles virus vaccine strain Edmonston (#M89920); DMV, dolphin morbillivirus (#Z47758); PMV, porpoise morbillivirus strain 53; phocine distemper virus, PDV (#X75960); and canine distemper virus, CDV strain Bussell (#Z54156). Seal numbers refer to P gene fragments from Caspian seals with identification numbers corresponding to those in the Table. A maximum likelihood tree was generated on P gene nucleotide sequences by means of the DNAML program of the Phylip 3.75 software package with 100 bootstraps and three jumbles.

respectively (7). Eight of these seals had serum IgM antibodies, and 12 had IgG antibody titers ranging from 40 to 640. These serologic data confirm recent and geographically widespread canine distemper virus infection in the Caspian seal population.

In recent years, several morbillivirus epizootics have occurred in pinniped and cetacean populations in the Northern Hemisphere (3). Canine distemper virus infection, the primary cause of high death rates in Baikal seals (*Phoca siberica*) in 1987-88 (8), was associated with a die-off in crab-eating seals (*Lobodon carcinophagus*) in Antarctica in 1955 (9). In both these pinniped populations, viral infection was thought to have been transmitted through contact with domestic dogs. The origin of the canine distemper virus that infected the Caspian seals is unknown, but there are anecdotal reports of contact between seals and terrestrial carnivores in this region (6). Further studies are required to determine if the latter species are infected with a canine distemper virus genetically similar to that found in the seals. The epidemiology of canine distemper virus infection, including its effects on the Caspian seal population, also remains to be investigated.

High levels of chemical contaminants have been recently identified in tissues of Caspian seals (10). As some of these substances have been shown to have immunotoxic effects in seals at the reported concentrations (11), further work is under way to determine whether pollutants contributed to these deaths.

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Nontoxigenic *Corynebacterium diphtheriae*: An Emerging Pathogen in England and Wales?

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Confirmed isolates of nontoxigenic *Corynebacterium diphtheriae* in England and Wales increased substantially from 1986 to 1994. Ribotyping of 121 isolates confirmed in 1995 showed that 90 were of a single strain isolated exclusively from the throat; none had previously been identified in toxigenic strains from U.K. or non-U.K. residents. The upward trend in nontoxigenic *C. diphtheriae* probably represented increased ascertainment, although dissemination of a particular strain or clone may have been a factor.

Clusters of cases of sore throat associated with isolation of nontoxigenic *Corynbacterium diphtheriae* were detected in gay men attending a genitourinary medicine clinic, military recruits, and children from a religious community in England and Wales in the late 1980s to mid-1990s (1-4). To determine the public health importance of the increase in cases, the Public Health Laboratory Service's (PHLS's) Streptococcus and Diphtheria Reference Unit and the PHLS Communicable Disease Surveillance Centre obtained more complete clinical information on nontoxigenic isolates referred in 1995 and 1996. Isolates received in 1995 were further characterized by molecular typing (ribotyping).

The Study

Laboratories in England and Wales routinely submit isolates of *C. diphtheriae* to the PHLS Streptococcus and Diphtheria Reference Unit for confirmation and toxin determination by both phenotypic (Elek and other immunoassays) and genotypic (polymerase chain reaction) methods (5,6). Routine screening of throat swabs with selective culture media for *C. diphtheriae* was encouraged in public health laboratories in England and Wales (7). Clinical and epidemiologic information was obtained from questionnaires sent to referring laboratories and from laboratory request forms. Responsibility for completion and return of the enhanced surveillance questionnaires was taken by laboratory staff, in consultation with senior medical microbiologists and attending physicians with access to laboratory and medical records. The questionnaires, which included history of recent travel, symptoms and signs of illness, general medical history, clinical management (particularly antibiotic treatment, contact tracing, and treatment of contacts) and bacteriologic and virologic investigations, were sent retrospectively for isolates received by the PHLS diphtheria unit from January to June 1995 and prospectively through 1996 (8). Isolates confirmed as nontoxigenic *C. diphtheriae* during 1995 were ribotyped. The isolates were referred from laboratories in England, Wales, Scotland, the Channel Islands, and the Isle of Man. Analysis of ribotype patterns was done by using Taxotron (Institut Pasteur, France) software, as previously described (9).

In 1995 and 1996, PHLS confirmed 265 isolates from residents of England and Wales as nontoxigenic *C. diphtheriae* (Figure 1). The isolates were submitted by 80 laboratories located throughout each country; 28 (35%) were public health laboratories. Each laboratory submitted 1 to 27 isolates. Questionnaires were

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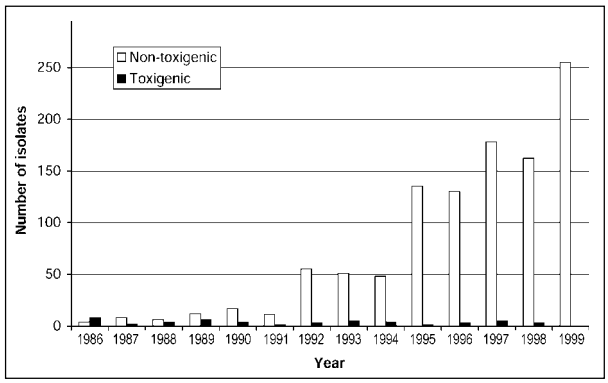

Figure 1. Annual number of isolates of *Corynebacterium diphtheriae* confirmed by the Public Health Laboratory Service's Streptococcus and Diphtheria Reference Unit, from residents of England and Wales, 1986-1999.

returned for 236 (89%) isolates. No pharyngeal membrane or systemic toxicity was reported. The age range of patients whose isolates were tested was 1 to 87 (median 20) years.

Two hundred forty-seven (93%) isolates were from the throat, ten (4%) from skin lesions, one from blood, one from a nose swab, and one from bronchial washings; five were from unrecorded sites. Biotype var g*ravis* accounted for 223 (84%), var *mitis* for 33(12%), and var *belfanti* for 9 (3%) isolates.

Of the 247 throat isolates, 238 were obtained as a result of clinical evaluation of patients with sore throats, and 9 were obtained from contacts of these patients. Of the 238 isolates obtained during evaluations, more than 25% were from male attendees at general outpatient or genitourinary medicine outpatient clinics (Table 1). Of isolates from females patients, 7% were from general outpatient clinics, and none were from genitourinary medicine outpatient clinics. Most isolates were from 15- to 24-year-old patients (Table 2). Of 238 throat isolates, 29 (12%) were from patients who had traveled outside the United Kingdom in the preceding 3 months, 20 to Western Europe, 4 to Australasia, 2 to Africa, 2 to the Indian subcontinent, and 1 to the Caribbean. Fever, lymphadenopathy, or both were reported in association with 72 (30%) of throat isolates. Nontoxigenic *C. diphtheriae* was reported as the predominant organism in 171 (72%) of the 238 throat swabs (Table 3) but was mixed with beta-hemolytic streptococci in 67 (28%). Penicillin was prescribed for 100 patients and a macrolide for 66, for a total of 166 (70%) patients treated according to current U.K. guidelines (10). Other antibiotics were prescribed for 7 patients and none for the rest. Viral throat cultures, reported for 10 (4%) of the 238 patients, were negative.

Table 1. English and Welsh patients with sore throats whose throat swabs yielded nontoxigenic *Corynebacterium diphtheriae,* by sex, 1995 and 1996

	Male patients		Female patients		Not recorded		Total	
Clinical setting	No.	%	No.	%	No.	%	No.	%
General practice	50	52	98	74	3	38	151	63
Outpatients	15	15	9	7	1	13	25	11
GUM clinic[a]	11	11	0	0	1	13	12	5
Inpatients	8	8	6	5	0	0	14	6
Other	2	2	5	4	0	0	7	3
Not recorded	11	11	15	11	3	38	29	12
All settings	97	100	133	100	8	100	238	100

[a]GUM = genitourinary medicine.

Table 2. English and Welsh patients with sore throats whose throat swabs yielded nontoxigenic *Corynebacterium diphtheriae*, by age, 1995 and 1996

	Age									
	<15		15-24		25-34		35+		All ages	
Clinical Setting	No.	%	No.	%	No.	%	No.	%	No.	%
General practice	20	13	103	68	24	16	4	3	151	100
Outpatients	1	4	15	58	7	31	2	8	25	100
GUM clinic[a]	0	0	6	50	4	33	2	17	12	100
Inpatients	4	29	8	57	2	14	0	0	14	100
Other	0	0	2	29	4	57	1	14	7	100
Not recorded	5	17	14	47	8	30	2	7	29	100
All settings	30	13	148	62	49	21	11	5	238	100

[a]GUM = genitourinary medicine.

Table 3. Pathogens in mixed growth with nontoxigenic *Corynebacterium diphtheriae* in throat swabs from English and Welsh patients with sore throats, 1995 and 1996

Pathogen	No.	%
Lancefield Group A streptococci	26	11
Lancefield Group C streptococci	30	13
Lancefield Group G streptococci	11	5
None	171	72
Total	238	100

Positive serologic results for infectious mononucleosis were reported in 10 (4%) patients. Seven isolates were associated with HIV infection, eight with psoriasis, one with gonorrhea, two with malaria, and one with cytomegalovirus infection and Crohn disease. Four of five laboratories referring 10 or more isolates reported that they had screened all throat swabs with selective media for *C. diphtheriae* during 1995 and 1996. These laboratories were located at two teaching hospitals in central London (25 and 19 isolates) and two public health laboratories, one in the northwest of England (13 isolates) and the other in Wales (10 isolates). These 67 isolates were obtained from screening 32,345 throat swabs during the survey period. This rate corresponds to an overall rate of two isolates per thousand throat swabs (1.2 to 2.9 isolates per thousand throat swabs for each individual laboratory). Of the 10 skin isolates, 2 were var *gravis*, 7 var *mitis*, and 1 var *belfanti*. Nine were associated with travel outside the United Kingdom in the previous 3 months: to Africa (three patients), the Indian subcontinent (one patient), the Caribbean (two patients), and Southeast Asia (three patients). The positive blood culture was biotype var *mitis*, obtained from a 2-year-old with congenital heart disease whose illness was diagnosed as endocarditis 3 weeks after returning from Pakistan. The isolate from the nose was var *belfanti* mixed with *Klebsiella aerogenes*, taken from a 23-year-old man of Pakistani origin, who had a 3-month history of rhinitis but had not traveled in the preceding 3 months. The isolate from bronchial washings was var *belfanti*, associated with a malignant lung tumor in a 68-year-old man.

Taxotron analysis was undertaken in 121 (90%) of 135 specimens obtained for isolation from referrals from laboratories in all eight health regions in England and Wales during 1995; 115 of these had been obtained through clinical evaluation and 6 through contact tracing (Table 4). Travel outside the United Kingdom in the preceding 3 months was reported in association with isolates from 17 patients (Tables 4 and 5). Eight additional isolates that had been submitted by laboratories in Scotland, the Channel Islands, and the Isle of Man were ribotyped (Table 4). Twenty-three distinct patterns were detected and were designated A to W (Figure 2 and Tables 4 and 5). Ribotypes A, B, C, and D were biotype *gravis*; ribotypes E, F, G,

Table 4. Throat isolates of nontoxigenic *Corynebacterium diphtheriae* submitted to the Public Health Laboratory Service's Streptococcus and Diphtheria Reference Unit, from U.K. residents,[a] 1995

Site of isolate	Travel outside U.K. within previous 3 months	No. ribotyped	Ribotypes
Isolates from residents of England and Wales			
Throat	No	96	78A,3B,1C,1D,5F,1G,1I,1J,1L,1N,1O,1P,1T
Throat	Yes	11	7A,1B,1E,1F,1U
Skin	Yes	5	1D,1H,1J,1M,1W
Blood	Yes	1	1Q
Bronchial washings	No	1	1S
Nose	No	1	1V
Total 115			
Isolates from contacts of residents of England and Wales			
Throat	No	6	5A,1Q
Total		121	
Isolates from residents of Scotland, Channel Islands, and Isle of Man			
Throat	No	8	4A,1K,1L,2R
Total U.K.		129	

[a]U.K. = England, Wales, Scotland, Channel Islands, and Isle of Man.

Table 5. Nontoxigenic *Corynebacterium diphtheriae* isolates from patients who traveled outside England and Wales in the previous 3 months, submitted to the Public Health Research Laboratory's Streptococcus and Diphtheria Reference Unit, 1995

Residence[a]	Destination	Ribotype	Site
London	Australia	A	Throat
London	Sierra Leone	A	Throat
Northwest	Cyprus	A	Throat
Northwest	Holland	A	Throat
Northwest	Canary Islands	A	Throat
Northwest	Spain	A	Throat
Wales	Germany	A	Throat
London	France	B	Throat
London	Vietnam	D	Skin
London	Gambia	E	Throat
London	Canary Islands	F	Throat
London	Sudan	H	Skin
Southeast	Philippines	J	Skin
London	Ghana	M	Skin
West Midlands	Pakistan	Q	Blood
London	Morocco	U	Throat
West Midlands	Jamaica	W	Skin

[a]English Health Regions and Wales.

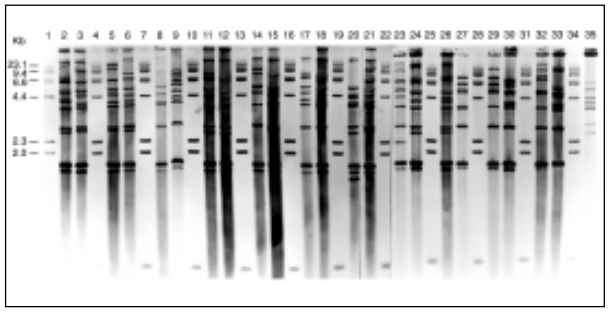

Figure 2. *Bst*EII rRNA gene profiles of nontoxigenic *Corynebacterium diphtheriae* from isolates submitted to the Public Health Laboratory Service's Streptococcus and Diphtheria Reference Unit from U.K. residents,* 1995. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, and 34 contain lambda *Hin*dIII digests as size standard (sizes indicated on left). The remaining tracks show ribotypes A to W: lane 2, 95/13 (A); lane 3, 95/281 (B); lane 5, 95/384 (C); lane 6, 95/358 (D); lane 8, 95/220 (E); lane 9, 95/258 (F); lane 11, 95/277 (G); lane 12, 95/515 (H); lane 14, 95/173 (I); lane 15, 95/171 (J); lane 17, 95/382 (K); lane 18, 95/167 (L); lane 20, 95/340 (M); lane 21, 95/428 (N); lane 23, 95/424 (O); lane 24, 95/453 (P); lane 26, 95/418 (Q); lane 27, 95/23 (R); lane 29, 95/338 (S); lane 30, 95/18 (T); lane 32, 95/324 (U); lane 33, 95/389 (V); lane 35, 95/21 (W).
U.K. = England, Wales, Scotland, Channel Islands, and Isle of Man.

H, I, J, K, L, M, N, O, P, and Q were biotype *mitis*; and ribotypes R, S, T, U, V, and W were biotype *belfanti*.

Ribotype A was isolated only from the throat and accounted for 90 (74%) of 121 isolates from residents of England and Wales (Table 4). The isolates represented 78 of the 96 throat isolates from specimens obtained during clinical evaluation and not associated with recent travel, 7 of 11 throat isolates taken at investigation and associated with recent travel, and 5 of 6 throat isolates obtained at contact tracing (Tables 4 and 5). Ribotype A also accounted for 4 of 8 throat isolates obtained at investigation from residents of Scotland, the Channel Islands, and Isle of Man (Table 4).

The remaining ribotype strains accounted for one to five isolates and were associated with a single health region or with travel outside the United Kingdom (Tables 4 and 5). Ribotype E was isolated from the throat of one person who had recently traveled to Gambia (Table 5). The five ribotypes from skin isolates were related to travel (Table 5); ribotypes H, M, and W were present in single isolates, while ribotypes D and J were also detected in nontravel-related throat isolates. Ribotype Q was isolated from blood of the 2-year-old with congenital heart disease and from the throat of a 4-year-old sibling. The nose isolate from the 23-year-old patient of Pakistani origin was ribotype V, and that obtained from bronchial washings of the 68-year-old man was ribotype S. All ribotypes from contacts were the same as their index case isolates. Ribotypes A, G, N, Q, P, L, and H formed a cluster with a genetic distance of <0.2, indicating greater than 80% genetic homology. Ribotypes L and H exhibited more than 90% genetic similarity.

Conclusions

One invasive infection with *C. diphtheriae* was associated with known risk factors. A single case of endocarditis without recognized risk factors was reported in England immediately before the survey (11). This picture is similar to that of an Australian series in which three of seven cases of invasive infection had no predisposing risk factors (12). Most isolates were from throat swabs of young adults in primary care. The preponderance of female patients reflects the pattern of age- and sex-specific consultation rates for acute pharyngitis and

tonsillitis (ICD 9th Revision codes 462 and 463) seen in general practice (13). Men attending genitourinary medicine clinics accounted for 5% of throat isolates, but there were no isolates in female patients from such settings. This is consistent with clustering previously noted in gay men but could be due to laboratory practice in hospitals serving large gay populations.

It is not known whether nontoxigenic *C. diphtheriae* strains were responsible for the illnesses that prompted the study. More than 25% of the 238 throat isolates obtained at investigation of sore throats were associated with beta-hemolytic streptococcal infection, infectious mononucleosis, or another illness; negative results for viral culture of the throat and for infectious mononucleosis were reported in a small proportion of the remainder. Community-based carriage studies and case-control studies, supported by comprehensive virologic investigation, will be required to obtain more complete information on the pathogenicity or copathogenicity of nontoxigenic *C. diphtheriae* in throat isolates.

Current U.K. guidelines state that when identified, nontoxigenic *C. diphtheriae* be regarded as a potential pathogen and be treated with penicillin or erythromycin if the patient has symptoms (10). Treatment was generally in accordance with these guidelines, but contact tracing, undertaken in 68 (26%) patients, and administration of a diphtheria immunization booster to 18 (8%) patients were not recommended. The few nontoxigenic *C. diphtheriae* isolates associated with chronic skin ulcers were mainly associated with recent travel to tropical zones.

A total of 23 distinct ribotypes were observed. However, ribotype A accounted for most isolates, was isolated exclusively from the throat, and was detected in isolates obtained throughout the United Kingdom. Seven of eleven throat isolates associated with a recent history of travel were also ribotype A, and it is possible that these were acquired in the U.K. Ribotype A predominated and appeared to circulate freely within the U.K. in 1995, which suggests that this strain may have some advantage in terms of transmissibility or pathogenic potential.

If nontoxigenic strains of *C. diptheriae* vary in factors associated with increased transmissibility of pathogenic potential, toxigenic strains may also vary in these factors. Toxigenic strains with these factors could be more likely than toxigenic strains without these factors to produce epidemics. This type of relationship may explain the appearance of an epidemic clone in the Russian diphtheria epidemic of the 1990s.

The marked variation in number of nontoxigenic *C. diphtheriae* isolates referred by laboratories in different regions probably reflected differences in the use of selective culture media for *C. diphtheriae* and practice in referral of isolates to PHLS. Increased professional awareness of the risk for imported diphtheria during the 1990s would have been expected to have increased both of these factors and may explain most, if not all, of the increase in the number of nontoxigenic *C. diphtheriae* isolates ascertained by the PHLS Streptococcus and Diphtheria Reference Unit during this period (Figure 1).

It has been suggested that nontoxigenic strains could become toxigenic by acquiring the *tox* gene, assuming that the chromosomal diphtheria toxin repressor gene (*dtx*R) is functional (14-16). However, no reports of membrane or systemic toxicity were received for any of our isolates, and ribotype patterns in the U.K. isolates for toxigenic and nontoxigenic strains differed. The rise in nontoxigenic strains from 1985 to 1996 and thereafter has not been accompanied by a rise in toxigenic isolates (Figure 1). These observations suggest that conversion to toxin production had not occurred despite continuing circulation of nontoxigenic strains. However, documented introductions of toxigenic *C. diphtheriae* into the U.K. are extremely rare.

Results from the four laboratories that routinely screen all throat isolates with selective culture media indicated a low isolation rate. This may not be seen as a cost-effective activity by many laboratories; less biased and cost-effective surveillance data could be obtained by undertaking selective culture for *C. diphtheriae* in population-based samples, accompanied by strict compliance with reporting.

Our data confirm the known association of nontoxigenic strains with localized disease and with occasional cases of invasive infection, particularly endocarditis. There was no evidence that nontoxigenic *C. diphtheriae* would have posed an increasing threat to public health in England and Wales during the survey period.

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Meningococcemia in a Patient Coinfected with Hepatitis C Virus and HIV

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We describe the first reported case of meningococcemia in a patient coinfected with hepatitis C virus and HIV. Hypocomplementemia secondary to hepatic dysfunction may have enhanced the patient's susceptibility to meningococcal infection.

Coinfection with hepatitis C virus (HCV) and HIV is an emerging public health problem. While coinfection with HIV can accelerate the progression of HCV (1,2), the impact of dual infection on other infectious diseases is unknown. We describe the first reported case of meningococcal infection in a patient coinfected with HCV and HIV.

Case Report

A 45-year-old woman was admitted to our hospital emergency department in September 1999 with influenza-like symptoms (36 hours) and fever, headache, and myalgias (12 hours). The patient's medical history included ongoing injection drug use, coinfection with HIV and HCV, an episode of *Staphylocccus aureus* cervical osteomyelitis, three culture-confirmed episodes of *Streptococcus pneumoniae* pneumonia, and HIV-associated thrombocytopenia. Her most recent CD4 count was 149 cells/µL, and in April 1999, her plasma HIV RNA level was 2,000 viral copies/ µL. She had received pneumococcal polysaccharide vaccine in 1996. Because of ongoing injection drug use, she had never received antiretroviral therapy. Her medications included trimethoprim/sulfamethoxazole, paroxetine, nifedipine, furosemide, and methadone.

The patient was lethargic (oral temperature 40.3°C, blood pressure 82/45 mm Hg, pulse rate 100 beats per minute, and respiratory rate 24 breaths per minute). Physical examination showed meningismus, bilateral conjunctival hemorrhages, diffuse petechiae, and tender palpable purpura on the lower extremities. Neurologic examination was nonfocal. Initial laboratory findings were as follows: hematocrit, 46%; platelet count, 69 x 10^9/L; leukocyte count, 6.0 x 10^9/L; creatinine, 1.6 mg/dL; aspartate aminotransferase, 61 U/L; alanine aminotransferase, 28 U/L; total bilirubin, 0.8 mg/L; alkaline phosphatase, 96 U/L; prothrombin time/international normalized ratio,14.1 seconds/1.3; partial thromboplastin time, 29.4 seconds. Urinalysis showed 14 red blood cells and 28 leukocytes per high-power field, no casts, and 3+ proteinuria. A later urinalysis showed that both the proteinuria and cellular elements had resolved. No schistocytes were visible on peripheral blood smear. A skin lesion biopsy performed in the emergency room revealed a thrombotic vasculopathy without evidence of rickettsiae (by direct immunoflourescence) or other microorganisms.

Because of concern for possible bacterial meningitis, the patient was immediately given 2 g ceftriaxone intravenously. After computed tomography of the brain revealed no acute intracranial process, lumbar puncture was performed. The cerebrospinal fluid was cloudy, with 7,675 nucleated cells/mm (92% neutrophils and 3% band forms). Protein was 382 mg/dL, and glucose was less than 20 mg/dL. Gram stain showed 3+ gram-negative diplococci. Cultures of the cerebrospinal fluid and blood yielded pure growth of serogroup Y *Neisseria meningitidis*.

The patient received a 10-day course of intravenous ceftriaxone, 2 g every 12 hrs. Whole hemolytic complement (CH50) drawn on hospital

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day 5 was 13 units (23-52 units). Other complement assays included a C3 value of 105 mg/dL (86-208 mg/dL) and a C4 value of 8 mg/dL (13-47 mg/dL. Results of a cryoglobulin screen were positive. Computed tomography of the abdomen revealed nodular liver and splenomegaly consistent with cirrhosis. The patient was given meningococcal vaccine near the end of her hospital course and was discharged with no sequelae. On follow-up with her primary physician, she had no evidence of complications from the meningococcemia. A repeat CH50 drawn 6 months after hospitalization was <10 units (23-52 units).

Conclusions

To our knowledge this is the first reported case of disseminated meningococcemia in a patient coinfected with HIV and HCV. Because coinfected patients constitute an increasing percentage of patients infected with HIV (2), several features of this case bear emphasis.

First, hepatic dysfunction from conditions such as HCV is an important risk factor for meningococcal disease (3). This increased risk is likely due to decreased hepatic synthesis of complement (3). Because hypocomplementemia occurs commonly in patients infected with HCV, particularly when cirrhosis or cryoglobulinemia is present (4), these patients are at increased risk for meningococcal infection (3). Patients who are coinfected with HIV and HCV may be at even greater risk for meningococcal infection because of accelerated hepatic destruction. For example, patients coinfected with HIV and HCV have a higher progression to hepatic fibrosis (1) and a 3.5-fold increase in hepatic cirrhosis (2), when compared to patients with HCV alone. Given that up to 9% of HIV-infected patients may be coinfected with HCV (2), a group of patients with potentially increased susceptibility to meningococcal infection may be emerging.

Second, unlike the interaction between HIV and *S. pneumoniae, Salmonella*, and other recognized bacterial opportunistic pathogens, the interaction between HIV and meningococcus is unclear. Fewer than 50 cases of meningococcal infections in HIV-infected patients have been reported in MEDLINE (5-14). These reports present conflicting results on the impact of HIV infection on the risk for meningococcal disease. For example, while one recent prospective cohort study reported an increased risk for meningococcal disease (relative risk 23.8, confidence interval 7.4-76.7; $p < 0.001$) in HIV-infected patients in the Atlanta metropolitan area (12), a case-control study in Africa showed no link between HIV infection and epidemic meningococcal disease (14). Despite the low rate of reported infection, asymptomatic colonization with meningococcus occurs in as many as 15%-23% of tested cohorts of both HIV-infected patients (15) and healthy young adults (16).

There are limitations to our report. Because our patient had several pneumococcal infections before the meningococcemia, immunologic defects other than hypocomplementemia (such as advanced HIV) may have contributed to susceptibility to both of these bacterial pathogens. Only limited data may be inferred from a single case. For example, assuming that the overall rate of meningococcal disease in the United States is 1/100,000, that 1% of the U.S. population is HIV positive, and that 9% of these patients are coinfected with HCV, only two to three cases of meningococcal infection would be expected to occur among coinfected patients if no additional risk for meningococcal infection were present. Because the true incidence of meningococcal infection among coinfected patients is unknown, future cohort studies will have to establish the impact of coinfection with HIV and HCV on the risk for meningococcal infection.

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Genotypic Analysis of Multidrug-Resistant *Salmonella enterica* Serovar Typhi, Kenya

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We report the emergence in Kenya during 1997-1999 of typhoid fever due to *Salmonella enterica* serovar Typhi resistant to ampicillin, tetracycline, chloramphenicol, streptomycin, and cotrimoxazole. Genotyping by pulsed-field gel electrophoresis of *Xba*I-digested chromosomal DNA yielded a single cluster. The multidrug-resistant *S.* Typhi were related to earlier drug-susceptible isolates but were unrelated to multidrug-resistant isolates from Asia.

Salmonella enterica serovar Typhi causes approximately 10 million cases of typhoid that result in 600,000 deaths each year, mostly in developing countries (1). The antibiotics that form the mainstay of therapy in developing countries are chloramphenicol, ampicillin, and cotrimoxazole. Multidrug-resistant (MDR) strains of *S.* Typhi (resistant to all the above antimicrobial drugs) have caused outbreaks in the Indian subcontinent, Southeast Asia, and the Middle East since 1987 (2). Genetic studies have shown that resistance is encoded on an HI1 incompatibility plasmid and is transferable (3). MDR *S.* Typhi has not caused problems in Africa, except in South Africa (4), nor in South and Central America (5), and most isolates have remained fully susceptible. During 1997-1999, a number of isolates of MDR *S.* Typhi were identified from patients with typhoid in Nairobi, Kenya. We have examined their genotypic relationship to each other, to sensitive strains from Nairobi, and to MDR *S.* Typhi from Southeast Asia.

The Study

We analyzed isolates of *S.* Typhi obtained in the Kenyatta National Hospital from blood cultures of 16 adults with typhoid from 1988 to 1993; from 22 cultures of 19 adults and 3 children from 1997 to 1999; and from 17 representative MDR *S.* Typhi strains collected from 1990 to 1995 (6) from Pakistan (7), Hong Kong (4), Bangladesh (3), Kuwait (1), and India (1). We did not have access to isolates from 1994 to 1996, when no active surveillance was conducted. MICs of ampicillin, co-amoxyclav, cefuroxime, cotrimoxazole, chloramphenicol, gentamicin, streptomycin, tetracycline, nalidixic acid, and ciprofloxacin were determined by the E-test method (AB Biodisk, Solna, Sweden).

Macrorestricted (using *Xba*I) chromosomal DNA from the *S.* Typhi isolates was separated by pulsed-field gel electrophoresis (PFGE) with a CHEF DRII system (Bio-Rad Labs, Richmond, VA). The gels were stained with ethidium bromide and photographed on an ultraviolet transilluminator. Banding patterns were compared (8), and dendrograms of relatedness were constructed by data clustering using the unweighted pair grouping arithmetic averaging method (Molecular Fingerprinting Program version 1.4.1, BioRad). Conjugation experiments, plasmid extraction and electrophoresis, and incompatibility grouping were performed as described (7).

All 16 *S.* Typhi isolates from 1988-1993 were fully sensitive to all the drugs tested (MIC 0.012-0.016 mg/L for ciprofloxacin to 1-3 mg/L for chloramphenicol). In contrast, 18 (82%) of the 22 *S.* Typhi from 1997-1999 were resistant to ampicillin, tetracycline, and chloramphenicol (MICs all > 32 mg/L), as were the 17 isolates from Asia. The first two MDR *S.* Typhi from Kenya were detected from blood cultures from two

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adults in March 1997. Active surveillance is ongoing, and multidrug resistance is detected in approximately 65% of all *S.* Typhi isolates to date. As we did not have access to isolates from 1994-1996, we cannot be certain that MDR *S.* Typhi did not emerge earlier than 1997.

All the Kenyan MDR *S.* Typhi isolates came from indigenous patients with no known history of recent travel outside the country. The Kenyan MDR isolates remained sensitive to co-amoxyclav, cefuroxime, gentamicin, nalidixic acid, and ciprofloxacin. The Kenyan MDR *S.* Typhi all transferred their full resistance phenotype to *Escherichia coli* K12 on 98- to 100-MDa plasmids of inc HI1 (or inc HI1 cross-reacting with inc FIIA).

All 22 MDR and 16 sensitive *S.* Typhi were analyzed by PFGE. As all 22 MDR isolates were similar by PFGE, only two representative strains were selected for further analysis. In addition, 5 representative sensitive *S.* Typhi and 11 representative MDR strains from Asia were analyzed for similarity by using dendograms. The sensitive *S.* Typhi (1987-1992) had a number of different genotypes. The MDR *S.* Typhi were identical, but differed from the sensitive isolates by more than seven bands, indicating they were different strains. However, on the dendogram comparing MDR *S.* Typhi from Asia and the *S.* Typhi from Kenya (both MDR and sensitive), the Kenyan isolates formed one cluster, with the nearest (but genotypically quite distinct) other cluster being the Pakistani MDR *S.* Typhi (Figures 1 and 2).

Conclusions

The emergence of an MDR *S.* Typhi strain in Kenya is of concern because resistance to first-line antibiotics that are also commonly used for treatment of other bacterial infections in hospitals may pose a major challenge to health care. Although these newly emerged MDR *S.* Typhi are sensitive to nalidixic acid and ciprofloxacin, their MICs are five and ten times higher, respectively, than those of the sensitive *S.* Typhi from 1988-1993. Although fluoroquinolones are not widely available in Kenya, they may be needed to treat MDR *S.* Typhi, and resistance will lead to problems with treatment, as in Asia (9). Multidrug-resistant *S.* Typhi isolates from Kenya produced an indistinguishable PFGE pattern that was related to those of sensitive strains but unrelated to those of MDR *S.* Typhi from Asia. This finding implies that the Kenyan MDR *S.* Typhi are most likely to have arisen from sensitive isolates by acquisition of resistance plasmids from antibiotic-resistant enteric bacteria. Plasmids of incompatibility group HI1 are those most frequently found in *S.* Typhi, but we did not detect them in any of our nontyphoidal salmonellae with the same plasmid-encoded resistance (7).

We observed the emergence of *S.* Typhi resistant to all first-line drugs used for treatment of typhoid in Kenya and in many other African

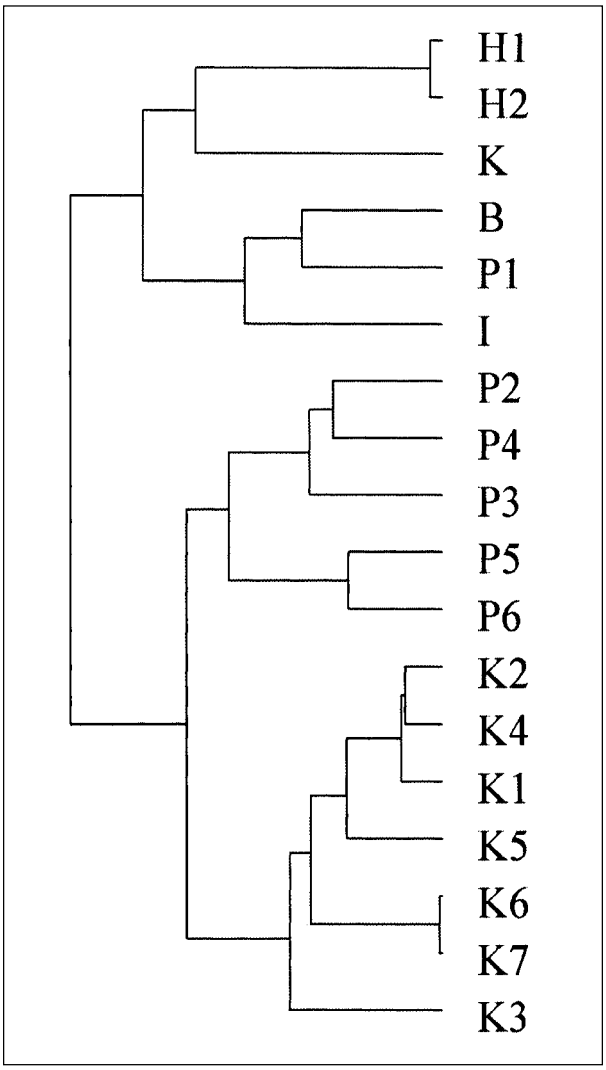

Figure 1. Dendogram showing genetic relatedness of *Salmonella* Typhi from Kenya and Asia. H1 and H2: MDR *S.* Typhi from Hong Kong; K: MDR *S.* Typhi from Kuwait; B: MDR S. Typhi from Bangladesh; P1-P6: MDR *S.* Typhi from Pakistan, I: MDR *S.* Typhi from India. K1-K5: sensitive *S.* Typhi from Kenya; K6 and K7: MDR *S.* Typhi from Kenya.

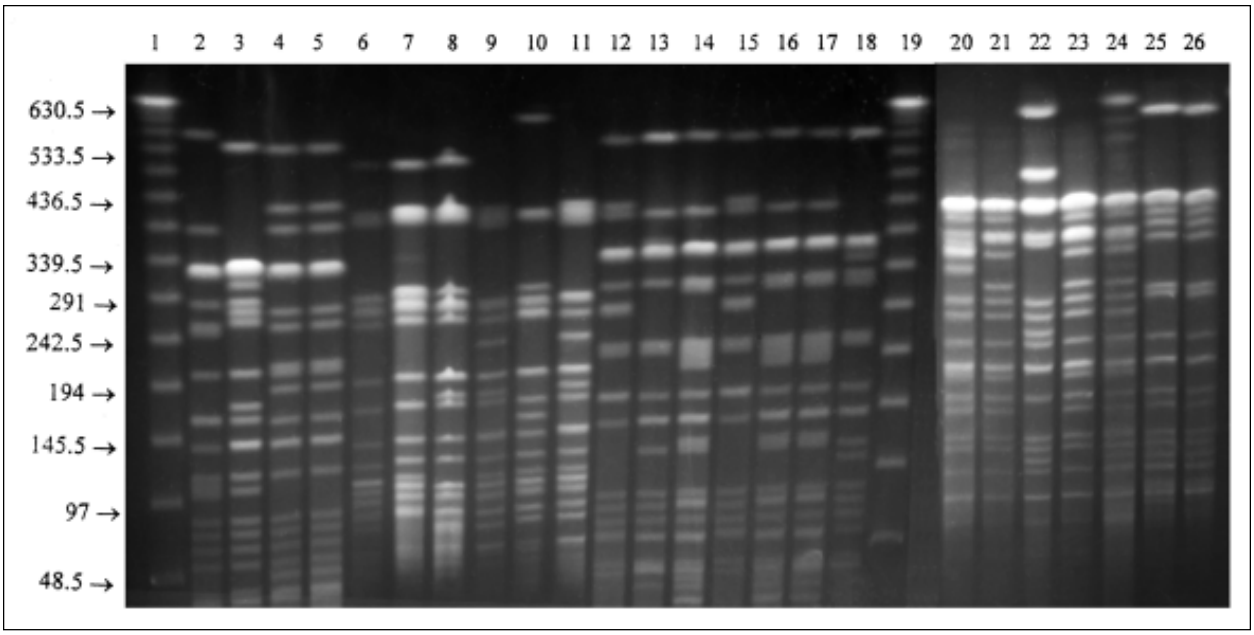

Figure 2. *Xba*I restriction endonuclease fragment patterns of representative *Salmonella* Typhi isolates from various countries. Lanes 1 and 19, molecular size standard; Lane 2, B1 from Bangladesh; Lane 3, I1 from India; Lanes 4 and 5, K1 and K2 from Kuwait; Lanes 6, 7, 8, and 9, M1, M2, M3, and M4 from Malaysia; Lanes 10, 11, 12, 13, and 14, Q1, A2, A3, A4, and A5 from Quetta; Lanes 15, 16, 17, and 18, R1, R2, R3, and R4 from Rawalpindi, Pakistan; Lanes 20-24, K1-K5: sensitive *S.* Typhi from Kenya; and Lanes 25 and 26, K6 and K7: multidrug-resistant *S.* Typhi from Kenya.

countries. Laboratories in Kenya should perform surveillance by routinely testing *S.* Typhi for susceptibility to first-line treatment drugs and to nalidixic acid to detect quinolone resistance. Effective surveillance for this newly emerged MDR *S.* Typhi in Africa and other developing regions of the world where MDR *S.* Typhi has not yet emerged would ensure prompt diagnosis, susceptibility testing, and appropriate antimicrobial chemotherapy.

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Lessons Learned from a Full-Scale Bioterrorism Exercise

During May 20-23, 2000, local, state, and federal officials, and the staff of three hospitals in metropolitan Denver, participated in a bioterrorism exercise called Operation Topoff. As a simulated bioterrorist attack unfolded, participants learned that a *Yersinia pestis* aerosol had been covertly released 3 days earlier at the city's center for the performing arts, leading to >2,000 cases of pneumonic plague, many deaths, and hundreds of secondary cases. The exercise provided an opportunity to practice working with an infectious agent and to address issues related to antimicrobial prophylaxis and infection control that would also be applicable to smallpox or pandemic influenza.

The sequence of events and the exact date of the exercise were not specified. However, the probable weekend and possible bioagents were suggested, which enabled us to begin preparations approximately 8 weeks ahead. Preparations included temporary appointments to the governor's 19-person Expert Emergency Epidemic Response Committee, which was created by enactment of a bioterrorism and pandemic influenza response law on March 15, 2000; recruitment of 25 epidemiologic and emergency management personnel from the 1,050 employees of our department and assignment to disaster response teams (e.g., surveillance, field investigation, and emergency management coordination); and establishment of a command center by reserving conference rooms and installing telephone, computer, and television equipment. Colorado's bioterrorism and pandemic influenza response law was not enacted to prepare for the exercise, but proved extremely useful. We recommend that state health agencies review their statutory authority and evaluate whether these laws would be adequate to deal with the threats of bioterrorism and pandemic influenza. During the exercise, we were provided information either from other participating agencies or from exercise controllers, and it was our task to investigate and respond. The staff reviewed mock medical records, analyzed laboratory specimens, interviewed patients, conducted meetings and group conference calls to assess surveillance data and decide on the next steps, drafted public health and executive orders, made written requests to federal officials for specific assistance, participated in news conferences, and packaged mock antibiotics for distribution at a prophylaxis clinic. By the end of day one, 783 cases and 123 deaths from plague had been reported from 16 hospitals (three participating hospitals and 13 simulated facilities). By the end of day two, 1,871 cases and 389 deaths were attributed to pneumonic plague, with 307 patients requiring ventilatory support. Cases were reported from six states outside Colorado. By the end of day three, 3,700 cases and 950 deaths were reported, including at least 780 secondary cases.

The exercise required state health department personnel to develop new working relationships. Although hospitals and local and state health agencies often collaborate with the Centers for Disease Control and Prevention in controlling an epidemic, we were unaccustomed to working closely with the Federal Bureau of Investigation, the U.S. Attorney for the District of Colorado, the Federal Emergency Management Agency, the Regional Office of the U.S. Public Health Service, and the Colorado Office of Emergency Management. Although lines of authority were clear, much time was spent in consultation and debate through scheduled bridge calls. Many persons joined these calls, and decision-making became inefficient, although not impossible. In a true incident, a central location for face-to-face meetings should be large enough to accommodate representatives from all agencies involved, but one difficulty encountered with arranging such meetings was that each agency seemed most comfortable in its own command center.

Another lesson we learned concerned our own organization. In addition to the surveillance, field investigation, and emergency management coordination teams, we needed teams to address laboratory testing, mass fatalities, legal problems, information technology, infection control, public and professional communications, and antibiotic and vaccine administration. During a disaster, no routine agency business can be conducted, as all employees are involved in the public health response. Finally, activities cannot depend on the direction of one or two key persons, such as the executive director and the state epidemiologist; other skilled, informed persons must be able to

assume leadership roles. An electronic database documenting events, decisions, and requests for resources should be maintained. These logs enable staff to monitor the epidemic and the public health response rapidly.

In Colorado, where plague is endemic, we are familiar with the public health management of single plague cases, but the magnitude of the simulated epidemic and the fact that infection was spreading from person to person after a short (2- to 3-day) incubation period quickly overwhelmed the available resources. The challenge to our surveillance system was not in detecting the outbreak but rather in maintaining surveillance at each of the 22 acute-care hospitals in metropolitan Denver. Our hospital surveillance system usually relies on reporting by infection control practitioners, but during the exercise these practitioners had many additional responsibilities. In a true bioterrorist attack, emergency response teams of state or local health department employees should be set up and sent to each hospital to monitor cases and provide information to a central command center.

As more cases were identified, an anticipated issue emerged: who should receive antimicrobial prophylaxis? The governor's committee debated whether to limit prophylaxis to close contacts of infectious cases or offer it more widely (e.g., to all health-care workers, first responders, and public safety workers and their families) to gain the support and participation of key workers. The committee decided on the latter approach, but not unanimously.

The process of isolating plague patients until they are no longer contagious and identifying close contacts is typically straightforward. Isolation, however, was not possible during this exercise. The hospitals had too many patients and worried-well persons and too few health-care workers and empty rooms to permit isolation of pneumonic plague patients. Case reporting was delayed, and there were too few trained public health workers to conduct interviews and locate contacts in a timely manner. As a result, an executive order was issued quarantining all persons in metropolitan Denver in their homes. With infection control in the general population supposedly managed by the order, we could turn our attention to securing additional supplies, staff, beds, and equipment for the hospitals.

However, quarantining two million persons is not simple. Essential workers must be identified, be given prophylaxis and protective barriers, and be permitted to do their jobs. Other members of the community can stay in their homes only a few days before they need fresh supplies of food. Therefore, a one-time, blanket quarantine order is unlikely to be successful and cannot be enforced unless these and many other issues are addressed. The hospitals were quite demanding in their requests for reinforcements, and we made great efforts to assist them. However, by day three of the exercise it became clear that unless controlling the spread of the disease and triage and treatment of ill persons in hospitals receive equal effort, the demand for health-care services will not diminish. This was the single most important lesson we learned by participating in the exercise.

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Preliminary Characterization and Natural History of Hantaviruses in Rodents in Northern Greece

To the Editor: Hantaviruses (family Bunyaviridae), cause hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus pulmonary syndrome in the Americas. In Greece, hantaviruses are endemic, and small outbreaks or sporadic cases of HFRS have been observed; Dobrava virus is the predominant hantavirus and causes severe HFRS (1,2), while Puumala virus is probably the causative agent of the milder forms of this syndrome (Papa et al., unpub. data). Hantaan virus, associated with *Apodemus agrarius*, and Seoul virus, associated with *Rattus norvegicus*, have never been detected in Greece. Dobrava virus is hosted by *A. flavicollis* in the Balkans (3,4) or by *A. agrarius* in northern Europe (5).

We surveyed two sites where Dobrava virus-caused HFRS was reported (2). The first was Nevrokopi, a small town in the Rhodope Mountains, 12 km from the Greek-Bulgarian border (41° 20.184' N, 23° 49.479' E, elevation approximately 650 m). The second site included Pramanta, a small village in the Pindos Mountains, approximately 67 km southeast of Ioannina (39° 31.722' N, 21° 5.872' E, elevation 790 m), and Matsuki, a small village near Pramanta (39° 33.909' N, 21° 9.713' E, elevation 1080 m). Animals were trapped and sampled outdoors according to established safety guidelines (6). Blood, lung, kidney, and spleen samples were kept in liquid nitrogen until transferred to -70°C freezers for storage. At Nevrokopi, 57 small mammals were captured during 887 trap nights for an overall trap success rate of 6.4%. At Pramanta and Matsuki, 13 small mammals were captured during 400 trap nights (3.3% trap success). The total of 70 captured mammals comprised seven species of rodents and one insectivore. *A. flavicollis* was the most commonly captured species (87% of captures).

Whole-blood specimens were tested for hantavirus immunoglobulin G by indirect immunofluorescence assay and enzyme-linked immunosorbent assay, using Hantaan 76-118 as antigen. Total RNA was extracted from homogenized tissues, and nested reverse transcriptase-polymerase chain reaction (PCR) was performed with two sets of nested primers (2): one set designed to detect the partial G1 coding region of hantaviruses associated with rodents of the subfamily Murinae (Hantaan, Dobrava, and Seoul viruses), and another to detect the N coding region of hantaviruses associated with rodents of the subfamily Arvicolinae (Prospect Hill virus). Eight *A. flavicollis*, all from Nevrokopi, were positive for hantavirus infection by serology or molecular methods, for a 13% overall prevalence in *A. flavicollis*. Some rodents positive by serology were negative by PCR and vice versa. One of three *R. rattus* captured at Pramanta was positive for hantavirus infection by indirect immunofluorescence assay.

PCR yielded products from tissues from seven *A. flavicollis*. A 270-bp segment of the G1 gene was sequenced and analyzed phylogenetically. A mean sequence similarity of 99.1% (range 98.5%-100%) was observed among the seven rodents. These sequences differed by 9.5% from Dobrava virus sequences of HFRS cases from northwestern Greece and by 8.5% from Dobrava virus sequences of HFRS cases from Dobrava-Slovenia. Similarly, sequences from Nevrokopi human samples were closer to Dobrava virus from Slovenia than to such virus from northwestern Greece. The nucleotide difference was 21% when the rodent sequences were compared to an Estonian sequence from *A. agrarius*. The deduced amino acid sequences of all seven Dobrava virus G1 fragments were identical. This analysis showed that the evolutionary relationship among Dobrava virus subtypes was closely correlated with that of the rodent reservoir and suggests that this virus is stably maintained in the rodent population.

All seropositive *A. flavicollis* were sexually mature adults; six (75%) of eight were male, compared to 26 (49%) of 53 seronegative animals (χ^2=0.97, p=0.32). Six seropositive animals of 8 (75%) had scars, compared to 14 (26%) of 53 seronegative animals (χ^2 =5.4, p=0.02). Finally, 14 (44%) of 32 male *A. flavicollis* had scars, compared to 5 (17%) of 29 females (χ^2 =3.8, p=0.05). Scarring has been significantly associated with Seoul virus antibody in wild rats and has been suggested as the primary mechanism by which hantaviruses are amplified epizootically (7). The higher prevalence of scars among male *A. flavicollis* and especially among males with evidence of hantavirus infection supports the hypothesis that hantaviruses are transmitted

when aggressive male animals fight. Although this pattern has been observed for several host species of New World hantaviruses, this is the first known demonstration for Dobrava virus and *A. flavicollis*. Of the two female rodents with evidence of hantavirus infection, one had scars, and one did not. The latter was positive by PCR on lung tissue but did not have detectable antibody in blood, which perhaps indicates very recent infection.

The seropositive *R. rattus* from Pramanta is the first evidence of hantavirus infection in *Rattus* within Greece. No *Rattus* captured during previous expeditions had hantavirus antibody (8). The low antibody titer (1:32) and failure to amplify viral RNA by PCR from this animal could indicate infection with a heterologous hantavirus with low cross-reactivity. Perhaps more likely, the antibody detected in this 39-g juvenile rat may represent waning maternal antibody. Transfer of protective maternal antibody to *R. norvegicus* pups by Seoul virus-infected dams has been demonstrated (9).

Our data implicate *A. flavicollis* as the reservoir of Dobrava virus in northern Greece and demonstrate the common occurrence of that species in both sylvatic and peridomestic habitats. These preliminary results underscore the need for continued, more intensive reservoir studies in Greece.

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Imported Dengue in Buenos Aires, Argentina

To the Editor: After more than 70 years without reports of cases, an outbreak of dengue (type 2) occurred in the northwestern region of Argentina from January to May 1998; 818 cases of denguelike illness were reported (incidence rate: 45/10,000 inhabitants) (1). The outbreak was restricted to a few cities of the Chaco Salteño Region.

The last dengue epidemic in Argentina (in 1926) (2) affected the Mesopotamia Region and Rosario City. An earlier widely distributed epidemic in 1916 occurred in the coastal region along the Uruguay River (Corrientes and Entre Ríos provinces), reached Parana City (along the Parana River), and affected approximately 50% of the city's population (3). Both outbreaks began in Paraguay. No cases were detected in Buenos Aires.

High numbers of *Aedes aegypti* are reported in all places where surveillance for these vectors is conducted in Argentina. The Breteau rate (a measure of vector density; the number of positive containers is divided by the number of inspected houses) in the Federal District

averaged >40% in the first trimester of 2000 and was 30% to 80% in suburban districts in 1999 (R. Boffi, Ministerio de Salud de la Nación; N. Schweigmann, University of Buenos Aires, pers. commun.).

In Argentina's neighboring countries, dengue is a serious public health problem. From December 1999 through March 2000, Paraguay reported 42,000 dengue cases, 9 of dengue hemorrhagic fever (4). Brazil has reported cases of dengue and dengue hemorrhagic fever, and Bolivia has reported dengue and a large yellow fever outbreak (4). From December 1999 to March 2000, 85 patients with denguelike illness arrived in Buenos Aires from one of these countries' dengue-epidemic areas and were seen at F. J. Muñiz Hospital in Buenos Aires. An enzyme-linked immunosorbent assay-capture immunoglobulin M test (commercial kit) (5) and a plaque reduction neutralization test on cell culture were performed (6). Laboratory diagnosis of dengue infection was made in 38 cases. Twenty-five cases were in female patients, and 13 were in male patients; the age range of patients was 8 to 74 years (average, 39 years). All patients were Argentinean residents; 18 (47.4%) lived in the Federal District, and 20 (52.7%) in the suburban area (west and south). Except for one patient who had traveled to Saint Thomas Island, the patients traveled from Paraguay (Asunción, Ciudad del Este, Luque, and other cities). The patients had been out of Argentina 4 to 60 days (average, 17 days). Twenty-six (68.4%) patients had viremia in their place of residence (Federal District or suburbs). In Buenos Aires, 20 patients had viremia for 5 days, 3 patients for 4 days, and 3 patients for 3 days. Ten patients (26.3%) had mild febrile illness; 23 (57.1%) had classic dengue fever; and 5 (13.2%) had dengue fever with hemorrhage. Four patients had epistaxis, and one woman had self-limited, abnormal vaginal bleeding of 24 hours' duration.

Considering *A. aegypti* infestation rates and the large population of this area, (3 million in the Federal District and 8 million in the suburban areas) (7), the probability of an outbreak is high. Historically, the highest rates for *A. aegypti* in this area are reported in April and May (8). In 1997, 1,608,062 tourists arrived from countries that have dengue transmission (1,135,168 from neighboring countries, 358,286 from Paraguay) (9). Approximately 40% of these tourists arrived by plane. In 1998, >700,000 Argentineans left the country through Buenos Aires to travel to countries where dengue transmission occurs (7). Migration through bordering areas, especially in tropical regions of northern Argentina, is underreported.

The number of imported dengue cases in Buenos Aires and other cities in Argentina detected in the current period is substantially higher than the number detected in previous years. Argentina is at risk for an outbreak of dengue, and the health system of the country should be preparing for it.

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American Robins as Reservoir Hosts for Lyme Disease Spirochetes

To the Editor: We read with great interest the article by Richter et al. (1) reporting the competence of American robins as reservoir hosts for Lyme disease spirochetes. The article demonstrates that *Turdus migratorius* is a reservoir for *Borrelia burgdorferi* sensu lato under experimental conditions. However, we want to draw attention to certain statements in the article regarding current knowledge of the ecology of Lyme borreliosis in Europe.

First, in the discussion the statement "The standard of proof (implied xenodiagnosis)... has not previously been applied to candidate avian reservoirs" is inaccurate. In fact, this method was applied a few years ago to pheasants (*Phasianus colchicus*) in the United Kingdom (2) and to European blackbirds (*Turdus merula*) in Switzerland (3) to investigate their respective reservoir competence. Even though these articles were cited by Richter et al., the use of xenodiagnosis detailed in them was not mentioned.

A second statement in the introduction claims that "Although spirochetes have been isolated from naturally infected European blackbirds (*T. merula*) (Humair et al., 1998), a laboratory study failed to demonstrate reservoir competence of these birds (Matuschka and Spielman, 1992); the reason for this discrepancy remains unclear." However, the reservoir competence of European blackbirds has been clearly demonstrated by tick xenodiagnosis (3).

Another statement cited in the introduction that pheasants "...cannot contribute to transmission because larval ticks seem not to feed on them, either in the laboratory or in nature (Kurtenbach et al. 1998a, 1998b)" is incorrect; no such statement occurs in the two papers by Kurtenbach et al. (2,4). Moreover, Randolph and Craine have clearly demonstrated that pheasants contribute to *Borrelia* transmission to ticks (5).

Finally, in the statement "Although certain genospecies of the Lyme disease spirochetes are said to be more mouse-adapted than others (Humair et al. 1995), no experimental evidence is available to support this concept" the term concept is inappropriate. *B. afzelii* and *B. burgdorferi* have been found associated with both Muridae and Sciuridae in various ecologic situations (4,6-10). *B. garinii* and *B. valaisiana* have been observed preferentially associated with certain avian hosts and associated ticks in particular ecologic situations (3,4,11-13). All these published results (3,4,6-13) demonstrate that the relationships between genospecies of *Borrelia* and hosts observed in some areas of Europe have gone beyond concept and are facts. Because the explanation of a phenomenon is not immediately obvious one cannot assert that the phenomenon does not exist or that the evidence can be denied. *Borrelia* sensitivity to serum complement may explain the existence of a preferential relationship between host and *Borrelia* genospecies (14).

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American Robins as Reservoir Hosts for Lyme Disease Spirochetes

To the Editor: The article by Richter et al. (1) presents interesting results, not only on the ability of American robins to transmit Lyme disease spirochetes but also on the birds' tolerance to reinfection after the original infectivity has waned. Even more interestingly, spirochetes that had been transmitted by these avian hosts were then transmitted by laboratory mice. However, important research on wildlife hosts of the various genetic strains of Lyme spirochetes is not fully acknowledged. Therefore, the new results are not put into the context of existing information, missing the opportunity for much interesting comparison between the American N40 strain of *Borrelia burgdorferi* sensu stricto, the subject of this work, and European strains of this and other *Borrelia* genotypes.

Chipmunks (United States [2]), two species of squirrels (United Kingdom [3] and Switzerland [4]), and hedgehogs (5) are missing from the list of wildlife hosts, and the competence of sheep is denied despite evidence to the contrary (6). Moreover, European blackbirds, which have been shown to transmit spirochetes to xenodiagnostic larval ticks (7), are dismissed as transmission hosts on the basis of earlier negative transmission results from Matuschka (8), which came from two birds and pre-dated knowledge of the genetic diversity and apparent host specificity of *B. burgdorferi* sensu lato. Pheasants are also dismissed as not contributing to transmission because, according to the authors, larval ticks do not feed on them. Although significantly fewer larvae than nymphs feed on wild pheasants, in the summer similar numbers of larvae feed on pheasants (median 7, range 0-64 on cock birds; median 0, range 0-7 on hens) as on rodents (3,9,10). Laboratory and field data (9,11) analyzed within a general transmission framework (10) suggest that pheasants can act as a natural reservoir for spirochetes of some genotypes.

A growing body of evidence, both observational and experimental, suggests that certain *B. burgdorferi* s.l. genotypes (e.g., *B. afzelii*) are transmitted much more efficiently by mammals and that other genotypes (e.g., western European *B. garinii*) are transmitted more efficiently by birds (4,7,11-14). Given the apparent lack of host specificity of *B. burgdorferi* s.s. N40, the new results would add to recent advances in explaining Lyme spirochete ecology if they were put in the context of these consistent independent findings. It is now understood that Lyme spirochetes circulate through populations of mixed species of hosts, each species making different contributions to the overall persistence of the pathogen because of their differential transmission competence and infestations by each tick stage (11,15). Larval and nymphal ticks quest at different heights (16), and this behavior changes in response to microclimate, resulting in differential attachment rates to various vertebrate species (17). In such a population of hosts, any one species can contribute a basic reproduction number (R_0) of less than unity but still play an important role in maintaining enzootic cycles (10,18).

Finally, some aspects of Richter et al.'s experimental results need clarification. Was the laboratory colony of ticks screened regularly for infection? Given the very high transmission rates recorded in this study (86% transmission by robins and 97.5% by mice), reassurance that all infections were derived from the experimental procedure would be helpful. In addition, the tolerance of repeated tick feeding by robins is not as high as claimed; 82% of 32 nymphs at the third infestation is (not quite significantly) lower than 96% of 48 and 98% of 40 at the first two infestations (Yate's corrected $\chi^2 = 5.5$, 2df, $0.1 > p > 0.05$). Recent evidence suggests that

repeated infestations of ticks on mice, even without obvious reduced feeding success, result in reduced transmission of spirochetes between mice and ticks (19).

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Response to Dr. Randolph and Drs. Gern and Humair

To the Editor: We define reservoir competence of a host for a vector-borne pathogen in terms of three component questions: How susceptible is the putative reservoir host when the pathogen is delivered by the bite of an infected vector tick? How effectively does the pathogen proliferate and develop in this host? And how infective is the resulting infected host to vector ticks and for how long (1,2)? Drs. Gern and Humair insert the parenthesis (implied xenodiagnosis) into a citation of our text, thereby, equating reservoir competence with a simple xenodiagnostic test that partially addresses only the third component of this definition. At best, such a test records degree of infectivity to vector ticks at some arbitrary and often unknown point in time, a consideration that persuades us to limit our citations referring to reservoir competence. Conclusions derived from xenodiagnosis performed on field-derived animals differ from those that are obtained by an experimental study. With regard to acknowledging relevant research, we did cite the study on pheasants (3) in which these birds were infected in the laboratory by tick-borne spirochetes and subsequently infected only about a quarter of vector ticks. The cited study on blackbirds (4), on the other hand, used ticks solely to diagnose

infection in field-derived birds that had been infected in nature. Although a few of these animals proved to be infectious to xenodiagnostic ticks when tested 1-3 days after capture, this study failed to quantify susceptibility or to determine intensity and duration of infectiousness to vector ticks. Our rigorously standardized study (1) is the first to establish experimentally that birds are highly competent as reservoir hosts for Lyme disease spirochetes.

Drs. Gern and Humair disagree with our statement that "larval ticks seem not to feed on [pheasants], either in the laboratory or in nature." However, a field study on pheasants states that "no fully engorged larvae … were recovered from thirty adult male pheasants shot in a Dorset woodland" (5). The previously cited experimental study on these birds similarly demonstrated that larval infestations generally fail, and stated that "In fact, most of the introduced larvae died while attempting to feed on the pheasants" (3). Inflammatory responses directed against feeding larvae were advanced as a possible explanation for the observed failure to feed to repletion. The larval stage of the American vector similarly seems to feed poorly on chickens (6). Passerine birds, however, seem to serve effectively as hosts for the larval stages of this complex of ticks, and we find that larvae attach readily to American robins (1). Numerous larval *Ixodes ricinus* ticks feed on European blackbirds in nature, and larval ticks attach readily and repeatedly to such birds in the laboratory (7,8). Therefore, the limited attractiveness of gallinaceous birds to larval *Ixodes* ticks may render them less important than certain passerine birds as natural reservoir hosts for Lyme disease spirochetes.

The transmission cycle of the agent of Lyme disease tends to be more complex in Europe than in North America. The host range of the European vector tick, *I. ricinus*, is broader than that of its American cousin (*I. dammini*, frequently cited as *I. scapularis*), and the European pathogen in humans is more diverse, comprising several genospecies. Our study aimed to define the competence of the American robin, *Turdus migratorius*, as a reservoir host for rodent-infecting *Borrelia burgdorferi* sensu stricto—not *B. burgdorferi* sensu lato as stated in Drs. Gern and Humair's letter—and used American *Ixodes* ticks as vectors. The mode of perpetuation of the agents of human Lyme disease in Europe is peripheral to the subject of our article.

Dr. Randolph and Drs. Gern and Humair express commitment to the concept that different European spirochetal genospecies perpetuate simultaneously in distinct kinds of vertebrate reservoir hosts. Their concept requires that a larval *I. ricinus* tick that acquires a rodent-specific genospecies from a rodent host must, in its nymphal stage, again feed on a rodent. If this nymphal tick were to feed on a bird, the rodent-specific spirochete would not perpetuate because this nonpermissive host would function zooprophylactically. A suggested avian-specific spirochete would perpetuate reciprocally. According to the MacDonald concept of vectorial capacity (9), such a relationship would be unlikely if pathogens requiring different reservoir host populations were to be transmitted simultaneously by the same vector population. The studies cited in support of this concept rest on correlative evidence derived from field data. No confirmatory experimental proof demonstrates an especially intense association of *B. afzelii* with rodents and *B. garinii* or *B. valaisiana* with birds. Indeed, European larval ticks acquire *B. afzelii* as well as *B. garinii* infection from field-derived passerine birds (10). Various other observations also contradict the suggested close association between genospecies and particular kinds of hosts (11-13). One of the studies (14) cited as evidence for genospecies specificity was published even before the genospecies were differentiated; the other "consistent independent findings" derive from the laboratories of Drs. Randolph and Gern. Our findings that birds serve as competent hosts for an apparently mammal-perpetuated spirochetal genospecies would seem to contradict the concept of separate genospecies perpetuation. No rigorous evidence is yet in hand to support the theory that the same population of vector ticks perpetuates different European spirochetal genospecies differentially in particular kinds of reservoir hosts.

Dr. Randolph suggests that our experiments may have been confounded because Lyme disease spirochetes may have been inherited persistently within the laboratory colony of ticks used in our studies. Although an early observation points toward the possibility of such a mode of transovarial transmission

(15), subsequent experimental evidence suggests that vertical transmission rarely, if ever, occurs (16). Inherited infection in nature would be exceedingly infrequent because spirochetes infect less than 1% of naturally questing larvae, both in North America and in Europe (17, 18), and some of these larvae may have acquired infection by feeding partially on an infected host. We routinely seek evidence of spirochetal infection in each cohort of larval ticks used in our experiments but have never found spirochetes in a nonfed, laboratory-reared larva. Our reported frequency of experimental transmission of Lyme disease spirochetes from reservoir mice to vector ticks corresponds to that reported elsewhere (19-21).

Dr. Randolph's statistical analysis of our data confirms that the feeding success of nymphal ticks on robins exposed repeatedly to ticks varies nonsignificantly, supporting our conclusion that nymphal ticks readily feed repeatedly on tick-exposed robins. Although repeated nymphal infestations may protect inbred laboratory mice from tick-borne spirochetes (22), natural reservoir hosts, such as white-footed mice and American robins, remain susceptible to such spirochetes, regardless of prior exposure to ticks (1, 23).

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Conference Summary

3rd Conference on New and Reemerging Infectious Diseases
University of Illinois, Urbana-Champaign

The 3rd Conference on New and Reemerging Infectious Diseases was hosted on April 20-21, 2000, by the Department of Veterinary Pathology at the University of Illinois at Urbana-Champaign.

Topics included chloroquine resistance in malaria; exotic pathogens, illustrated by West Nile virus; effects of exposure to heat and acid pH on *Leishmania donovani* amastigotes; method for purification of polar tube proteins of Microsporidia; zoonotic spread of rotaviruses and caliciviruses; unforeseen effects of pathogen eradication programs; and panzootic outbreaks of morbillivirus infection in marine mammals and viral and fungal diseases in amphibians.

This year's conference was dedicated to the memory of parasitologist Norman D. Levine, who was a professor at the University of Illinois for more than 45 years. Next year's conference is planned for April 19-20, 2001.

Upcoming Events

Fifth International Symposium on Hemorrhagic Fever with Renal Syndrome, Hantavirus Pulmonary Syndrome, and Hantaviruses
Les Pensieres, Veyrier-du-Lac, in the French Alps
June 13-16, 2001

Organized by the Merieux Foundation, the program will include sessions in the following areas: ecology and epidemiology, viral genetic analysis, molecular and cell biology, clinical aspects and pathogenesis, laboratory diagnosis and immune response, and antiviral and vaccine development.

The deadline for abstracts is March 10, 2001. For information, contact Betty Dodet at bdodet@fond-merieux.org. The website address for information and registration is http:/www.fond-merieux.org.

Erratum, Volume 6, Number 4

Additional changes were needed in the letter, First Report of Human Granulocytic Ehrlichiosis from Southern Europe (Spain), by José Oteo et al. on p. 431, column 2 and p. 432, references. The corrected sentences and references follow.

First paragraph, second sentence: "Samples were sent to Dr. Barral at the Instituto Vasco de Investigacion y Desarrollo Agrario (NEIKER) who kindly performed the assay using a set of primers based on the published sequence of the 16s rRNA of *E. phagocytophila* (E1: 5'-GGC ATG TAG GCG GTT CGC TAA GTT -3' and E2: 5'-CCC CAC ATT CAG CAC TCA TCG TTT A-3') (10)."

Second paragraph, second sentence: "The prevalence of *E. phagocytophila* genogroup in the tick *Ixodes ricinus* is high (24.1% of nymphs, determined by PCR) (11) in La Rioja, and evidence of HGE infection in patients at risk has been reported (12)."

Corrected references

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We regret any confusion these errors may have caused.

Japanese color woodcut print advertising the effectiveness of cowpox vaccine

In 1849, 18 years before the Meiji Revolution, cowpox vaccination was first successful in Nagasaki, which was the only city open to foreign visitors during the Tokunaga Era. This method became widespread throughout Japan. Dr. Ryusai Kuwata (Bunka 8/1811-Keio 4/1868) from Edo (modern Tokyo) made this color woodcut print to advertise the effectiveness of the vaccination to protect against smallpox; he used this picture at the Osaka Vaccination Clinic. The white cow in the print represents vaccination.

It was known that among persons and families who tended cows, the clinical signs of smallpox were never serious. Following this clue, Dr. Kuwata tried vaccinating children with the contents of eruptions from cowpox. As a result, the children's illness was mild and not transmitted to others. Dr. Kuwata, who was the pioneer of vaccination in Japan, vaccinated more than 70,000 people. He died with a vaccination needle in his hand in 1868, when he was 58 years old.

[Translation of front covertext]
Use of Vaccine Has Spread from Osaka to Edo (Tokyo) for the Benefit of Mankind

I wonder who in the world named Smallpox a God? It is no more than a demon that deviated from the path of mankind.

There are approximately five ways of treating smallpox; one is called the vaccinia method. In the 1790s, a Dutchman [sic] named Edward Jenner announced this method. It is said that when this method was first used, a vaccine made from cow's blood was directly injected into patients. It was quite astonishing that this vaccine, which was made from blood drawn from cow udders and was used on children, cured smallpox.

The first form of this vaccine was the Dutch form, but the Chinese form was used after 1806. The Chinese form is currently being used in Japan, but many forms and methods are used throughout the world. In this manner, vaccination has become a method of treating smallpox.

We began using the Dutch form of the vaccine in the latter half of the 1800s. Thanks to the patronage of our loyal customers, the use of our vaccine has spread widely.

(Translator's note: At the end of this advertisement, there is a poem, the gist of which is, "Parents should do whatever they can to ensure the well-being of their children.")

[Translation of back cover text]
What You Should Know about Vaccinations

1. Babies should be vaccinated on the 70th day after birth, regardless of the season. However, babies may be vaccinated sooner if an outbreak of smallpox has already occurred nearby.

2. After receiving the vaccine, patients should not become infected with smallpox, but patients who come into contact with the smallpox virus before being vaccinated should provide a vaccination document that states "smallpox" and "vaccine" to the physician within 10 days after the vaccination.

3. If patients become infected with another disease during the vaccination period, they should, at their own responsibility, request to be examined by other physicians and receive medicine for the disease.

4. During the vaccination period, a patient may bathe every day in a bath of warm water up to the hips.

5. Patients must return to the original medical institution and undergo another examination 8 to 10 days after receiving the vaccination. If a patient fails to receive the second examination, the "vaccination complete" certificate will not be issued.

6. A person who has received the "vaccination complete" certificate must be vaccinated again 6 years later and must receive a third vaccination 6 years after that. In either case, the patient must have a physician confirm that the vaccination was successful; provided, however, that if an outbreak of smallpox occurs nearby during that period, another vaccination may be administered before the end of the 6-year period.

Cover text translation by Bonnie and Masahito Mikami

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy Reviews, and Dispatches. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

Title page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use WordPerfect or MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is 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 D 61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

Types of Articles

Perspectives, Synopses, Research Studies, and Policy Reviews: Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change; human demographics and behavior; technology and industry; economic development and land use; international travel and commerce; and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses: This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

Research Studies: These articles report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (e.g., "Here is what we found, and here is what the findings mean").

Policy Reviews: Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

Dispatches: These brief articles are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

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.