EMERGING INFECTIOUS DISEASES

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Emerging Infectious Diseases: The New Zealand Perspective

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Cover: The Arrival of the Maoris in New Zealand (1898) by Louis J. Steele and Charles F. Goldie. Auckland Art Gallery Toi o Tamaki, bequest of Helen Boyd, 1899.

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Emerging Infectious Diseases in an Island Ecosystem: The New Zealand Perspective

John A. Crump,* David R. Murdoch,† and Michael G. Baker‡
*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Canterbury Health Laboratories, Christchurch, New Zealand; and ‡Institute of Environmental Science and Research, Porirua, New Zealand

Several unique features characterize infectious disease epidemiology in New Zealand. Historically, well-organized, government-run control programs have eliminated several zoonoses. More recently, however, communicable disease control has been mixed. Rates of rheumatic fever, tuberculosis, and enteric infections are high, and rates of meningococcal disease are increasing. These diseases are overrepresented in New Zealanders of Polynesian descent, who generally live in more deprived and overcrowded conditions than do those of European descent. Measles and pertussis epidemics are recurring because of inadequate vaccine coverage, despite a well-developed childhood immunization program. A progressive response to the HIV epidemic has resulted in relatively low rates of infection, particularly among injecting drug users; however, the response to other sexually transmitted infections has been poor. A key challenge for the future is to build on successful strategies and apply them to persisting and emerging infectious disease threats in a small, geographically isolated country with limited economic resources.

Unique Historical and Epidemiologic Features

New Zealand (known as Aotearoa in Maori), a South Pacific nation with a population of 3.8 million, is the largest island group in Polynesia (Figure). It shares strong biologic similarities with other islands in Polynesia, although it is often wrongly grouped with Australia. New Zealand has several unique features of special interest in the study of emerging infectious diseases. These include unusual native fauna, lack of native terrestrial mammals, and recent incursions of exotic fauna. With exotic fauna came a limited range of zoonoses that were successfully controlled and excluded by a strict quarantine system. Furthermore, New Zealand has unusually high rates of some endemic infectious diseases and delayed impact from infectious diseases emerging in other parts of the globe.

New Zealand developed from the margin of the southern landmass of Gondwana. Separation from Australia and Antarctica occurred 100 to 75 million years ago (1). The country is one of the most geographically isolated and remote temperate islands in the world. Until recently, this isolation allowed a peculiar native fauna to evolve in the absence of natural predators and incursions by exotic species. The only native mammals of New Zealand are two genera of bats (*Chalinolobus* spp. and *Mystacina* spp.). Native bird and insect species evolved to fill ecologic niches that in other countries are occupied by mammalian species. Native parasitic arthropods matched the limited range of terrestrial fauna. It is thought that relatively few microorganisms capable of infecting humans existed in New Zealand before the arrival of the first settlers.

The first evidence of humans in New Zealand dates to approximately 700 years ago. The colonizing Polynesian population is now known as the Maori. Strong oral and artistic traditions maintained by the Maori are not particularly revealing of early health history.

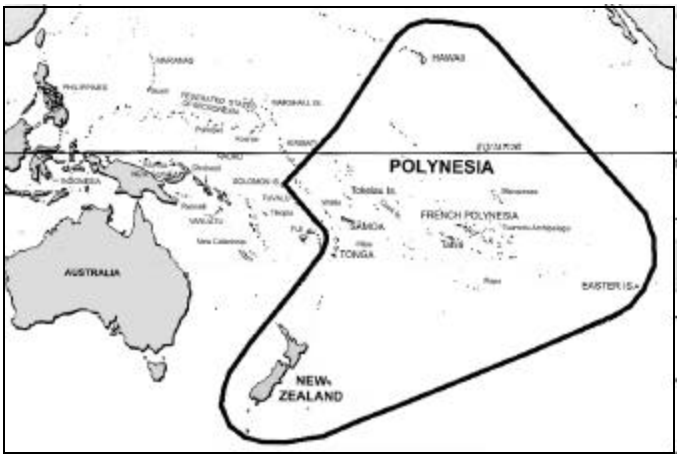

Figure. New Zealand and the region of Polynesia.

Address for correspondence: John A. Crump, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Mailstop A38, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA; fax: 404-639-2205; e-mail: zcn0@cdc.gov

European exploration began in 1642 and continued until 1769. Introduced epidemic disease occurred among the Maori population from the 1790s. The introduction of infectious agents such as *Influenza virus* (2) parallels similar introductions elsewhere in the world. Colonization, primarily from Great Britain, followed in the 1790s. Migrants brought a range of infectious diseases endemic in Europe.

New Zealand's native fauna does not include recognized intermediate hosts for human pathogens. The introduction of exotic terrestrial mammals created a new potential for zoonotic disease. In the 14th century, early Polynesian immigrants introduced the Polynesian rat (*Rattus exulans*) and, much later, the dog. However, the arrival of Europeans with a vast range of exotic species 200 years ago brought about one of the most massive recent introductions of new species into a virgin environment.

British explorer Captain James Cook introduced pigs (*Sus scrofa*) and goats (*Capra hircus*) to New Zealand in 1773. The brown rat (*R. norvegicus*), the black rat (*R. rattus*), and the house mouse (*Mus musculus*) were introduced 150 to 200 years ago. Various animals followed, including hedgehogs (*Erinaceus europaeus*), three species of the genus *Mustela* (stoat, weasel, and ferret), various species of deer (including *Dama dama*), chamois (*Rupicapra rupicapra*), thar (*Hemitragus jemlahicus*), hares (*Lepus europaeus*), rabbits (*Oryctolagus cuniculus*), wallabies (*Macropus* sp.), and the Australian brush-tailed possum (*Trichosurus vulpecula*). In addition, domestic animals, including cats, cattle, sheep, and horses, were introduced (3,4).

Along with exotic fauna came a variety of ectoparasites, some of which were potential vectors for arthropod-borne disease. These included the dog flea (*Ctencephalides canis*), the cat flea (*C. felis felis*), the human body louse (*Pediculus humanus*) and various animal louse species, several culicine mosquito species (including *Aedes notoscriptus*, *A. australis*, and *Culex quinquefasciatus*), a limited number of tick species (including *Ixodes holocyclus*), and the oriental rat flea (*Xenopsylla cheopis*) (5).

Several factors limited the range of zoonoses introduced to New Zealand. These included a small number of animal sources (almost exclusively from the British Isles, Australia, and Chile) and the selection of only healthy stock for transportation. Although settlement and trade often came via Australia, the extended sea voyage to New Zealand during the early European period provided a form of enforced quarantine for some diseases (3,6). Only diseases that could persistently circulate in the crowded conditions of these voyages and those that persisted in a chronic state in humans or animals were imported. The opportunity to take advantage of an island free of most mammalian diseases was recognized by agriculturalists and government, and strict quarantine practices were rapidly put in place. Since primary production has been the main contributor to New Zealand's economy, there is a strong interest in preventing the importation of animal diseases, many of which are potential zoonoses. New Zealand maintains one of the most strict quarantine systems in the world.

This system has been highly successful but has not been immune to biocriminal acts, such as the illegal importation of *Rabbit hemorrhagic disease virus*, probably from Australia, first detected in New Zealand in August 1997. In the absence of natural predators, rabbits thrive in New Zealand and cause considerable damage to grazing pasture. The covert introduction of this rabbit virus appears to have been motivated by frustration among farmers. Although this biosecurity breach has not had detectable consequences for human health, it illustrates the potential for agents to escape even the most vigilant quarantine systems (7).

Emergence and Control of Zoonotic Diseases

With the early importation of exotic animals came a limited but important range of zoonotic diseases. These diseases emerged over the past century with the development of an agriculture-based economy. At its peak in the 1980s, New Zealand had 27 production farm animals per capita. Although this figure has fallen to approximately 18 per capita, the occupational hazards for zoonotic disease in New Zealand agricultural workers are higher than in countries where similar diseases occur but the ratio of humans to animals is lower (3). New Zealand has been successful in the control and elimination of some zoonoses; however, others remain problematic.

The threat of introducing plague from the infested ports of post-penal Australia led to the establishment of the New Zealand Department of Health in the late 1800s. Despite improved quarantine, plague did become established in New Zealand as an epizootic of rats in 1900. Human cases occurred from June 1900 until May 1911. Most cases occurred in Auckland, and only one occurred in South Island at the port of Lyttelton. The disease was controlled by a strict port health inspection system, surveillance of arrivals, fumigation of luggage, rat surveillance, and improved building sanitation. Spread was also minimized as the result of low human population density (8).

Anthrax was introduced into New Zealand in the mid-1890s from Calcutta in unsterilized bone dust fertilizer. Outbreaks declined when public health workers, whose infrastructure had been strengthened by the plague effort, imposed sterilization regulations on imported bone dust. The last case of anthrax was recorded in New Zealand in 1954. It is believed that anthrax spores and bacilli are unlikely to persist in New Zealand soils because of high competitive microbial activity (6). As a precaution, several properties remain under active surveillance for the disease.

Historically, *Brucella abortus* was endemic in New Zealand cattle herds and was an important occupational pathogen in farmers and animal slaughterers. A successful animal vaccination and surveillance system resulted in the last indigenous bovine case of brucellosis in New Zealand in 1989; no further indigenous human cases have been recorded (9). *B. suis* and *B. melitensis* are absent from New Zealand, and *Francisella tularensis* is believed to be absent.

Echinococcosis was probably well established in New Zealand before 1873, when it became notifiable. Annual human incidence reached 7 per 100,000 persons between 1900 and 1925. The risk for disease was five to six times higher for New Zealanders of Polynesian descent. Arecoline hydrobromide was introduced in 1908 for treatment of dogs, and an official education program began in 1938. Neither intervention affected the incidence of hydatid disease. In the late 1950s, a massive national effort was undertaken, including the establishment of 800 local voluntary committees throughout New Zealand, education, promotion, peer pressure, dedicated "hydatids officers," and the introduction of

the Hydatids Act of Parliament that imposed a levy on dog owners and compulsory dog registration. Canine-stool surveillance and use of arecoline continued until 1972, when it was combined with niclosamide treatment (administered every 6 weeks) and, in 1978, praziquantel. By 1990, active surveillance showed that only three farms were still not free of *Echinococcus granulosus*. In 1999, New Zealand was pronounced provisionally free of hydatids. Human cases of hydatid disease now represent distant past infection, and the New Zealand hydatid control program has been widely regarded as a success (10).

Leptospirosis is endemic in New Zealand and is a frequent cause of disease in farmers and meat workers (11). Of more than 180 recognized serovars of *Leptospira*, only 8 have been isolated in New Zealand. These are *L. interrogans* serovars *australis, canicola, copenhageni,* and *pomona,* and *L. borgpetersenii* serovars *balcanica, hardjobovis, tarassovi,* and *ballum*. Of these, serovars *australis* and *canicola* have been isolated only once each, probably reflecting imported disease in the absence of an endemic animal reservoir. Serovars *hardjobovis, pomona,* and *tarassovi* account for >90% of human leptospirosis cases in New Zealand. Approximately two thirds of disease in dairy farmers is due to serovars *hardjobovis* and one third to serovar *pomona*. Swine farmers are more often infected with serovars *pomona* and *tarassovi*. A readily available animal vaccine, combined with improved milking facilities and sanitation, has contributed to a reduction in human cases. The ongoing annual incidence rate of leptospirosis of 5 per 100,000 persons probably reflects underuse of the animal vaccine. Although serovar *balcanica* is maintained in the possum, transmission of *balcanica* from possums to production animals appears uncommon.

Mycobacterium bovis disease is now rare among New Zealanders, partly because of pasteurization of dairy products. However, the elimination of *M. bovis* from cattle herds has proved difficult despite widespread efforts by the veterinary community. New Zealand's maligned Australian brushtailed possum population, estimated at approximately 70 million, emerged as a sylvatic *M. bovis* reservoir in the 1970s and provided a major source of transmission to cattle and deer. Cattle herds in regions of endemic disease are kept under movement control, and a "test and slaughter" policy is in place. The prospect of eliminating animal disease is daunting.

Several zoonotic diseases are notable by their absence. Q fever has been carefully sought for but never found in New Zealand (12). The absence of the disease despite large animal herds, a large farming community, and high levels of animal slaughter is a tribute to careful quarantine, lack of an efficient arthropod vector, and probably an element of good fortune. New Zealand is free of bovine spongiforme encephalopathy because of strict control of animal feed and animal importation. Rabies is also absent from New Zealand, probably reflecting the prolonged duration of the early sea voyage, which exceeded its incubation period in animals; the paucity of indigenous biting animals in the early colonial period; and subsequent strict quarantine practices.

Endemic and Epidemic Infectious Diseases

The control of infectious diseases in New Zealand during the 1980s and 1990s has been mixed, characterized by persisting and emerging threats, as well as successes and failures (Table).

Table. Key infectious disease threats facing New Zealand

Infectious disease category	Important examples	Contributing factors
Vaccine-preventable diseases	Measles Pertussis Influenza	Inadequate vaccine coverage
Respiratory-transmitted diseases	Meningococcal disease Tuberculosis Rheumatic fever	Socioeconomic deprivation and crowding
Enteric diseases	Campylobacteriosis Salmonellosis Cryptosporidiosis Shiga toxin-producing *Escherichia coli* Yersiniosis Marine biotoxins	High density of reservoir animals Long coastline (in the case of marine biotoxins)
Zoonotic disease	Leptospirosis	High levels of infection in animal populations
Diseases from contaminated environments	Legionellosis	Uncertain
Travel-associated and imported infectious diseases	Hepatitis A Tuberculosis HIV/AIDS	High rates of overseas travel Relatively high levels of immigration
Vector-borne diseases	*Ross River virus* *Dengue virus*	Suitable vector mosquitoes and potential NZ habitats
Blood-borne diseases	Hepatitis C	Continuing sharing of injecting equipment by injecting drug users
Sexually transmitted infections	*Chlamydia* Gonorrhea	Increased levels of sexual activity among young people
Hospital-acquired infections	MRSA *Acinetobacter* spp. *Serratia* spp.	Disease burden poorly defined in NZ and no national HAI surveillance system
Diseases caused by antibiotic-resistant organisms	Penicillin-nonsusceptible pneumococci VRE MDR-TB	Microbial evolution and incorrect use of antimicrobial agents

Abbreviations used in this table: NZ, New Zealand; HAI, hospital-acquired infection; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; MDR-TB, multidrug-resistant tuberculosis.

Infectious Diseases of Poverty and Overcrowding

Certain endemic bacterial diseases have emerged as major causes of illness and death, particularly among New Zealanders of Polynesian descent. These are focused around

urban south Auckland and rural parts of North Island, where populations live under difficult socioeconomic conditions.

A serogroup B meningococcal disease epidemic began in 1991 and has persisted for >10 years. Disease rates exceed 12 per 100,000 persons per year, well above those in other industrialized countries (13). Household crowding is an important risk factor, at least for children (14). Identification of the epidemic strain as phenotype B:4:P1.4 provides the opportunity to test the efficacy of a strain-specific serogroup B meningococcal vaccine (15).

After declining for many decades, tuberculosis rates reached a plateau during the 1980s and began to rise during the 1990s. In 1999, 450 cases were reported (12.4 per 100,000), the highest incidence for 20 years (16). Rates among New Zealanders of Polynesian descent are 5 to 15 times higher than among persons of European descent. Immigration from other areas in Oceania (Australia and the Pacific Islands) and high-incidence countries accounts for approximately half the cases each year.

Group A streptococcal disease and its complications are also overrepresented in New Zealanders of Polynesian descent. An average of 100 initial attacks of rheumatic fever have been reported annually in New Zealand over the past 10 years (17). The contributions of environmental and biologic determinants are yet to be established, but there is evidence that both may play a role. Improved management of group A streptococcal disease and secondary prophylaxis for rheumatic fever are research priorities and areas in which New Zealand has special expertise.

Staphylococcal disease is also overrepresented in New Zealanders of Polynesian descent. A recent study of *Staphylococcus aureus* bloodstream infections in Auckland and Christchurch found a rate of community-acquired *S. aureus* bacteremia of 10 per 100,000 per year. Compared with the risk for infection in persons of European descent, the relative risk was 1.8 for indigenous New Zealanders of Polynesian descent (Maori) and 4.0 for nonindigenous New Zealanders of Polynesian descent (18).

Diseases from Contaminated Environments

Legionella infection is an endemic cause of pneumonia, at least in South Island (19), and accounts for 10% of hospital admissions for community-acquired pneumonia. Cases are generally sporadic, and illness is due to various species of *Legionella*.

Vaccine-Preventable Diseases

New Zealand has a well-developed childhood immunization program, including universal infant hepatitis B immunization and a two-dose measles, mumps, and rubella schedule. However, coverage levels remain low, with barely 60% of children fully immunized by the age of 2 years (20). Consequences include recurring measles (21) and pertussis epidemics (22). Hepatitis B has been overrepresented among those of Polynesian descent (23); hepatitis B surface antigen positivity rates in this population are approximately 5%, compared with 0.5% in New Zealanders of European descent. Annual influenza epidemics, which usually peak around July, also have a large health impact. Free annual vaccination has recently been introduced for those ≥65 years of age and those with chronic medical conditions. However, influenza vaccine coverage remains at only 55% in this age group (24).

HIV and Other Sexually Transmitted Infections

New Zealand, like Australia, adopted a very progressive response to the arrival of HIV infection and introduced routine control measures early in the epidemic. As a consequence, the prevalence of HIV infection has remained relatively low, particularly among injecting drug users (25). Most cases of HIV/AIDS are in men who have sex with men. The response to other sexually transmitted infections has been far less adequate; consequently, rates of gonorrhea and chlamydia infection appear to be rising (26).

Enteric Diseases

Enteric infectious diseases rates in New Zealand are among the highest in industrialized countries (27). Culture-confirmed campylobacteriosis occurs at a rate of 305 per 100,000 per year and *Yersinia enterocolitica* at a rate of 14 per 100,000 per year. Furthermore, febrile blood transfusion reactions due to *Y. enterocolitica* occur at the rate of 1 per 65,000 transfusions, reflecting the high number of blood donors with occult bacteremia. These infections occur despite implementation of guidelines to prevent yersiniosis arising from transfused blood (28). Salmonellosis also occurs at relatively high rates in New Zealand. However, the global pandemics of *Salmonella* Enteritidis and *Salmonella* Typhimurium DT104 have not emerged in New Zealand. It is likely that they have been excluded by quarantine practice on animals and animal feed. Giardiasis and cryptosporidiosis (29) rates are also very high. The high rates of endemic enteric infections are not fully understood. The high ratio of domestic production animals to humans and frequent use of rural water supplies in New Zealand have been raised as hypotheses. Perhaps surprisingly, Shiga toxin-producing strains of *Escherichia coli* have emerged relatively recently, but the incidence is now rising rapidly (30). One factor that may contribute to the delayed appearance of this pathogen may be New Zealand's system of pastoral agriculture. Cattle and dairy herds graze year round in open fields, and there is virtually no use of contained feedlots.

New Zealand has a growing aquaculture industry particularly focused on the cultivation of green-lipped mussels (*Perna canaliculus*). As is the case in most temperate regions where commercial shellfish cultivation occurs, nonbacterial forms of seafood poisoning present a potential threat to the industry and human health. A well-developed program of environmental monitoring for dinoflagellate blooms provides an early warning system that prevents potentially toxic shellfish from entering the food chain (31).

Antimicrobial Resistance

Some global infectious disease threats tend to emerge later in New Zealand than in most other industrialized countries. Delayed occurrence also applies to problems of antimicrobial resistance. One notable exception was the early recognition in 1988 in Auckland of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infection. The relatively susceptible Western Samoan phage patterns predominated (32,33). Multiple drug-resistant MRSA strains remain relatively uncommon, particularly in South Island, where routine screening of staff and patients from

areas with endemic MRSA is still an effective hospital infection control practice.

The first vancomycin-resistant *Enterococcus* isolated in New Zealand was reported from Waikato Hospital in 1996. Studies confirm that vancomycin-resistant *E. faecium* and *E. faecalis* are rare (34). The rate of sterile-site isolation of penicillin-nonsusceptible *Streptococcus pneumoniae* was as low as 2.0% in 1993 but rose to 15.1% in 1999. Multiple drug-resistant tuberculosis is very rare and is exclusively imported (35).

Emerging Infectious Diseases

The disease threats described above are likely to persist unless effective control measures are introduced. In some cases, (e.g., enteric diseases), more research is needed to identify effective prevention and control measures. More often, the measures are well defined but require the political will and resources to introduce them, e.g., to raise immunization coverage, create an environment that encourages safer sexual practices, and address such social determinants as overcrowding. Other disease threats are less predictable and may be caused by the emergence of new organisms, changes of known organisms, and the introduction of previously absent organisms.

Emergence of New Organisms

To date, human pathogens found in New Zealand have been identified elsewhere. A possible exception is a rickettsial disease first reported from North Island in the 1990s (36,37). Cases were often reported in possum hunters and clustered around the Kaukapakapa region. The rickettsial species, yet to be determined, is probably from the typhus group and may be transmitted to humans from Australian brush-tailed possums by a flea vector. The implication of these animals as a reservoir of an emerging rickettsial disease would only add to the spectra of infectious disease problems and environmental damage that some exotic mammals cause. New Zealand expends considerable resources in pest control.

Travel and Risk for Imported Diseases

New Zealanders have among the highest per capita international travel rates in the world. Recent data suggest a short-term annual departure rate of 0.31 per capita (38). This compares with 0.22 per capita for Australia and 0.05 per capita for the United States. Oceania and Asia represent the two most popular short-term travel destinations. In addition, New Zealand accepts in excess of 50,000 immigrants annually, predominantly from the same regions. These highly mobile populations provide an ongoing risk for importation of exotic disease. Some of these diseases are capable of becoming established in New Zealand.

Vector-Borne Disease

No cases of vector-borne disease transmission within New Zealand have been identified. Arboviruses are present, but indigenous circulation of an arbovirus that causes disease in humans has not been documented. The Sindbis-like alphavirus, *Whataroa virus*, is established in bird populations on the west coast of South Island, where human infection without disease has been determined (39,40). The unclassified tick-borne arbovirus, *Johnston Atoll virus*, has been documented in gannet colonies (41).

The potential for introduction of arboviruses currently absent from New Zealand poses an important public health threat (42). The greatest threat is from Australia, a nation with a high incidence of arboviral disease and frequent traveler exchange with New Zealand. Travelers viremic with *Ross River virus* (RRV) and *Dengue virus* arrive with some frequency in New Zealand. Competent exotic vector mosquitoes for both these viruses (i.e., *A. notoscriptus, A. australis*, and *C. quinquefasciatus*) are now established in New Zealand. The efficient RRV vector, *A. camptorhynchus* (the southern salt-marsh mosquito), was also recently introduced from Australia (43). *A. albopictus*, a competent dengue vector, was detected in tires imported from Japan in 1993. This cold-hardy mosquito has not become established in New Zealand but could pose a threat in the future. For climatic reasons, *A. aegypti* is probably precluded from New Zealand, except for the extreme north. *C. annulirostris* from Australia (vector of *Murray Valley encephalitis virus*) and *A. polynesiensis* from the Pacific are vectors of RRV and would be of concern if introduced into New Zealand. Furthermore, a low level of community awareness of and involvement in mosquito control caused by the traditional absence of these diseases would compound the public health effort required to control outbreaks (42).

Conclusions and Global Relevance

New Zealand provides a unique model for the study of emerging infectious diseases because of its unusual and sparse indigenous fauna, geographic isolation, and relatively recent exposure to humans and exotic animals. Although zoonoses have provided and continue to provide disease control challenges, the economic and structural capacity of New Zealand has allowed several diseases to be eliminated or controlled. Strict agricultural quarantine practices have prevented the reintroduction of these and other zoonotic diseases and helped control human disease. New Zealand's facilities reflect the capacity and limitations of a quarantine system functioning under near optimal circumstances and illustrate the vulnerability of even this system to biocriminal acts. Leptospirosis and bovine tuberculosis provide ongoing challenges.

Group A streptococcal disease, staphylococcal disease, meningococcal disease, and enteric infections are of particular importance in New Zealand; disparities in disease incidence between ethnic groups are of concern. The reason for the high incidence of these infections is being investigated. These and other key communicable disease indicators suggest that further investments in research and control are warranted. A rickettsial disease, probably of the typhus group, is currently emerging in New Zealand. Vectors are established that are capable of transmitting a number of anthropod-borne infections present in neighboring regions and pose a threat should the organisma be imported. Other infectious diseases consistently emerge up to 10 years later in New Zealand than in other industrialized nations. Among these are Shiga toxin-producing strains of *Escherichia coli* and a number of multidrug-resistant bacteria. Here, New Zealand is in the unique position of being able to test recommendations already established in other countries, relatively early in an epidemic.

Although New Zealand invests considerable resources in preventing the introduction of exotic animal and plant diseases, human communicable disease control and research have been inadequately supported. This can be attributed in part to economic constraints and policy but also to complacency that may have arisen from the country's ecologically privileged status for many infectious diseases.

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Dr. Crump, a New Zealander, is an infectious disease physician and medical microbiologist. He is currently an Epidemic Intelligence Service Officer in the Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia.

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Implementing a Network for Electronic Surveillance Reporting from Public Health Reference Laboratories: An International Perspective

Nancy H. Bean and Stanley M. Martin
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Electronic data reporting from public health laboratories to a central site provides a mechanism for public health officials to rapidly identify problems and take action to prevent further spread of disease. However, implementation of reference laboratory systems is much more complex than simply adopting new technology, especially in international settings. We describe three major areas to be considered by international organizations for successful implementation of electronic reporting systems from public health reference laboratories: benefits of electronic reporting, planning for system implementation (e.g., support, resources, data analysis, country sovereignty), and components of system initiation (e.g., authority, disease definition, feedback, site selection, assessing readiness, problem resolution). Our experience with implementation of electronic public health laboratory data management and reporting systems in the United States and working with international organizations to initiate similar efforts demonstrates that successful reference laboratory reporting can be implemented if surveillance issues and components are planned.

The environment for infectious disease surveillance systems is rapidly changing, and the need to obtain current information about diseases in a specific population is increasing. Disease surveillance in many countries is often fragmented or out-of-date, slow, nonstandardized, inflexible, and not well integrated with respect to both laboratory and epidemiology functions. Emerging diseases (1,2), changes in antibiotic resistance (3), and threats of terrorism with biologic agents (4) have heightened the awareness of surveillance needs worldwide. In this environment, public health officials in many countries desire to improve their surveillance for infectious diseases and look to electronic reporting systems to address deficiencies. Indeed, electronic data reporting from public health laboratories to a national-level surveillance database provides a means for public health officials to identify problems rapidly and take action to prevent further spread of disease (5). However, implementing an electronic surveillance system is considerably more complex than simply applying new technology. Issues such as personnel, funding, politics, and public health policies also must be taken into account.

Well before adopting an electronic surveillance system, those involved in planning it may want to address issues regarding public health systems in general and how these apply specifically to their own country or laboratory. For example, laboratory methods for characterizing and subtyping agents are rapidly changing; new technologies for surveillance can make even recent developments appear obsolete; personnel, public health policies, and politics change, to name a few factors. With these considerations in mind, planners will want to consider flexible laboratory surveillance systems. Planners also need to determine exactly what the surveillance system is to accomplish, whether it is to detect outbreaks, analyze trends, or generate hypotheses (6-10).

Address for correspondence: Nancy Bean, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop C09, Atlanta, GA 30333, USA; fax: 404-639-2780; e-mail: NBean@cdc.gov

Benefits of Electronic Reporting for Surveillance

Electronic surveillance systems meet three broad surveillance objectives: they generate hypotheses, monitor trends, and detect clusters and outbreaks (11). Electronic data transmission enables these objectives to be met very rapidly and often more accurately than with other reporting systems, thus extending the benefits to actually controlling the spread of illness. While the information needs for tomorrow and capacities to meet them can change overnight, the underlying surveillance principles and objectives are constant.

Generating Hypotheses

One role of surveillance is to provide hypothesis-generating data (e.g., demographic characteristics of patients, risk factors for illness, or antimicrobial resistance patterns of infecting organisms). Surveillance databases should not be expected to provide answers to all questions about a particular disease or topic but to comprise a minimum data set to suggest hypotheses about events under surveillance.

Monitoring Trends

Surveillance systems that collect consistent data over extended periods can provide valuable information about spatial, temporal, and demographic changes in disease incidence. For example, the emergence or reemergence of pathogens, changes in antimicrobial resistance, or changes in target populations can be detected rapidly by examining electronic surveillance data. Information on trends or patterns provides a reliable basis for decision making about preventing and controlling disease.

Detecting Clusters or Outbreaks

Before the initiation of electronic reporting, surveillance data may have signaled only that a cluster had occurred; this signal was often of little value in outbreak control since the outbreak may have been over before it was recognized. Detecting clusters often depended on alert laboratorians or epidemiologists recognizing increases in disease occurrence on the basis of their increased workload or their memory rather than actual data. With electronic reporting, data can be transmitted so rapidly that an outbreak can be detected and investigated while it is ongoing and interventions can be implemented. Statistical evaluations of surveillance data reported electronically can be more timely and accurate, and they will have greater value in detecting and curtailing outbreaks early (5,10,12-16).

Planning

Planning might begin with general considerations on how to address surveillance. Even at this stage, perceptions and opinions of planners are likely to differ. The difficulty of arriving at consensus on topics such as the appropriate flow of data can be demonstrated by the discussions surrounding the National Salmonella Surveillance System (17), which have been ongoing since the system converted to an electronic format in 1990. Although it may not be possible to bring closure to all general surveillance questions (6,18), several need to be asked early in planning: What is appropriate and necessary to report? Should international (regional) centers be established for receiving and distributing reports? How can regional centers obtain participation of member countries and ensure appropriate levels of discretion regarding potentially damaging information traveling along the network?

Planners should also research what will be required to support an electronic surveillance reporting system in their country. The suggestion that a country build a network for rapid reporting, analysis, and communications is usually received with enthusiasm (19). Later, when the resources (including personnel) required become apparent, enthusiasm can wane. In every case, inevitable situations exist that could undermine a country's ability to implement and sustain a system. Obtaining software and beginning to report without first planning and establishing the support base for the system spells failure (6). The following issues should be considered by all involved in planning electronic systems.

Availability of Data at All Levels

The purpose of laboratory disease surveillance is to collect appropriate data for each site, analyze these data, and act on the information in conjunction with other public health groups. Although summary data are occasionally collected, they allow for only limited analyses, and sites receiving only summary numbers have little, if any, way to evaluate the data quality (aggregated data are less amenable to editing at sites beyond the reporting site). In contrast, data pertaining to individual disease events provide a basis for multiple analyses, and receiving sites can develop edit routines to detect errors in data reported. Thus, data should be collected at a level of specificity that will provide decision makers the information they need to take action.

Analysis of laboratory data should be encouraged at all levels (11,18). At the local level, public health officials are accustomed to dealing with known levels of specified diseases and are more likely to detect an abrupt change in frequencies as their case follow-up load increases or their demands for certain laboratory services increase. At successively broader-reaching levels of the public health system, there is less front-line knowledge of specific local disease events. When public health data are analyzed and interpreted at all participating levels and appropriate databases are built to satisfy the needs for data at each surveillance level (11), the interval from disease event to problem recognition can be substantially reduced. This is particularly true in the networks of international surveillance. Multinational centers may encourage and assist ministries of health to evaluate their data for unusual clusters and trends, and then assimilate reports of national disease problems into regional assessments. In the United States, for example, reports of *Salmonella* isolates are examined for national outbreaks at the Centers for Disease Control and Prevention (CDC) and also by personnel in state health departments for outbreaks within states.

Continuous System Support

An electronic reporting system needs well-defined, continued support. Experts on disease are needed to establish surveillance relationships, definitions, and ongoing surveillance; and technical experts need to establish system tools and implement business rules (6,8,20). Two other levels of commitment are necessary. First, personnel at the receiving site should establish a cooperative relationship with reporting sites; reach agreement on disease definitions, reporting frequencies, and data elements; and provide feedback to reporting sites (18). Second, reporting sites should agree to use a common surveillance reporting tool that is coordinated and supported by the central site. At CDC, disease specialists (epidemiologist, laboratorians, and statisticians) work together to accomplish the first task, while system trainers and computer specialists manage the laboratory reporting "help desk" to answer system questions by telephone and visit reporting sites to provide training and answer technical questions. The Caribbean Epidemiology Center (CAREC), a Pan American Health Organization (PAHO) comprising 21 member countries, is an example of such an international reporting infrastructure. The CAREC central data-receiving site provides disease reporting specifications, training, and support to member countries, i.e., the reporting sites.

A critical component of system support is building consensus among partners. In the United States, the Council of State and Territorial Epidemiologists (CSTE), the Association of Public Health Laboratories (APHL), CDC, local health departments, and other agencies are often active partners in developing national surveillance systems. CSTE

and APHL meet annually to discuss surveillance issues, system implementation policies, and data dissemination. CAREC meets annually for a joint meeting of laboratorians and epidemiologists from each member country to define surveillance directions, set policy, and develop consensus plans.

Resource Needs

Paper-based systems require resources for data processing (encoding, editing, and data input), reporting, and storage. In contrast, electronic reporting imposes the additional larger burden of training (21), technical assistance, and hardware requirements (Table 1). Resources to sustain a reference laboratory reporting system vary by site and depend on such factors as number of diseases reported, complexity of the system, and number of sites involved

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Table 1. Comparison of paper-based vs. electronic reporting systems for addressing specific surveillance components

Surveillance component	Paper system	Electronic system
Data input	Questionnaire	Data input/import
Reporting	Mail delivery	Electronic transmission
Processing	Coding, editing, storage, input	Maintaining reporting sites
Analysis; interpretation	Merging data; trend analysis	Cluster detection; trend analysis
Feedback	Periodic reports	Electronic feedback

Resource requirements for electronic surveillance may have the largest impact on three areas: personnel, funding, and workload. Probably the most important of these is personnel since the outcome of reference laboratory reporting is largely dependent on the will of the involved personnel to make the system succeed. This highlights the need to assure acceptance of electronic reporting by everyone involved in the planning stages (22). Obtaining support and guidance for the system from organizations representing reporting sites can also encourage acceptance by participating laboratories. For example, APHL was instrumental in developing and implementing electronic reporting from state public health laboratories in the United States (9). During the initial phases, CDC was in constant contact with APHL to determine system specifications and resolve data issues.

Funding resources can vary greatly by reporting site (6,11,22-24). In the United States, state public health laboratory resources are not consistent, ranging from the underfunded state laboratory with outdated computers to the highly funded laboratory with state-of-the-art computer equipment and networks. In the public health community, competition for funding is strong; in some instances, organizations with the most resources can prepare the most effective funding proposals, thereby obtaining more funds than those with more urgent needs. Similarly, in developing countries, resources to replace antiquated equipment for basic laboratory functions may not exist, and establishing electronic reporting may be impossible (24). Obtaining hardware for a laboratory system may depend on external funding (22). For implementing a network hub, resources must be allocated for hardware, software, and personnel. Creating such networks may be very valuable in terms of increased frequency and speed of communication among participants and greater ease of data sharing.

The workload of laboratories in the United States and other countries is overwhelming, and data management and reporting are considered less urgent than simply meeting the day-to-day demands. Thus, system designers need to minimize additional work while demonstrating added value. Rapid reporting of public health laboratory data also imposes additional burdens on epidemiologic resources. As clusters are detected electronically, epidemiologic assessments are needed and interventions are often necessary (5), further increasing workloads.

High-Quality Data at All System Points

Electronic laboratory reporting provides the opportunity for large numbers of records to populate a database rapidly, regardless of data quality. Changes in data origin, acquisition, and reporting practices at reporting sites may remain invisible to the receiving sites. Such variations may cause subtle data misrepresentations, which can adversely affect data quality and cause public health officials to take erroneous action. Recently, for example, an unusual number of multiresistant pathogens were electronically reported to CDC in a single week. The report caused concern and prompted immediate public health action in the belief that a dangerous statewide outbreak was occurring. Further data evaluation revealed a data entry error by untrained personnel at the site of origin. This example highlights the need for constant evaluation of data by trained personnel at both reporting and receiving sites.

Multinational surveillance centers face even more such issues, since data quality varies from country to country. One benefit of having these centers would be to improve a country's existing infrastructure or assist in building one sufficient to provide quality data. Long-term surveillance benefits are much greater when electronic reporting systems use the established public health infrastructure rather than bypassing it (19,25). Systems bypassing public health infrastructures will likely have data of no higher quality than those reported through official channels.

CAREC is an example of a multicenter international organization that has taken a leadership role in improving infrastructure in participating countries. The 21 CAREC member countries vary dramatically in their public health laboratory resources. CAREC has been instrumental in providing hardware, software, and training to these laboratories. A week-long training workshop on laboratory reporting that CAREC conducted for member country epidemiologists and laboratorians strengthened the laboratory data management infrastructure and led to a greater commitment to electronic reporting.

Balancing International Data Sharing and Country Sovereignty

Each country should maintain jurisdiction over its data and the extent to which data originating within its borders are distributed and published. Political and economic issues concerning data reporting for surveillance purposes should not be overlooked. The consequence of a regional center's dis-

closing data outside a country while a serious disease problem is occurring within the country might be economically and politically devastating. For example, a report of a food-associated disease can prevent acceptance of food products exported from the country and may inflict havoc on its tourism industry (14,26). Local participants in an electronic surveillance system should be notified immediately if the national site detects an unusual cluster in the area.

Considering the sensitive nature of some disease problems, a two-pronged international public health surveillance network would be acceptable in many places and is often used, i.e., a formal surveillance network and an informal communications network (12,13,27-29). The formal network comprises information in the public domain that is open via authorized access and might include controlled access by the public. The informal network, providing information and data summaries not appropriate for public knowledge but needed to alert appropriate public health officials about potential (possibly unconfirmed) disease problems, serves a restricted group of users and is open only to key public health officials participating in the surveillance effort. The formal network provides the public with limited information that has been confirmed and is considered correct and final, and the informal network, based on possibly preliminary and incomplete data, serves the function of quietly alerting national officials about a potential disease problem outside their country that might affect their own population. Such a strategy must have exceptions or be modified in some situations. For example, a PAHO subregional center (20) may provide laboratory services for member countries that do not individually have resources to perform these functions. In this case, the data flow is reversed, since data from individual countries are generated at the center's laboratory (e.g., by *Salmonella* serotyping) instead of being reported to the center. The center is responsible for analyzing the data and rapidly returning appropriate results to the country. The center and the individual country become coowners of the data, but their responsibilities differ: The center is responsible for sending data and analysis results to the country; the country is responsible for interpreting the results and acting appropriately.

Clearly, an information international network has the benefit of providing public health officials a worldwide perspective on disease trends and international outbreaks. This in turn provides the mechanism for public health officials to rapidly identify worldwide problems and take international action to prevent further spread of disease.

Strong Leadership

Because the topic of disease surveillance can be politically sensitive, adding electronic reference laboratory reporting tends to raise political interest (6,18). Competing organizations within the public health system may have conflicting interests that hinder effective implementation of electronic systems. These issues should be identified in the planning phase; personnel representing different interests should participate in the planning and consensus on key issues. Even so, internal issues will arise that can substantially diminish efforts to implement an effective system. Leaders in the planning process should be persons or organizations knowledgeable about electronic systems, focused on the goals and purposes of surveillance, and able to resolve conflicting viewpoints.

In the United States, CDC in conjunction with CSTE is integrating the numerous approaches now used to report the list of notifiable and reportable diseases. Although the list itself is a unifying point of agreement among the state and federal governments (30), approaches to building databases for these reports vary widely, as do solutions for how diseases should be reported (23). By assuming the leadership and problem-solving roles and pursuing a collective effort to integrate these approaches, these groups anticipate reducing the reporting burden at local, state, and federal levels (31).

Components of Successful Electronic Reporting

A successful electronic surveillance system has many components. Surveillance systems and software for surveillance are not equivalent. The following items should be considered in initiating such a system.

Authority

Public health reference laboratory reporting should be supported from the highest office with the authority to mandate public health offices and officials to participate (6,22) (e.g., Ministry of Health). Without the mandate of official authority (including funding and infrastructure), surveillance systems may be built but operate successfully for only a short time. In a multinational surveillance system, all participating countries should have such a clear mandate; it should not be assumed.

Standardizing Disease Definitions

Standardizing disease definitions among reporting sites is a critical component for data analysis. This role is taken by the central receiving site (32). The public health community has developed standards for use in case and laboratory electronic reporting (33-35); these could be implemented in international reference laboratories and clinical laboratories. In some international communities, the public health reference laboratory may also serve as the clinical laboratory.

Although data about cases can be standardized by carefully constructed disease definitions, standardization of laboratory data may be more difficult because data quality is affected by the variability of laboratory competency, methods, workload, equipment, and other factors. Periodic quality checks of laboratory procedures may help increase data quality.

Planning

Planning is a critical component in setting up a reference laboratory surveillance system. Identifying system stakeholders (both laboratory and epidemiology) is often a first step. Hosting a stakeholders' planning meeting can provide a means to address and define system goals, objectives, business rules, functions, and surveillance approaches (e.g., sentinel vs. comprehensive). These meetings also provide an avenue to evaluate and plan for political and technical issues surrounding integration; a workable timetable for system implementation including system pilot testing; data confidentiality, ownership, and dissemination rights at all reporting sites; quality control assessment; identification of

personnel for system management; and creation of a system help desk.

Data Elements

Data collected for surveillance differ from research data. Surveillance should provide the minimum necessary data fields to understand the current disease situation. The tendency to ask for data about every factor potentially related to each disease condition should be curtailed, as it will burden both the plan and reporters.

System Support

Initially, a support plan may be developed for each site to address personnel turnover, personnel responsibilities, and backup procedures. After reporting has begun, all system users should have a point of contact for problem solving. If the central reporting site is also the developer of the surveillance software, user-support personnel fill this role and can be a continuing resource for identifying needs for future enhancements. User groups can also be a means for system or software developers to receive system specifications and enhancements. Support personnel for electronic reporting can be generally grouped according to function (Table 2). Although numbers of persons will vary, personnel performing each function should be specifically identified (6,21,36). In some settings, one person may be assigned more than one task. When a data transmission system is down and surveillance is interrupted, resources and support should be available at each site to make system recovery immediate (6). Delays can deliver an implicit message to participants that timeliness is not important

The responsibility for analyzing electronically reported data and returning results rapidly to the reporting sites may be specifically designated. The complexity of analysis and volume of data will determine the need for statistical, programming, and epidemiologic resources (36). At the central receiving site, these resources should be allocated in the initial planning stage and should not be diverted to other areas after its implementation.

The support structure in state and international public health laboratories varies according to the funding and the complexity of the data management system. For example, in smaller laboratories with limited computer resources, one person may serve many roles (e.g., site supervisor, data manager, programmer). In a larger laboratory (the central receiving site), many people perform these roles. In CDC's Division of Bacterial and Mycotic Diseases (in which approximately 100,000 isolates per year are reported electronically) one computer programmer, one epidemiologist, one statistician, and one health communicator are actively involved with data analysis, system support, and feedback to 100 reporting sites.

Assessing Country Readiness

Assessing a country's readiness for electronic surveillance before a system is implemented involves many elements. The existing laboratory infrastructure should be evaluated to ensure commitment to the project. Potential participating laboratories (reporting sites) should also be evaluated to determine technical capabilities and requirements (e.g., access to an Internet service provider, access to deicated quality phone lines, computer knowledge, determination of hardware requirements, and availability of computer maintenance support). Assessment of professional staff support to determine political concerns (e.g., data sharing between laboratory and epidemiology sites), and evaluation of staff personnel requirements to provide effective maintenance of surveillance are also important. Ongoing ources of financial and political support should be contacted to determine if long-term commitment to the surveillance system exists among those in authority to support the projects.

Table 2. Functional roles in electronic laboratory reporting for surveillance

Functional role[a]	Location	Functions
Central system administrator	Central receiving site	Coordinate sites' uses of system; train site supervisors; define users to system; solve problems; distribute upgrades; expand system for new diseases
Site supervisor	Reporting site	Define users to system; train subordinate site supervisors; solve problems; distribute upgrades; expand system for new diseases
Technical resource	Central receiving site Reporting site	Resolve hardware and communications problems
Data manager	Central receiving site Reporting site	Input or import data; receive data from subordinate sites; review data for quality; export data for analysis
Programmer	Central receiving site Reporting site (optional)	Perform programming tasks for data analysis and feedback
Statistician	Central receiving site Reporting site (optional)	Perform analyses and consult with epidemiologists
Epidemiologist	Central receiving site Reporting site (optional)	Interpret analyses with statistician and laboratorians; take appropriate actions

[a]More than one task may be assigned to each person.

Site Selection and Cooperation

Each public health site has a different surveillance environment, with varying levels of interest and expertise as well as differing perspectives about surveillance and software tools, funding levels and sources, political atmospheres, and disease problems affecting daily workloads. Such differences should not be overlooked in enlisting sites.

The central site should coordinate selection of reporting sites on the basis of what they can contribute to the surveillance effort and their desire to participate. Persons at the

central receiving level who have particular interest and knowledge about the disease should enlist the appropriate sites, provide definitions and consultation, and assist in outbreak or other investigations. At CDC, each implementation of a new laboratory reporting module (a set of specifications for each reporting condition) is spearheaded by an epidemiologist with close assistance of technical staff. Electronic reporting will benefit substantially when two persons with distinct job functions work together to prepare reporting sites. For example, having epidemiologists prepare personnel at the participating sites for their expected role in the system before contact is made by the technical support team should help to ensure that site epidemiologists and laboratorians have been given adequate specifications and that the capabilities of the site to participate have been determined.

Site Preparation and Training

As each reporting site joins the electronic reporting system, all persons participating at that site should be trained to perform the duties associated with surveillance and the system tools (11,22,36). Various approaches to system training may be used.

In one approach, trainers from the central site visit the reporting sites to train laboratorians and epidemiologists to use the system and assist with issues specific to their environments. This strategy was demonstrated in PAHO-sponsored electronic laboratory training conferences in Trinidad, Venezuela, and Argentina. Although it involves substantial travel costs to the central site, more people are usually trained than if each site sends personnel to the central receiving site, and the training is conducted in the reporting site's environment.

A second approach is for participants from reporting sites to visit the central receiving site for training, as occurred when personnel from the Ireland Food Safety Authority visited CDC for training. This approach is efficient for the central receiving site because training can be coordinated into a single large session without incurring travel time.

A third hybrid of these approaches, especially appropriate in the international setting, provides a training workshop at the central site attended by representatives from the reporting sites and follow-up with onsite training. Bringing a group together at the central site creates an environment that induces interactions among participants that may not likely occur otherwise. All these approaches, however, have a common goal: to train people who can then train others at their sites.

Often, initial training is lost as the organization experiences personnel turnover, system enhancements, or infrequent use of the system. The central receiving site may want to be alert to signs that retraining might be needed. A plan could be devised initially to provide training to representatives from all sites, follow-up to ensure that the training was effective, and retraining when necessary.

Software/Hardware Distribution

Often, surveillance systems are funded in part by the central receiving site (24) or by sources outside the public health domain. Hardware provided by these sources may arrive at sites with no provisions for its disposition. The support team should ensure that equipment from either source arrives at the correct location and is tested and operational, that software is properly installed, and that equipment is not diverted to another project.

Software selection presents additional considerations. Software designed for collecting and reporting data about only a single disease can be designed in every detail to accommodate the needs for that particular surveillance effort. However, the broader perspective for surveillance will embrace software that is designed for multiple disease reporting purposes and that can be easily adapted for many different environments and reporting requirements. In either case, software should be regarded by its users as easy to use and comprehensive in its functions (8). The tendency is to begin laboratory reporting by shopping for software that might perform data management without concern about surveillance goals. Rather, one may want to select software on the basis of the functions it can bring to the system (10,15). If a software package has been developed and successfully implemented for laboratory reporting, then implementation or modification of existing software may be more efficient.

Pilot Test

Successful laboratory implementations (9) include a pilot phase in which questionnaires, equipment, and personnel are tested. Pilot phases ensure that data being reported from sites meet system specifications. Later, as new disease conditions are added to the system, they should also be tested.

Problems identified during the pilot testing phase can range widely—from poor software performance, communication problems, and data issues to funding and resource problems. Each problem should be addressed and its solution retested before actual reporting is begun. In the United States, pilot testing of the reporting system was done in five state public health laboratories. When problems reported to CDC were resolved, these laboratories were able to assume a leadership role in implementing the system in other state laboratories.

Feedback

Often, surveillance systems are dependent on voluntary or mandatory reporting without compensation (34). To assure that the system's value is recognized, central receiving sites should provide meaningful feedback to the reporting sites (18). Feedback should provide information needed by the sites and stimulate sites to input data in a timely way. Feedback on recent trends and current multisite clusters of disease should take precedence over bulky reports and detailed tabulations, which, although useful for fiscal accounting or periodic disease assessment, provide little incentive for timely reporting.

Summary

Our experience with implementing electronic public health laboratory data management and reporting systems in the United States and with working with international organizations to initiate similar efforts demonstrates that successful reference laboratory reporting can be implemented if surveillance issues and components are adequately planned for. The public health can benefit when data arrive at analysis sites so rapidly that outbreaks can be detected, responses initiated, and interventions imple-

mented in time to prevent cases that would otherwise have occurred. Resources focused on an electronic reporting systems for public health laboratories and efforts to sustain the system are worthwhile when the system rapidly makes data available to describe current disease situations. Initiating electronic reporting opens a new paradigm for conducting surveillance—one that is highly challenging but increasingly necessary.

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Dr. Bean is the Chief of of Biostatistics and Information Management Branch and project leader of the Laboratory Information Tracking System in the National Center for Infectious Diseases, Centers for Disease Control and Prevention. Her research interests include reference laboratory infrastructures, outbreak detection, design and implementation of reference laboratory information management systems, and electronic laboratory surveillance.

Dr. Martin is the former Chief of the Biostatistics and Information Management Branch and former project leader of the Laboratory Information Tracking System at the National Center for Infectious Diseases, Centers for Disease Control and Prevention. His research interests include reference laboratory infrastructure; outbreak detection; design and implementation of reference laboratory information management systems; electronic laboratory surveillance; and *Salmonella* stereotypes trend analysis.

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Potential Infectious Etiologies of Atherosclerosis: A Multifactorial Perspective

Siobhán O'Connor,[*] Christopher Taylor,[†] Lee Ann Campbell,[‡] Stephen Epstein,[§] and Peter Libby[¶]

*Centers for Disease Control and Prevention, Atlanta, GA, USA; †National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA; ‡University of Washington, Seattle, Washington, USA; [§]MedStar Research Institute, Washington Hospital Center, Washington, DC, USA; and [¶]Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

Coronary heart disease (CHD) contributes substantially to illness and death worldwide. Experimental studies demonstrate that infection can stimulate atherogenic processes. This review presents a spectrum of data regarding the link between CHD and infection. In addition, the need for improved diagnostic tools, the significance of multiple pathogens, and potential intervention strategies are discussed.

Cardiovascular disease (CVD) from all causes accounts for 29% of deaths worldwide and ranks second only to infectious and parasitic diseases (1). Deaths from CVD are often premature, and millions of nonfatal events result in disability. Atherosclerosis, a major component of CVD, has properly been considered a public health problem of industrialized countries, accounting for an estimated one third of deaths overall (1). In the United States alone, atherosclerosis reportedly affects one in four persons, causing approximately 42% of all deaths. Approximately half of these are due to atherosclerotic coronary heart disease (CHD) (2-4). Atherosclerotic CVD now threatens developing countries as well, perhaps reflecting greater exposure to certain risk factors with rising standards of living (5). In India, for example, higher socioeconomic status correlates with increases in risk factors for and rates of CVD (5). Thus, the need for public health measures to limit its impact is expanding worldwide.

Many persons with atherosclerosis, however, lack identifiable traditional risk factors. The well-recognized influences of smoking, diet and exercise, hypercholesterolemia, hypertension, diabetes, and some genetic factors account for much less than 100% of disease. Despite continued reductions in the prevalence of modifiable risk factors, the steady, two-decade decrease in cardiovascular mortality rates in western industrialized countries has reached a plateau (2-4). In contrast with India, death rates for chronic (including atherosclerotic) and infectious diseases in the United States are inversely related to education and income (4-6). These observations fuel renewed interest in a link between atherosclerotic CVD and as-yet poorly defined environmental exposures, including infectious agents. If epidemiologic and laboratory evidence eventually supports the association, atherosclerosis could emerge as another noncommunicable chronic condition related to infection.

Address for correspondence: Siobhán O'Connor; Centers for Disease Control and Prevention, 1600 Clifton Rd. NE, Mailstop C-12, Atlanta, GA 30333, USA; fax: 404-639-3039; e-mail: sbo5@cdc.gov

How strong is the link? This review examines several candidate infectious agents, referring to reports and reviews by investigators summarizing their own work and a body of research. It discusses the potential roles of less well-established factors, interactions between infection and traditional risk factors, and unanswered questions on etiology, pathogenesis, and appropriate interventions. Developing an appropriate research agenda and public health response requires clarification of these issues. The following discussion provides a foundation for future studies.

Infection as an Inflammatory Stimulus

Atherogenic processes resemble many aspects of chronic inflammation (7), a response that may be promoted by microorganisms (8-10). Accordingly, reviews have revisited the venerable hypothesis of an infectious etiology (8,9,11). Experimental animal studies have shown that bacterial and viral agents could contribute to atherogenesis. Both *Chlamydia pneumoniae* and cytomegalovirus (CMV), for example, are widely distributed, can infect blood vessel wall cells, and exhibit persistence, latency, and recurrence of infection. However, the potential mechanisms of infection-induced atherosclerosis remain speculative.

The earliest lesions of atherogenesis consist of arterial intimal accumulations of foam cells (primarily lipid-laden macrophages) and T lymphocytes intermixed with smooth muscle cells (7). Infection could indirectly influence this process without infiltrating the artery wall (Figure 1). Host defenses to extravascular infections usually elicit proinflammatory cytokines and stimulate increased expression of cellular adhesion molecules, enhancing leukocyte adhesion. These cytokines could elicit a second wave or "echo" from inflammatory cells already at sites of atherogenesis, such as arterial wall cells or macrophages (12). Circulating microbial products such as endotoxin can also produce an echo. Similarly, cytokines induced by extravascular infection (specifically interleukin-6) characteristically elicit hepatic synthesis of acute-phase reactants, some of which might promote

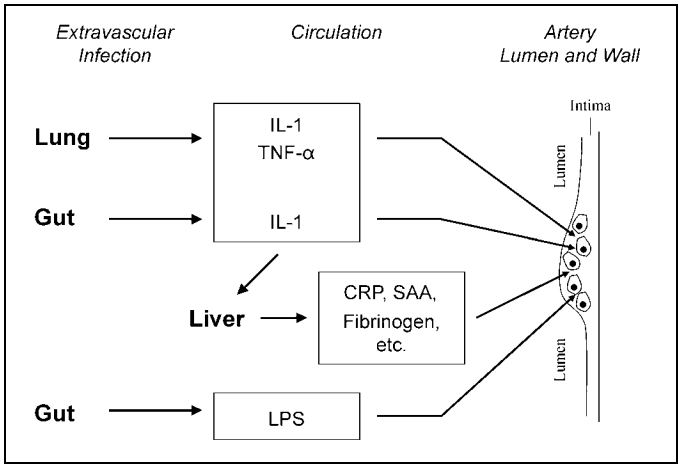

Figure 1. "Echo" hypothesis: activation of atheroma-associated cells by bacterial products and cytokines released in response to extravascular infection. a. Extravascular infection stimulates production of inflammatory cytokines, e.g. interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), that can elicit an echo cytokine response from inflammatory cells in residence at sites of atherogenesis. Circulating microbial products, e.g. endotoxin, can also elicit an echo response at the artery wall. b. Extravascular infection-elicited cytokines stimulate hepatic synthesis of acute-phase reactants. Some factors, e.g. fibrinogen, might influence complicated atheromata formation or arterial thrombosis.

atheromata complicated by thrombosis. Accordingly, levels of the acute-phase reactant fibrinogen correlate prospectively with risk for coronary events, and plasminogen activator inhibitor can promote clot stability by interfering with fibrinolysis (7-10). Still, direct infection of the arterial wall could promote evolution of atherosclerotic lesions or precipitate acute cardiovascular events. These potential effects are discussed below.

Chlamydia pneumoniae Association

Lines of evidence associating *C. pneumoniae* with atherosclerosis include seroepidemiologic studies, direct detection of bacterial components in atherosclerotic lesions, occasional isolation of viable organisms from coronary and carotid atheromatous tissue, and in vitro and animal experiments (reviewed in 8,9,13,14). Most cross-sectional and prospective studies have correlated seroprevalence with myocardial infarction, chronic CHD, or stroke (8,13,15). However, the diagnostic criteria used, cut-off titers to define seropositivity, adjustment for confounders (e.g., smoking), and sample sizes have varied notably. Indeed, as the interrelationships of smoking, *C. pneumoniae* infection, socioeconomic status, and atherosclerotic CVD are further investigated, epidemiologists continue to discuss which data adjustments are most appropriate (16-19). Interpretation of these studies is complicated by a lack of standardized serologic methods and by interlaboratory variations and poor reproducibility in microimmunofluorescence test results (20-22). Temporal variations in antibody and antigen titers and the high prevalence of infection–more than 50% of adults have been infected at least once–also complicate interpretation (13). Still, more than 38 studies have reported a positive association between antibodies to *C. pneumoniae* and atherosclerotic disease. Only four studies did not confirm this association, including two recent, large, well-controlled investigations in which anti-*C. pneumoniae* immunoglobulin (Ig) G titers did not correlate prospectively with risk for coronary events in men or women (18,23-25). Additionally, the prospective Atherosclerosis Risk in Communities Study correlated IgG titers $\geq$1:64 with incident CHD, but the relationship did not persist after the data were adjusted for several cardiovascular risk factors (26).

The strongest evidence associating *C. pneumoniae* with atherosclerotic CVD has been detection of bacterial components in atherosclerotic lesions. *C. pneumoniae* appears to have a tropism for atheromata. It is rarely found in normal arteries or infectious and noninfectious granulomas and is documented more frequently in atheromata than in lung or other tissue from the same patient (13). Over 30 peer-reviewed publications from investigators worldwide, using different diagnostic methods, have localized *C. pneumoniae* antigen, DNA, or both in atheromata; three reports did not find such components (8,27,28). Although detection rates have varied depending on the diagnostic methods used, the cumulative evidence supports existence of the organism in many lesions (8,27). These histologic findings, however, do not establish a causal role for *C. pneumoniae* in atherogenesis. Despite improved yields, culture of *C. pneumoniae* from atheromata remains difficult (8,13,29). Moreover, while the presence of a viable microbe or its components at a site suggests that it may initiate or exacerbate disease, it does not prove pathogenesis.

Detection of *C. pneumoniae* antigens or DNA in intimal thickening and fatty streaks of young adults and Alaskan Natives (the latter group at low risk for coronary atherosclerosis) supports an early microbial role in pathogenesis (30). Postmortem, the Alaskan retrospective study also positively correlated prior systemic infection with evidence of *C. pneumoniae* in atherosclerotic lesions; studies from Seattle reported a slightly higher detection rate in late-stage lesions (13). Recently, investigators reported a statistically significant relationship between the presence of *C. pneumoniae* and the severity of human coronary atherosclerosis (31). Although others observed no correlation between *C. pneumoniae* and disease severity in the same coronary artery, evidence of the bacterium was found in 80% of patients' arteries examined (32).

Detection of *C. pneumoniae* in plaques has not correlated well with serology (8,33), so investigators have attempted to predict endovascular infection through polymerase chain reaction (PCR) detection of microbial DNA in peripheral blood monocytes. The prevalence of *C. pneumoniae* DNA in these mononuclear cells has varied between studies (perhaps due to differences in both assay sensitivity and extraction procedures), but was 59% in coronary angiography patients compared with 44% in blood donors in one series; the rate appears to increase with age (33,34). Several but not all investigators have now correlated PCR-positive mononuclear cells with clinical CHD (34,35) or the detection of *C. pneumoniae* nucleic acid in atherosclerotic aortic tissue (36).

In vitro studies support hypotheses that *C. pneumoniae* might directly promote atherosclerosis (Figure 2). Infection of human endothelial cells augments their production of inflammatory cytokines and modulates expression of adhesion molecules, enhancing recruitment of inflammatory leukocytes to the vessel wall (8,37,38). Chlamydial endotoxins, much less virulent than those of enterobacteriaceae (e.g., *Escherichia coli*), can promote macrophage foam cell formation in vitro (8,39). Furthermore, *C. pneumoniae* heat shock

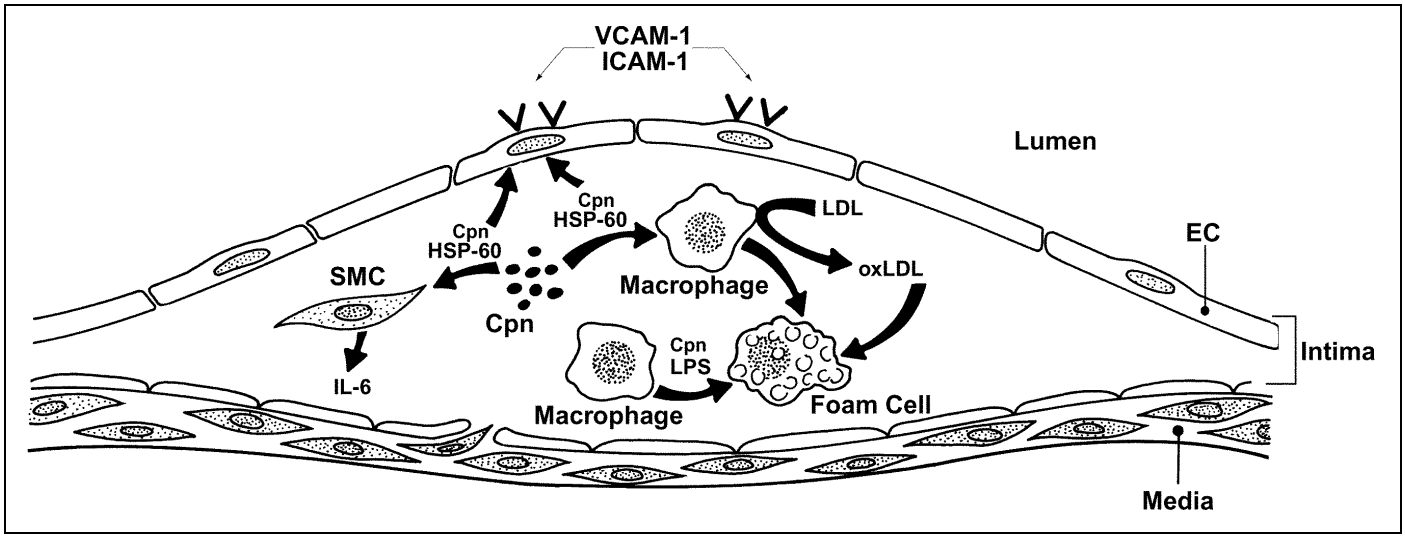

Figure 2. Possible direct effects of *Chlamydia pneumoniae* (Cpn) on atheromata. Cpn infection augments endothelial cell production of inflammatory cytokines and expression of adhesion molecules, e.g., vascular cell adhesion molecule (VCAM)-1, enhancing leukocyte recruitment to the arterial wall. Chlamydial endotoxin (LPS) may promote macrophage foam cell formation at the site. Chlamydial heat shock protein (HSP-60) may elicit proinflammatory functions from arterial wall macrophages, endothelium, and smooth muscle cells (SMC), or promote macrophage oxidation of lipoproteins.

protein (HSP-60) can activate both macrophage tumor necrosis factor-alpha and expression of matrix-degrading proteinases that may weaken atherosclerotic plaques, rendering them susceptible to rupture and hence thrombosis. HSP-60 can induce proinflammatory activities in macrophages and vascular cells (e.g., endothelium, smooth muscle cells) and promote oxidation of lipoproteins by macrophages (10,40).

Animal experiments have also explored the link between *C. pneumoniae* and atherosclerosis. Intranasal or intratracheal *C. pneumoniae* inoculation of New Zealand white rabbits fed a normal diet produced inflammatory changes of the aorta (41-43). Although one group did not observe foam cells in inflamed sites, another has reported foam cells in lesions resembling early atherosclerosis. Macrolide antibiotic therapy appeared to dampen the additive effects of hypercholesterolemia and serial *C. pneumoniae* infection (9,41,43); macrolide antibiotics with antichlamydial activity administered early in infection could prevent changes, but delayed treatment had little or no effect (41). These observations, along with diminished effects on cholesterol-induced lesions (in the absence of infection), suggest antimicrobial mechanisms of action.

Inflammatory changes without foam cell lesions and typical atheromata have also been observed in *C. pneumoniae*-infected mice fed a normal chow diet (13,44). However, *C. pneumoniae* infection does potentiate the atherogenic effects of hypercholesterolemia in three mouse models. Intranasal inoculation of apolipoprotein E-deficient mice fed a regular chow diet accelerated aortic arch plaque development (45); similar changes occurred in low-density lipid receptor-deficient mice on a high-cholesterol regime but not those on a regular diet (46). In C57BL/6J mice fed a high-fat, high-cholesterol diet, *C. pneumoniae* infection accelerated the development of atherosclerotic lesions in the aortic sinus (44,47).

Although experimental animal lesions do not mimic human atherogenesis exactly, similarities have prompted human intervention studies directed against *C. pneumoniae* infection. Three prospective therapeutic trials have been reported, but all had insufficient statistical power to resolve the question (reviewed in 23,48). One described a fivefold decrease in cardiovascular events among seropositive men treated with short courses of azithromycin after myocardial infarction (MI) (49). In a double-blind study of unstable angina or non-Q wave infarction, patients treated for 30 days with roxithromycin had a decrease in cardiovascular events that waned by 6 months (50). Additionally, when 302 MI survivors with *C. pneumoniae* antibody titers >1:16 were treated for 3 months with azithromycin, investigators observed no significant reduction in recurrent events at 6 and 24 months, although a gradual decline in serologic (inflammatory) markers of cardiovascular risk was initially seen (51,52). Three disparate retrospective, multivariate analyses of antibiotic use and CHD risk, using different methods and populations, also reported both positive and negative correlations between past antibiotic prescriptions and CHD or MI risk (48). At this time, any specific effects of treatment remain unclear (23).

Cytomegalovirus Links

Almost two decades ago, several investigators suggested a role for herpesviruses in CVD. Lesions resembling human atheromata developed in pathogen-free chickens infected with Marek disease virus, an avian herpesvirus; immunization with turkey herpesvirus was protective. In vitro, infected arterial smooth muscle cells accumulated cholesterol. Since then, seroepidemiologic, histopathologic, in vitro, and animal studies have investigated possible links between human atherosclerosis and human herpesviruses, primarily CMV (reviewed in 14,15,53-55).

Studies have linked CMV to three arterial diseases: primary atherosclerosis, post-angioplasty restenosis, and post-transplantation arteriosclerosis. For each, seroepidemiology has relied on single measures of viral IgG antibodies, which only indicate previous exposure. Similar to *C. pneumoniae*, the worldwide ubiquity of lifelong, latent CMV infections,

temporal variations in antibody or antigen titers, and viral reactivation could mask or falsely highlight causality. These factors may explain inconsistencies in cross-sectional, retrospective, and prospective studies attempting to correlate seroprevalence or higher IgG titer with primary atherosclerosis (10,14,15,24,56-58). Differing disease classifications (based on histopathology of vascular surgery, general autopsy or atherectomy specimens, angiography, noninvasive carotid imaging, or clinical history) and epidemiologic methods (small sample sizes, diminished statistical significance after data adjustment, nonuniform consideration of other risk factors) also limit interpretation (14,53,55). Results are conflicting even in prospective, nested case-control investigations focused on but not limited to male Caucasians. The Atherosclerosis Risk in Communities Study correlated preexisting high anti-CMV titers and traditional risk factors with carotid atherosclerosis, incident MI or CHD death (relative risk 1.76; 95% confidence interval 1.00-3.11) during a 5-year follow-up period (57). In contrast, the 12-year Physician's Health Study found no association between antibody prevalence and subsequent first MI or thromboembolic stroke, or elevated C-reactive protein (56), while the Cardiovascular Health Study correlated antibodies to herpes simplex virus type 1 but not CMV or *C. pneumoniae* with incident events in the elderly (59).

In post-angioplasty restenosis, preexisting and high-titer IgG CMV antibodies predicted restenosis (43% vs. 8%) independently of CVD risk factors, but the sample size was small and no follow-up titer analysis was included (53,60,61). Cross-sectional and prospective studies also link CMV to post-transplant arteriosclerosis (14,62-64), in which a subset of immunosuppressed recipients manifests accelerated disease in the first year.

Evidence of CMV in these disease entities, however, does not prove causality. Different techniques have detected viral antigens and nucleic acids in 0% to 94% of plaques (mean 40%-50%, usually localized to the periphery of advanced plaques), but also in most of the uninvolved arteries from the same patients and >50% of random arterial specimens (14,54,65). A recent PCR analysis of coronary arteries and venous bypass graft occlusions found no CMV in specimens but did detect *C. pneumoniae* (66). Reports of viral nucleic acids in post-transplantation arteriosclerotic lesions, using different techniques, are not uniform (62,63,67,68).

In vitro observations support a potential etiologic role for CMV in atherosclerotic CVD. Indirectly, virally provoked paracrine expression of chemokines and growth factors might stimulate migration of smooth muscle cells from the adventitia and media into the intima, along with smooth muscle cell proliferation, both important components of atherogenesis and restenosis (54). Direct infection of arterial smooth muscle cells appears to augment expression of the CMV chemokine receptor US28 (69). Investigators have also reported inhibited apoptosis and augmented cellular proliferation when the virally directed protein IE2-84 binds or inhibits transcriptional activity of p53 (54,70). Direct CMV infection may also enhance uptake of oxidized low-density lipoprotein by macrophages and smooth muscle cells, promoting foam cell development; through its immediate-early gene products, CMV may increase uptake of modified low-density lipoprotein and stimulate type A scavenger receptor gene expression by smooth muscle cells (54).

CMV animal experiments also do not duplicate human disease but can provide insight on causality. While CMV infection of rats several months preceding or during balloon injury to the carotid artery augmented neointimal thickening (54,71), infection 14 days after injury did not (72). In ApoE-deficient mice, systemic infection was reported to increase atherosclerotic lesion size (54); local infection accentuated subendothelial inflammation and coronary arteriosclerosis in rat cardiac and aortic allografts, apparently reduced by ganciclovir (DHPG) treatment (55,73). Thus, CMV remains potentially linked to the promotion of atherosclerotic CVD.

Roles for Other Microbes?

Oral Pathogens

During the past decade, several reports have suggested a relationship between chronic oral infections (e.g., periodontitis) and cardiovascular disease (74). Among the Pima Indians, periodontitis emerged as an independent risk factor for atherosclerotic events (74). Other studies described an association between tooth loss related to periodontal disease and CVD (75), while persons with diabetes have a higher incidence of infections (including oral) and atherosclerotic CVD. Pathogens potentially associated with CVD include *Porphyromonas gingivalis, Bacteroides forsythus, Campylobacter rectus, Fusobacterium nucleatum, Treponema* spp., and *Prevotella* species. At least two, *P. gingivalis* (42%) and *Streptococcus sanguis* (12%), were identified in the periphery of atherosclerotic plaques by immunologic stain (65). Another group detected $\geq$2 periodontal pathogens by PCR for 16SrDNA in 13 of 22 endarterectomy specimens (76).

Researchers have hypothesized that certain persons with a hyperinflammatory phenotype are at increased risk for both periodontitis and atherosclerotic CVD, with dietary and other factors possibly altering disease progression (reviewed in 74). Like *C. pneumoniae*, oral bacteria might affect atherosclerosis through direct invasion of vascular endothelial cells or indirectly through products that stimulate proinflammatory and prothrombotic functions of vascular cells. For example, *P. gingivalis* and *S. sanguis* both express a platelet-aggregation factor (74). However, the true links between oral bacteria and atherosclerotis are still to be determined, requiring further laboratory and carefully adjusted clinical studies.

Helicobacter pylori, Herpes Simplex Virus (HSV), and Other Pathogens

The literature linking *H. pylori*, HSV-1, and HSV-2 to atherogenesis is less extensive than for *C. pneumoniae* or CMV (9,14,15,25,53,59). *H. pylori* has not been detected in human atheromata, and available seroepidemiologic data conflict, weakening the proposed etiologic association (9,14,15,25). Social factors linked to CVD and *H. pylori* infection, including socioeconomic status, may confound these reported associations, and data were not adjusted appropriately in most studies (15,77). Nonetheless, an indirect role for *H. pylori* in the pathogenesis of atherosclerosis has not been disproved.

Unlike CMV, HSV-1 has a predilection for epithelial and neuronal tissues, not mononuclear cells. Seroepidemiologic and histopathologic study results vary widely (53,55-57,59).

For example, the prospective, nested Atherosclerosis Risk in Communities Study found no association between incident CHD and preexisting HSV-1 antibodies (57). Although consistent with most other analyses (25,53,56), this contrasts with positive HSV-1 results from the elderly Cardiovascular Health Study cohort (59).

In summary, laboratory investigations suggest but do not conclude that herpesviridae could directly and indirectly induce several endothelial cell responses involved in atherothrombosis (78). The long-term effects of commonly silent or chronic herpesvirus or other persistent infections render plausible a potential role in atherosclerotic CVD.

Key Issues for Future Research on Potential Infectious Etiologies of CVD

Chronic diseases, including atherosclerosis, have complex causal mechanisms. Accepting the infection hypothesis for atherosclerotic CVD depends on several key issues, including those discussed below.

Concept of Multiple Pathogens

Long-term exposure to proinflammatory, toxic, or transforming microbes and their products is a possible mechanism for infection-related atherosclerotic CVD, as is exposure to the aggregate effects of coinfections, detected or undetected. Screening for *C. pneumoniae*, CMV, and HSV-1, one group of investigators detected 2-3 microbes in 32% of atherosclerotic lesions, mostly in plaque shoulders and inflammatory infiltrates; 71% of the organisms identified were *C. pneumoniae*. Expanding the analysis, this group reported a range of 1-4 microbes in atheromata, including the oral pathogens *P. gingivalis* and *S. sanguis* (65). These findings do not confirm pathogenesis, but do highlight the possibility of synergy between organisms or a cumulative infectious effect in atherogenesis. As emphasized at a 1998 National Institutes of Health workshop, research on associations between infection and atherosclerotic CVD must consider the coinfluence of multiple infectious agents (79).

Investigators are testing the hypothesis of dose-response relationships between atherosclerotic CVD and the number of microbes to which a person has been exposed (54). For example, a recent prospective cohort related expression of antibodies against several pathogens to MI or death in patients with angiographically documented coronary artery disease (80). Perhaps cardiovascular risk increases with cumulative or earlier exposure to more or specific microbes (potentially atherogenic). *C. pneumoniae* infection of cells in vitro may activate a reporter gene controlled by the CMV major immediate-early promoter (81). In vivo co-infection might reactivate latent CMV infection, increase the activity of persistent virus, or both.

Integration of Infection into a Multifactorial View of CHD Risk

Infection as a potential CVD risk factor requires consideration in context. Although interventions targeting traditional risk factors have often reduced clinical disease, considerable impact persists. Perhaps microbes enhance the harmful effects of traditional causes; for example, foam cells in dyslipedemia-induced lesions might respond to bacterial inflammatory products. Genetic and gender predispositions could determine an association between periodontitis and atherosclerosis (74). Physical activity, smoking, and lipids could modulate immune status and thus individual susceptibility to acute, reactivated, or persistent infections that are potentially important in atherogenesis (4,22,79).

Gender and nutrition can each influence immune responses to infection and atherothrombotic disease. Supporting sex determination of atherosclerotic CVD, the Pathological Determinants of Atherosclerosis in Youth study found more extensive fatty streaks in the right coronary arteries and thoracic aortas of men, but greater abdominal aortic disease in women (82). Among primates, which have diet-induced atheromata resembling those of humans, premenopausal female monkeys develop less atherosclerosis than males (83); females with low levels of social stress developed the least disease, stressed males the most. Could gender affect susceptibility to or immune response following atherogenesis-inducing infection? Reports that sex steroid hormones (e.g., estrogen) can modulate endothelial cell production of microbial-induced proinflammatory factors such as cytokines, endothelin-1, angiotensin II, and nitric oxide suggest a single common pathway (84).

Nutritional effects on atherosclerosis also extend beyond hypercholesterolemia, hypertriglyceridemia, and obesity. Years of rigorous investigation have linked antioxidant consumption to coronary artery disease, and the antioxidant vitamin E suppresses viral infection (85). However, results of antioxidant vitamin supplementation on reduction of atherosclerotic risk have generally proven disappointing (85,86). Nevertheless, if nutrition affects inflammatory response to potentially atherogenic infections, adjunct therapies could exploit nutritional supplementation in high-risk populations.

Methods Limit Conclusions

Clarification of the above issues requires improved analytic tools. Current reagents and methods are not sufficient to prove or refute a potential infectious etiology in atherosclerotic CVD. Public health officials, laboratory investigators, and clinicians participating in two 1998 workshops emphasized that deficiencies in these tools weaken conclusions (22,79). As an example, culturing *C. pneumoniae* from clinical specimens is difficult, and serology has relied on tedious, reagent- and reader-dependent microimmunofluorescence; immunocytochemistry and PCR substrates and techniques vary between laboratories. Temporal fluctuations in bacteremia or viremia can alter PCR analysis of peripheral blood monocytes, and no assay yet differentiates the presence of any microbe from its role in pathogenesis. Study comparability and reproducibility will require standardized, sensitive, and specific reagents (20-22).

Adding to the ambiguity, human studies have focused on late-stage atherothrombotic disease, while animal studies have concentrated on earlier stages of atherogenesis (i.e., initiation). These issues are critical for translating experimental evidence to human disease because childhood exposures to ubiquitous infections are common, frequencies of reexposure are unknown, and the age at which microbes might influence atherosclerosis is uncertain. Additionally, available analytic tools do not characterize the roles of past, active, persistent latent, or recurrent infection. Recommendations from a recent international workshop sponsored by the Centers for Disease Control and Prevention and the Laboratory

Centre for Disease Control (Canada) seek to improve the reproducibility and comparability of *C. pneumoniae* investigations (20,21). In the future, more sensitive and specific assays, including microarray technologies (e.g., pathogen-specific identification chip) may also enhance specimen analysis (21).

Antimicrobial Therapy: Potential Benefits and Risks

If evidence substantiates a link between infection and atherosclerotic CVD, targeted antimicrobial therapy might mitigate atherosclerosis in persons at risk; controlling infections might decrease the impact of disease. Inappropriate antimicrobial therapy, however, could accelerate development of resistance in both associated and nontargeted organisms, without changing disease outcomes. Studies linking level of antibiotic use and prevalence of resistant bacteria in hospitals and communities justify these concerns; trends toward more frequent erythromycin- and penicillin-resistant pneumococci with higher antibiotic consumption reversed when macrolide use was reduced (87-89).

Although clinical antibiotic resistance in *C. pneumoniae* has not been described, certain characteristics–its sometimes asymptomatic, chronic, or recurrent nature–may favor this development (87). Indeed, laboratories have described antibiotic-resistant strains of *C. trachomatis*, a closely related organism. Antimicrobial delivery to intracellular *C. pneumoniae* is not well described, efficacy during different stages of the life cycle (e.g., infectious elementary bodies, replicative reticulate bodies) is unclear (8,87), and antibody titers do not reliably track infection (87). Whether antibiotics can eradicate or suppress *C. pneumoniae* replication, particularly in the persistent or latent state, remains conjectural. Clinically, azithromycin resistance in any *Chlamydia* spp. has not been extensively evaluated; however, a transient, rebound increase in resistant pneumococci has been observed following single-dose therapy for trachoma (90), and increased carriage of macrolide-resistant microbes has accompanied macrolide therapy for otitis media (91).

Widespread or long-term antibiotic use in atherosclerotic CVD could adversely affect beneficial commensals that provide nutrients and inhibit pathogen overcolonization, as well as increase antimicrobial resistance in unrelated microbes. Similar issues may emerge with antiviral therapies.

Vaccines

If antibiotics or antiviral agents do not limit the pathogenesis of acute, latent, or chronic active infections, vaccines are an alternative approach to prevention or treatment. For example, anti-*Chlamydia* vaccines have been sought for decades. Recent advances in immunologic techniques and description of the complete *C. pneumoniae* genomic sequence permit identification of antigens that may elicit protective immunity (92,93). However, poor understanding of host defense mechanisms still impedes the development and application of *C. pneumoniae* and other microbial vaccines. The essential components of humoral and cell-mediated immune responses associated with protection must be identified before prophylactic vaccine trials against any pathogen are implemented. To minimize the risk of stimulating autoimmunity, concerns for cross-reactivity during immunization must be addressed as vaccines against *C. pneumoniae* and other pathogens are pursued (94,95).

Conclusion

The current interest in infectious causes of atherosclerosis revives a venerable hypothesis. If true, the global prevention and intervention opportunities could be substantial (demonstrated with *H. pylori* in peptic ulcer disease). However, the seroepidemiologic data that triggered this renaissance lack robustness because of confounding factors (e.g., socioeconomic status, smoking) and publication bias towards more positive results. Indeed, more rigorously controlled observational and prospective studies appear to weaken the seroepidemiologic links between infection and atherosclerosis. Nonetheless, studies of the vascular biology of infection clearly demonstrate that agents such as *C. pneumoniae* and CMV can elicit potentially pathogenic functions of vascular wall cells and leukocytes in atheromata, supporting the hypothesis that such infections may potentiate atherosclerotic CVD. Results will soon be available from prospective clinical trials that critically examine whether macrolide antibiotic therapy reduces recurrent coronary events or modifies CHD. However, these trials will not determine eradication or suppression of one or more microbes, optimal timing of intervention, and duration of benefits, and thus fall short of providing convincing evidence for a causal relationship between infectious agents and atherosclerosis. These trials will neither define the portion of atherosclerotic CVD caused by infection nor indicate benefits or risks of vaccine therapy.

Routine use of antibiotics to prevent atherosclerotic events should await evidence from sufficiently powered and well-designed clinical trials. Premature, inappropriate use has potential adverse effects. Clearly, research on potential infection-atherosclerosis links must continue, complemented by a public health approach. Participants in recent workshops and symposia have evaluated gaps in the evidence and outlined ways to fill them (22,23,79,96,97). All agree on the need for more specific, sensitive, and standardized reagents and assays (20-22).

Research must also address interactions between infection, traditional risk factors, and other determinants of host susceptibility, such as gender and nutrition, in multifactorial atherosclerotic CVD (7,22,79,96). It will be critical to determine the age at which pathogenesis begins and to identify the attributable fraction of preventable or reversible disease and the subpopulations at risk. Such data might eventually warrant adoption of infection control strategies–minimized exposure, vaccination, and antimicrobial therapy–aimed at reducing the impact of symptomatic cardiovascular disease.

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Dr. O'Connor is Assistant to the Director of the National Center for Infectious Diseases, Centers for Disease Control and Prevention. Her research interests include identification, characterization, and prevention of infectious diseases that may trigger or determine chronic illness and disability.

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Changing Epidemiology of Q Fever in Germany, 1947-1999

Wiebke Hellenbrand, Thomas Breuer, and Lyle Petersen
Robert Koch-Institut, Berlin, Germany

The epidemiology of Q fever in Germany was examined by reviewing revelant studies since 1947 and by analyzing available surveillance data since 1962. The average annual Q fever incidence nationwide from 1979 to 1989 was 0.8 per million and from 1990 to 1999, 1.4 per million. The mean annual incidence from 1979 to 1999 ranged from a minimum of 0.1 per million in several northern states to 3.1 per million in Baden-Württemberg, in the south. We identified 40 documented outbreaks since 1947; in 24 of these, sheep were implicated as the source of transmission. The seasonality of community outbreaks has shifted from predominantly winter-spring to spring-summer, possibly because of changes in sheep husbandry. The location of recent outbreaks suggests that urbanization of rural areas may be contributing to the increase in Q fever. Prevention efforts should focus on reducing sheep-related exposures, particularly near urban areas.

Q fever is caused by the pleomorphic, obligate intracellular rickettsial agent *Coxiella burnetii*, which has an envelope similar to that of gram-negative bacteria. It is found worldwide except in New Zealand (1). Its most important reservoirs are ticks and ruminant animals such as cows, sheep, and goats (1-4). Although infection rarely causes major clinical symptoms in animals (5), it has been associated with infertility (6,7) and abortion (8,9), particularly in first-bearing newly infected parturient animals (10). Birth products from infected animals thus contain high concentrations of *C. burnetii* and can be an important source of environmental contamination (11). Transmission to humans and other animals by the aerosol route is facilitated by the tenacity of *C. burnetii*'s survival for months to years in a sporelike state on wool or fur contaminated with infected tick feces, in water, and in soil (12).

Acute Q fever in humans is characteristically a febrile, flulike illness associated with pneumonia or hepatitis (2,3,13-18). Rare complications include myocarditis, pericarditis, or meningoencephalitis (1,13,19). The death rate among persons with Q fever pneumonia is 0.5% to 1.5% (14). Up to half of patients may suffer protracted fatigue and weakness after acute disease (20,21). Rarely, more serious forms of chronic disease (most commonly endocarditis but also chronic hepatitis and vascular, osteoarticular, or pulmonary infections) may develop months to years after the acute infection (13,22,23). An increased long-term risk for arterial disease and death has also been observed (24).

Q fever was first recognized in southern Germany when several large outbreaks occurred in rural communities in 1947 to 1948 (25-28). Since then, it has been endemic in Germany. In the 1990s, several large outbreaks were recognized and investigated (5,29-36). This led us to review the epidemiology of this disease in Germany.

Methods

Clinically manifest Q fever has been statutorily notifiable since 1962 in the former West Germany and since 1979 in the former German Democratic Republic (GDR, East Germany). In 1991, the two reporting systems were amalgamated. The Robert Koch Institute in Berlin receives weekly reports of the number of persons with diagnosed (laboratory-confirmed or epidemiologically linked) Q fever from local health departments through the state health authorities. Local health departments receive notification of Q fever infections from hospitals and physicians in private practice. The Federal Office of Statistics prepares annual statistical reports of notifiable diseases. We reviewed surveillance data on Q fever from humans in Germany since 1962 from these sources.

The mean annual incidence rate was calculated for the periods from 1979 to 1989 and from 1990 to 1999 by taking the mean number of persons with Q fever reported per year during each period and dividing by the 1985 and 1995 German populations, respectively. The mean annual incidence rate for the period 1979 to 1999 was calculated by taking the mean number of persons with Q fever reported per year and dividing by the 1990 German population. Incidence rates for each of the 16 German states were calculated similarly.

We reviewed veterinary surveillance data based on passive, biannual reports of the number of domestic animal herds with laboratory-confirmed Q fever (37). To obtain further information on the epidemiology of Q fever in Germany, we searched databases (MEDLINE, from 1966; EMBASE, from 1974; AGROKAT, from 1960; CAB ANIMAL, from 1972; and CABVET SCI, from 1972) and reviewed the cited literature in the retrieved relevant articles. In addition, we contacted local health departments for unpublished details on recent outbreaks.

Address for correspondence: Wiebke Hellenbrand, Department of Infectious Diseases Epidemiology, Robert Koch-Institut, Stresemann Str. 90-102, 10963 Berlin, Germany; fax: 30-4547-3533; e-mail: HellenbrandW@rki.de

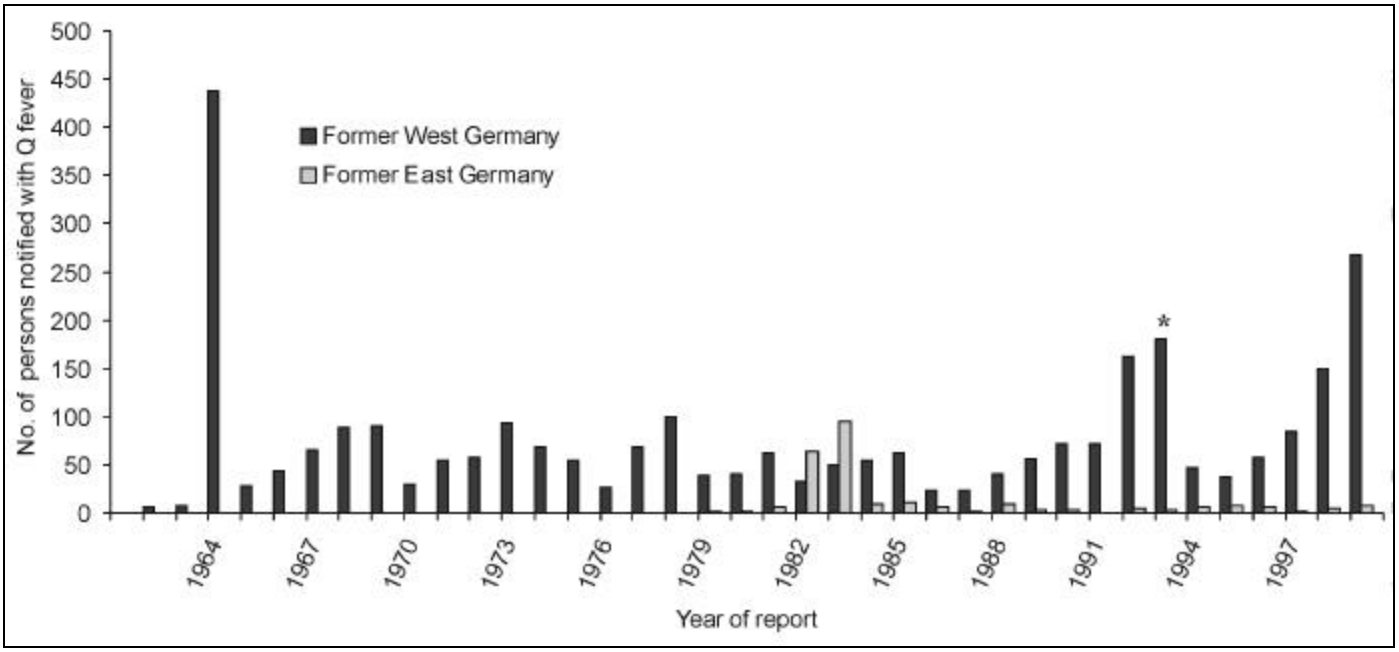

Figure 1. Number of reported cases of Q fever in Germany, 1962-1999.
*In 1993, 184 persons with Q fever were officially reported; 101 of these persons were part of the outbreak in Oberscheid, Hesse, and were only reported to the Robert Koch Institute and not to the federal Office of Statistics (36). However, a total of 97 symptomatic persons with serologically confirmed Q fever were described in a report of the outbreak in Dortmund, Northrhine-Westphalia, and 43 serologically confirmed symptomatic cases were reported in an outbreak among military recruits in Sontra, Hesse (Table 1). Thus a minimum of 94 persons with Q fever were not officially reported in 1993. (In Northrhine-Westphalia, 34 cases were reported; thus a minimum of 97 - 34 = 63 cases were not reported. In Hesse, 117 cases were reported, including 105 in conjunction with the Oberscheid outbreak [Table 1]; thus, a minimum of 43 - [117-105] = 31 cases were not reported from Hesse). Sources: (36), Robert Koch-Institute, and Federal Office of Statistics, Wiesbaden.

Results

Surveillance

Human Q fever surveillance data since 1962 indicate an irregular cyclic incidence pattern (Figure 1). Very few cases were reported from states constituting the former GDR (approximately one fourth of the German population), with the exception of the years 1982 and 1983, when an outbreak occurred in Thuringia (38). To consider the long-term incidence pattern between 1962 and 1999, data from West German states must be considered separately. Except for 1979 to 1991, when there is little cyclicity, the interval between the peaks is approximately 4 to 6 years. The peak in 1993 with 181 (278, if unreported cases from two outbreaks [30,35] are taken into account) and the peak in 1999 with 268 cases (West German states) were the highest since 1964, when 437 cases were reported.

The average annual incidence of Q fever in Germany from 1979 to 1999 was 1.1 per million population. From 1979 to 1989, the incidence was 0.8 per million population, and from 1990 to 1999, 1.4 per million population. The mean annual incidence rates calculated from 1979 to 1999 were generally higher in the southern German states–highest in Baden-Württemberg (4.1 per million), followed by Hesse (2.8 per million), Rhineland-Palatinate (0.9 per million), and Bavaria (0.8 per million) (Figure 2). The high average incidence observed in West Berlin (1.4 per million) is explained by an outbreak in 1992 (5,39). Had the outbreak not occurred, an incidence rate of 0.2 per million would have been observed in Berlin in this period.

Outbreak

We documented 40 outbreaks of Q fever in humans from 1947 to 1999 (Table 1). In all but three outbreaks, the disease was confirmed in at least some of the persons affected, either serologically or by transmission from human serum or sputum to mice or guinea pigs.

Sheep were implicated in the transmission of Q fever to humans in at least 24 of the documented outbreaks (in six rural community outbreaks the source was uncertain). Exposure to products of conception was explicitly considered the potential source of infection in 11 of the sheep-associated outbreaks. In the outbreak in Dortmund, Northrhine-Westphalia, a case-control study revealed that exposure to manure contaminated with the products of conception from infected sheep was associated with Q fever (35). Infectious dust produced by shearing of sheep whose wool was presumably contaminated with infected tick feces was suspected as another possible source of transmission, since shearing and disease occurrence were temporally associated in 3 of these 11 outbreaks and as the main source in one other outbreak. In 12 outbreaks, sheep located near or migrating through inhabited areas were implicated without specification of the presumed mechanism of transmission. Dry weather or wind blowing from areas where sheep were located to inhabited areas was thought to play a contributory role in at least 14 outbreaks.

Cattle were suspected as the source of infection in six outbreaks; four were community outbreaks (40-43). In the Niederrhein outbreak in 1958 (42,43), exposure to products of conception aborted by a seropositive cow and to other seropositive cows sold at an animal fair was considered to be the source of infection (42,43). Exposure to infected cows,

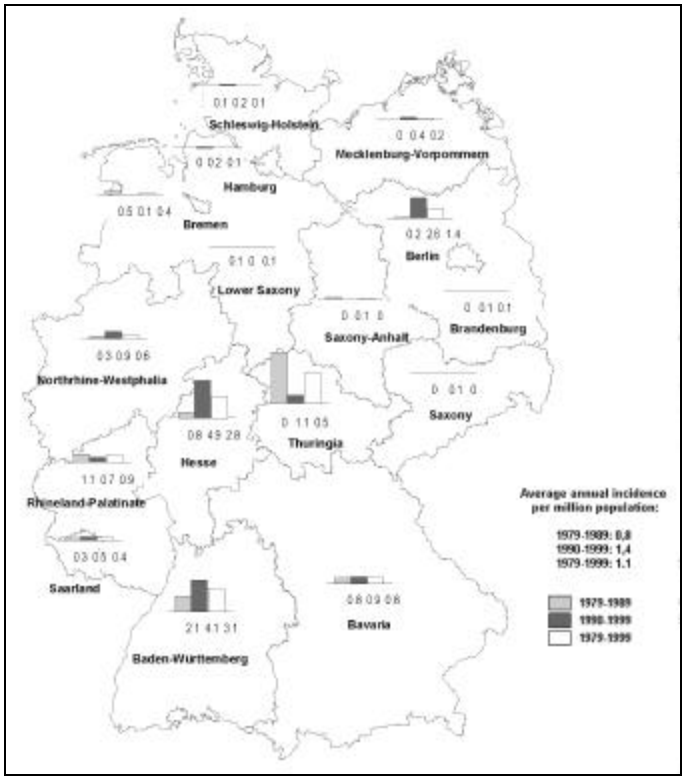

Figure 2. Mean annual Q fever incidence per million population in Germany.

some shedding *C. burnetii* in their milk, was implicated in the community outbreaks in Neulussheim, Zuzenhausen, and Schwaikheim in Baden-Württemberg (40,41). The other two cattle-associated outbreaks occurred in abattoirs (44,45). In one additional abbatoir-related outbreak, the source of infection could not be identified (46).

Two laboratory outbreaks occurred in 1947 and 1948 (26,47), but none have been reported since. Exposure to sputum of a patient who contracted Q fever in a laboratory that contained high concentrations of *C. burnetii* caused a hospital outbreak in 1948 (28).

The seasonality of community outbreaks in Germany has changed during the past decade compared to earlier years (Figure 3), with a marked decrease in winter outbreaks and an increase in summer ones. In contrast to earlier community outbreaks, which were almost exclusively rural, recent outbreaks have frequently involved people living in or near urban areas (5,30,34,35,48).

Seroprevalence Studies

Seroprevalence studies of *C. burnetii* antibodies in the German population have not been performed recently. Of 1,611 serum samples collected from blood donors from all 16 German states from 1983 to 1986, 22% were positive for *C. burnetii* antibodies using a phase I/II immunoglobulin (Ig) G enzyme-linked immunosorbent assay (ELISA) (49). In this survey, 15% of the 205 specimens from Hesse were positive. None of 1,075 sera from blood donors from Hesse had been positive for *C. burnetii* using complement fixation (CF) (positive titer 1:10) in a 1977 survey (50). This suggests a possible increase in exposure to *C. burnetii* during this interval, although ELISA has a higher sensitivity than the CF test (1). State-specific surveillance data from Hesse also suggest an increase in disease activity during this interval: The annual number of reported human Q fever cases was 0.6 cases per year between 1962 and 1977 but averaged 5.3 cases per year from 1978 to 1986. In a seroprevalence study among German federal armed forces personnel from 1985 to 1987 (51), 22% of 1,651 blood donors had antibodies to *C. burnetii* using the same ELISA as used by Schmeer et al. (49).

Q Fever in Animals

The extent of disease in animals over time is difficult to quantify. State veterinarians are required to report biannually the number of herds in which one or more animals have laboratory-confirmed Q fever (37). The average annual number of reported herds with Q fever was 71 between 1971 and 1979, 328 between 1980 and 1989, and 303 between 1990 and 1998. However, this does not permit inferences about the extent of infection, as neither the number of herds nor the number of animals tested is available. Furthermore, testing is not representative because the disease is generally asymptomatic in animals. Most testing occurs routinely in dairy cattle whose milk is destined for human consumption without pasteurization or in cattle destined for slaughter; otherwise, testing occurs only sporadically, as when the number of abortions increases, infertility is noted, or an outbreak of human disease is thought to be related to an animal source. In 1998, a survey requesting information on the number and results of Q fever testing in state veterinary laboratories in 13 of the 16 German states was performed (52). This showed that 7.8% of 21,191 tested cattle, 1.3% of 1,346 tested sheep, and 2.5% of 278 tested goats had evidence of *C. burnetii* infection by various tests (52). Again, the reasons for testing are not available; thus, the results cannot be considered representative of Q fever in animals in general and give only a rough indication of the extent of disease.

Seroprevalence surveys in cattle reveal that *C. burnetii* infection has been endemic in cattle in southern Germany since the 1950s; the seroprevalence reported in various surveys is generally >5% (53-55). Baden-Württemberg may be an exception (7), as a very low cut-off titer was chosen there, making comparison with other studies difficult (Table 1). Although different tests were used over time and between locations, seroprevalence in cattle in southern Germany appears to have remained fairly constant since the early 1950s. However, in other parts of Germany that have data available over time (e.g., Hesse, Northrhine Westphalia, Lower Saxony), seroprevalence was very low in the 1950s and 1960s but increased to levels comparable to those in southern Germany in the 1980s and 1990s (Table 1). In herds that had problems with infertility or abortions, seroprevalence rates were as high as 75% (6,7).

Seroprevalence of *C. burnetii* antibodies in surveys of sheep has generally been lower than in cattle (Table 2). This is likely partially due to the longer persistence of antibody titers in cattle (13). However, when surveys were performed in flocks of sheep implicated in human outbreaks, much higher seroprevalences were often found (Table 2), presumably reflecting more recent, acute infection with *C. burnetii.*

Discussion

The number of persons reported annually with Q fever in Germany rose markedly in the 1990s. Although this could

Table 1. Seroprevalence of antibodies to *Coxiella burnetii* in cattle in Germany

Survey location	Year(s)	No. of animals	No. of herds	Test used	Seroprevalence (%)	Seropositive herds (%)
Northern Germany						
Schleswig-Holstein (56)	1955-56	425	36	Microagglutination [a] (≥1:20)	0	0
Lower Saxony (57)	1970	400	N/A	CF [b] (≥1:20)	11	N/A
Lower Saxony (58)	1992-93	665	39	CF (≥1:20) CF (≥1:10)	4.7 9.6	76.9
Eastern Germany						
Former East Germany (59)	1980-89	95,464	N/A	CF (≥1:20), except 913 sera with ELISA)	8.3[c]	N/A
Middle/Western Germany						
Hesse (60)	1953	585	97	Transmission from milk to guinea pigs; CF (≥1:5)[d]	0	0
Northrhine-Westphalia (61)	1958-60	2,157	N/A	CF (≥1:20) CF (≥1:5)	0.1 0.5	N/A N/A
Northrhine-Westphalia (62)	1981-83	5,184	297	CF (≥1:20) CF (≥1:5)	3.8 6.7	 23.6
Hesse (6)	1982-83	3,200	591	ELISA[e] CF (≥1:20)	13.4 6.3	29.6
Northrhine Westphalia (63)	1989-90	3,500	155	ELISA IFT[f] (≥1:8)	13.3 12.9	57.4 53.5
Southern Germany						
Bavaria (53)	1953	1,000	N/A	CF (≥1:16) CF (≥1:32)	10.1 5.9	N/A
Bavaria (64)	1968	1,000	N/A	CF (>1:20)	8.4	N/A
Baden-Württemberg (7)	1984	2,109	125	CF (≥1: 5)	8.0	35.2
Northern Bavaria (54)	1983-84	3,384	246	CF (>1:20)	7.6	30.0
Southern Bavaria (55)	1991	1,095	21	ELISA	11.8	81.0

ELISA = enzyme-linked immunosorbent assay; CF = complement fixation; IFT = immunofluorescence testing; N/A = not available.
[a]The sensitivity and specificity of microagglutination are comparable to that of the ELISA (1).
[b]CF is less sensitive and less specific than ELISA, IFT, and microagglutination.
[c]17.3% among dairy cattle.
[d]According to a study by Schaal and Schäfer (62), 33% of cows seropositive at a titer of 1:10 and 65% positive at a titer of 1:20 shed *C. burnetii* in their milk.
[e]The ELISA used in Germany and in all studies listed in this table (except for [59], for which no information was provided) is based on the test developed by Schmeer et al. (49,65), which contains both phase I and phase II antigens and detects immunoglobulin (Ig) G as well as IgM-antibodies. The following antigens are included in this ELISA: Nine Mild Strain (phase II), Munich Strain (phase I and II), and Frankfurt Strain (mainly phase I). A net absorption cutoff of 0.200 read at 450 nm was used. In a large seroprevalence survey by Gouverneur (6), all of the sera testing positive by CF at a titer ≥1:20 tested positive by ELISA. However, while 13.4% of sera tested positive by ELISA, only 6.3% tested positive using CF at a titer ≥1:20. At a CF titer of 1:10, only 58% of positive sera also tested positive by ELISA. However, at this titer, 8.4% of sera tested positive by CF. This is still 37% less than found using the ELISA. In a smaller study by Schmeer et al. (49), all sera testing positive at a titer ≥1:16 tested positive by ELISA.
[f]The IFT is considered the reference method of Q fever serodiagnosis (1).

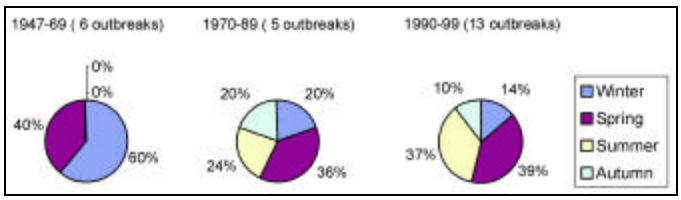

Figure 3. Seasonality of 24 sheep-associated community outbreaks in Germany, 1947-1969, 1970-1989, and 1990-1999. For each community outbreak in which sheep were implicated, the number of months' duration in each season was calculated. For each time period, the percentage of the total number of outbreak months occurring during each season was calculated. The "year-round" outbreak in Dettenhausen (Table 1) was excluded. Winter = January-March; spring = April - June; summer = July-September; autumn = October-December.

be due to enhanced awareness and better clinical recognition of the disease, the increased number of Q fever outbreaks, which are less likely to be clinically missed than sporadic cases, suggests that the increase is real. In fact, outbreaks in Baden-Württemberg, Hesse, Northrhine-Westphalia, and Berlin are responsible for most of the increase. The increased outbreak activity observed during the 1990s is reminiscent of the large outbreaks described between 1948 and 1954 (see online table of documented Q fever outbreaks in Germany from 1948 through 1999 at URL:http://www.cdc.gov/eid/vol7no5/hellebrand_table).

The seroprevalence of 22% found in blood donors and military personnel in the 1980s in Germany is probably not representative of the general population. We have no information on the proportion of rural versus urban residence for blood donors. However, a seroprevalence study of blood donors in Switzerland (using a phase II IgG immunofluorescence test with a cut-off titer of 1:20) revealed lower seroprevalences in urban areas (10.9%) as compared to rural areas (17%) (68). In addition, because soldiers often train on grounds that are grazed upon by sheep (69), their exposure to *C. burnetii* may be higher than the general population's. A seroprevalence of 4% was found in 942 blood donors in Marseille, France, in 1988 also using a phase II IgG immunofluorescence test (cut-off titer 1:25) (70). In the Netherlands, a much higher seroprevalence of 45.5% was found in sera collected from blood donors between 1968 and 1983, using a Phase II IgG immunofluorescence test (cut-off titer 1:16) (71). However, international comparisons are difficult because of varying test methods and cutoffs.

The relatively high seroprevalence of *C. burnetii* antibodies in Germans suggests that the true number of persons with Q fever exceeds the number reported. Because Q fever often presents as a flulike illness, it may not be correctly diagnosed unless there is heightened suspicion of Q fever, such as would occur during an outbreak. Even then, not all cases may be reported. In 1993, at least 94 persons with symptomatic Q fever during recognized outbreaks were not reported to the surveillance system (Figure 1).

The cyclic incidence peaks seen on the surveillance curve generally correspond to years in which one or more outbreaks were documented. Thus, the occurrence of outbreaks was largely responsible for the cyclic pattern, a finding similar to that observed in Israel (72). It is unknown whether the incidence of sporadic Q fever follows the same cyclic pattern as Q fever occurring in outbreaks.

The highest Q fever incidences in Germany were observed in Bavaria, Baden-Württemberg, Rhineland-Palatinate, Hesse, Northrhine-Westphalia, and Thuringia, with Hesse showing the greatest increase since 1990. Of the 34 community outbreaks, 33 occurred in these six states. The tick *Dermacentor marginatus*, which acts as a host for *C. burnetii* and feeds on sheep in large numbers, is endemic in the four most southern states (Bavaria, Baden-Württemberg, Rhineland-Palatinate, and Hesse) but has not been

Table 2. Seroprevalence of antibodies to *Coxiella burnetii* in sheep

Location	Year(s)	No. of animals	No. of herds	Test used	Seroprevalence (%)	Seropositive herds (%)
Surveys unrelated to human outbreaks						
Northrhine-Westphalia (61)	1958-60	2,199	N/A	CF (1:20)	0	N/A
Bavaria (64)	1968	1,000	N/A	CF (>1:20)	4.5	N/A
Thuringia (66)	1980-89	4,337	17	CF (≥1:20) CF (>1:10)	0.73 1.1	47
Surveys in herds implicated in human outbreaks						
Southern Germany (53)	1953	31	1	CF (≥16)	32.2	N/A
Rhineland-Palatinate (67)	1974	265	1	CF (≥1:20) CF (≥1:10)	7.9 12.5	N/A
Rollshausen, Hesse (33)	1996	20	1	ELISA	75.0	N/A
Giessen, Hesse (31)	1997	100	1	ELISA	~50	N/A
Dortmund, Northrhine-Westphalia (35)	1999	100	1	ELISA	57.0	N/A

N/A = not available; CF = complement fixation; ELISA = enzyme-linked immunosorbent assay.

found in the more northern states of Northrhine-Westphalia or Thuringia (59, 73-75). Excreta from infected ticks persist in animal fur as a highly infectious dust, permitting aerosol transmission within the flock as well as to humans (73). This can occur through direct or indirect contact or through shearing, as observed in the outbreaks in Baden-Württemberg in 1998 and 1999 (see online table). *C. burnetii* has also been isolated from the tick *Ixodes ricinus* in Baden (40), in Northrhine-Westphalia (61), in Hesse (69), and in Thuringia (38). A role for *I. ricinus* in the transmission cycle of *C. burnetii* in areas where *D. marginatus* is not endemic but Q fever incidence is relatively high, such as Northrhine-Westphalia and Thuringia, is plausible, although evidence is lacking thus far. A tick-independent cycle of *C. burnetii* has been observed in cattle (55).

In addition to infectious dust from tick excreta, contaminated birth products are an important source of human infection (2,12,13,76). Terhaag (77) first noted the temporal association of Q fever outbreaks with outdoor lambing in southern Germany. Exposure to infectious products of conception was implicated in 10 sheep-associated community outbreaks and one cattle-associated community outbreak in this study.

Cattle were implicated in only four community and three abattoir outbreaks, none of which occurred recently. Although the increase in seroprevalence in cattle in more northern parts of Germany has been accompanied by an increase in reported Q fever outbreaks in Hesse and Northrhine-Westphalia (but not Lower Saxony or Schleswig-Holstein), cattle were never implicated as a possible source of the outbreaks. This is likely because cattle do not migrate over large distances, they graze on pastures close to inhabited areas less frequently, and they are not shorn. Moreover, calving normally occurs indoors under more controlled conditions.

From 1947 to 1969, epidemic Q fever activity occurred mainly during winter and spring (Figure 3). From 1970 to 1989, outbreaks occurred less commonly during winter, and started to occur during summer and fall as well. Finally, since 1990, outbreak activity has decreased further during winter and increased during summer (Figure 3). The change in seasonality coincides with changes in sheep husbandry. The nomadic form of sheep husbandry practiced mainly in southern parts of Germany has become less common. In former West Germany, nomadic herds gradually decreased from 1,178 in 1968 (30% of sheep) to 917 in 1988 (27% of sheep) (78). By 1994 this had decreased further to 702 (18% of sheep, reunified Germany) (79). Limited data from the late 1940s reveal that 50% of sheep farming was nomadic in Bavaria, 55% in Baden, and 88% in Württemberg (80). In 1994, this had decreased to 28% in Bavaria and 56% in (now unified) Baden-Württemberg (79). Because winter lambing (80-82), with either spring (80,81) or winter (82) shearing, is practiced in this form of sheep husbandry, the decrease in nomadic sheep farming in Germany may be related to the decrease in outbreaks during winter.

Spring lambing and shearing are most common in other forms of sheep farming, which have increased proportionately in Germany (82). Lambing at other times of the year, however, is possible and is being increasingly encouraged to enable year-round provision of the market with fresh lamb meat (83). Increased lambing or shearing during the warmer, drier seasons could increase the risk of aerogenous spread of *C. burnetii* from birth products or wool to humans and may thus be one possible explanation for the recent seasonal shift as well as for the increase in Q fever outbreaks in the 1990s.

In addition, outbreaks have increasingly affected people living in or close to urban areas (30,34,35,48). This suggests that increased exposure of susceptible humans to sheep through urbanization into traditionally rural areas may be another factor contributing to the observed increase in outbreaks. The use of sheep for landscaping purposes on recreational and park lands has also increased in importance since the 1970s (79,84).

Taken together, the presented data suggest that conditions for the transmission of *C. burnetii* to humans mainly from sheep have become more favorable in recent years, leading to increased outbreak activity in Germany. Therefore, stricter implementation of preventive measures is essential.

Apart from attempting to prevent the disease in animals as far as possible (e.g., isolation of animals that abort, examination of aborted lambs [69,85]), preventive measures must aim at preventing human contact with potentially infectious dust in animal fur and with infectious products of conception. In areas endemic for *D. marginatus*, control of transmission from ticks to animals must be achieved by rigorous treatment of sheep with acaricides (86-88). Since 1981, acarcide treatment of sheep has been recommended in areas where *D. marginatus* is endemic (86). However, such treatment may not protect for the entire tick season (86), so avoiding close contact between tick-infested sheep and susceptible persons (particularly during shearing) remains important. Rigorous disinfection after lambing or calving, including adequate heat or chemical treatment of manure before its use as fertilizer as well as removal of birth products through a licensed institution (89-91), is essential to prevent transmission.

Finally, investigation of vaccination of sheep against *C. burnetii* as a preventive measure in Germany may be warranted, particularly in light of a recently developed chloroform-methanol residue vaccine with fewer side effects (13,92,93) and long-lasting antibody induction in sheep (94). Studies in cattle suggest that this could reduce both the degree and proportion of infectious placentas (7,54). Unfortunately, no Q fever vaccine is currently licensed for use in animals in Germany.

Conclusion

Human disease caused by *C. burnetii* appears to have increased during the past decade in Germany. Q fever is endemic in cattle throughout Germany and in sheep at least in southern, eastern, and western parts of the country. Sheep have most often been implicated in transmission of the disease to humans. Urbanization into rural areas; increased grazing of sheep on recreational and park lands, leading to increased opportunity for contact between susceptible persons and infected animals; and changes in sheep husbandry may have contributed to the observed increase in outbreaks. Thus, awareness of this disease as a threat to human health and compliance with preventive and control measures must be improved among farmers, veterinarians, and the public.

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Dr. Hellenbrand is a research assistant at the Infectious Diseases Epidemiology Unit, with research interests in Q fever, disease surveillance, and vaccine-preventable diseases.

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Cost-Effectiveness of a Potential Vaccine for *Coccidioides immitis*

Amber E. Barnato,* Gillian D. Sanders,† and Douglas K. Owens†‡
*University of Pittsburgh, Pittsburgh, Pennsylvania, USA; †Stanford University, Stanford, California, USA; and ‡VA Palo Alto Health Care System, Palo Alto, California, USA

Coccidioidomycosis, a systemic fungal infection, affects Americans living in the Southwest. We evaluated the cost-effectiveness of a potential vaccine against *Coccidioides immitis*. Using a decision model we developed, we estimate that among children, vaccination would save 1.9 quality-adjusted life days (QALD) and $33 per person. Among adults, screening followed by vaccination would save 0.5 QALD per person and cost $62,000 per quality adjusted life year gained over no vaccination. If the birth cohort in highly endemic counties of California and Arizona were immunized in 2001, 11 deaths would be averted and $3 million would be saved (in net present value) over the lifetime of these infants. Vaccination of adults to prevent disseminated coccidioidomycosis would provide a modest health benefit similar in magnitude to other vaccines but would increase net expenditures. Vaccination of children in highly endemic regions would provide a larger health benefit and would reduce total health care expenditures.

Coccidioides immitis, an infectious fungus, grows in the arid soil of the Central Valley of California, southern Arizona, and parts of Nevada, New Mexico, Utah, and Texas, as well as northern Mexico and parts of Central and South America. Regions endemic for *C. immitis* are home to approximately 20% of the U.S. population; an estimated 5 million persons live in the areas of highest endemicity (Figure 1) (1-3). Humans are infected by inhaling dust containing *C. immitis* arthroconidia. Dust storms (4,5) and activities associated with heavy dust exposure such as agricultural labor (6), excavating archeologic ruins (7), and military combat training (8,9) increase infection rates, total infectious load, and the proportion of symptomatic cases. Infection rates, as reflected by positive skin tests, have decreased since the 1940s and 1950s from as high as 8% per month to approximately 2% to 3% per year in highly endemic regions. This decrease is likely the result of reduced dust exposure attributable to lifestyle changes and urbanization. However, population growth in endemic regions has been steady and is projected to increase.

Recent epidemics in California and Arizona highlight the continuing public health threat and costs of coccidioidomycosis (10,11) and have led to efforts to develop a vaccine. An economic analysis of the 7,130 cases from 1991 to 1993 in California's Kern County demonstrated a cost to that county of $56 million (12). Because the U.S. training post for desert warfare is located in the Mojave Desert, an area endemic for *C. immitis*, a vaccine is a military as well as a civilian priority. A killed whole spherule vaccine that showed promise in animal trials was not well tolerated in humans (13). Current efforts of the Valley Fever Vaccine Project, a consortium of researchers funded primarily by the California Healthcare Foundation and the California Department of Health Services, focus on a number of antigens successful in mice models that may form the basis of a subunit vaccine (G. Rutherford, pers. comm.).

Despite the potential clinical value of a vaccine, the only study of the cost-effectiveness of a vaccination program was an Institute of Medicine (IOM) analysis for the purposes of setting priorities in vaccine development (14). The IOM concluded that vaccine development would cost more than $100,000 per quality-adjusted life year (QALY) gained, but included vaccine development costs and used a very simplified model. If the vaccine would not be cost-effective, current development efforts might be in vain. We present the results

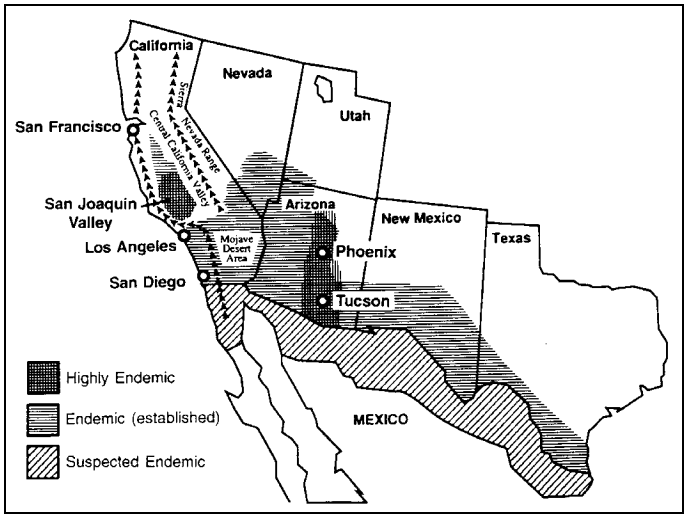

Figure 1. Areas in the United States endemic for *Coccidioides immitis*. Cross-hatching indicates the heavily disease-endemic area; single hatching indicates the moderately disease-endemic area. Reprinted with the author's permission from Kirkland (1).

Address for correspondence: Amber E. Barnato, University of Pittsburgh School of Medicine, Division of General Internal Medicine, 933W-MUH, 200 Lothrop Street, Pittsburgh, PA 15213, USA; fax: 412-692-4838; e-mail: barnato@post.harvard.edu

of a detailed cost-effectiveness analysis of a potential vaccine against *C. immitis*.

Data and Methods

We used a decision model to evaluate the health and economic consequences of withholding the vaccine, screening and vaccinating only those susceptible to infection (screening/vaccination), or vaccinating all eligible persons. Taking a societal perspective, we calculated the incremental cost-effectiveness of these strategies (formula in Appendix I, online; URL: http://www.cdc.gov/ncid/eid/vol7no5/barnato_appendix1.htm), and we discounted both costs and benefits at an annual rate of 3%. We performed one-way sensitivity analyses on all the model variables, as well as a three-way sensitivity analysis of the most sensitive variables, and best- and worst-case scenarios.

We based the estimates for input variables on the literature whenever possible (Appendix II Table). Our base-case estimates represent our judgment about the best estimate from the literature and discussion with experts. The ranges for costs represent variation by 25% above and below the base-case estimate, except where otherwise specified. The ranges for sensitivity analyses on quality-of-life estimates represent the 25th and 75th percentiles of patients' assessments of quality of life (15), except where otherwise specified. The authors had complete scientific and editorial independence from the funding agencies.

Decision Model

We developed a Markov model (Figure 2) with Decision Maker software (version beta 0.99.11.14.0a, 2000, S. Pauker, et al., Boston, MA) (16,17) to track hypothetical cohorts of patients who either did not receive the *C. immitis* vaccine, received it, or received it only if their skin test was negative. Patients who received the vaccine were at risk for pain at the injection site, mild to moderate fever, and anaphylaxis without death. Vaccinated patients were at decreased risk for extrapulmonary dissemination after primary infection. Patients neither immune from previous infection nor successfully vaccinated were at risk for *C. immitis* infection and serious sequelae. All patients were at risk for dying from other causes (18). We followed cohorts until death, with a Markov cycle length of 1 month.

Patient Population

We used epidemiologic and demographic data from two California counties (Kern and Tulare) and eight Arizona counties (Cochise, Gila, Graham, Maricopa, Pima, Pinal, Santa Cruz, and Yavapai) as proxies for the population features of highly endemic regions (2,19; Internal Revenue Service, unpub. data). We used two cohorts representing the weighted average age and prior probability of naturally acquired immunity among children (ages $\leq$17) and adults (ages 18 to 65) in 10 highly disease-endemic counties. New residents with no natural immunity were added to these cohorts. Children had an average age of 8.85 years, and 14.5% were naturally immune; adults had an average age of 39.51 years, and 47.5% were naturally immune.

Clinical Manifestations

Smith's classic studies in military recruits stationed in disease-endemic regions during World War II established that 60% of infected persons have no symptoms and 40% have a flulike illness (20). Asymptomatic infection and symptomatic infection with recovery confer probable lifelong immunity with a positive delayed-type hypersensitivity skin-test reaction to coccidioidin or spherulin. However, a fraction of per-

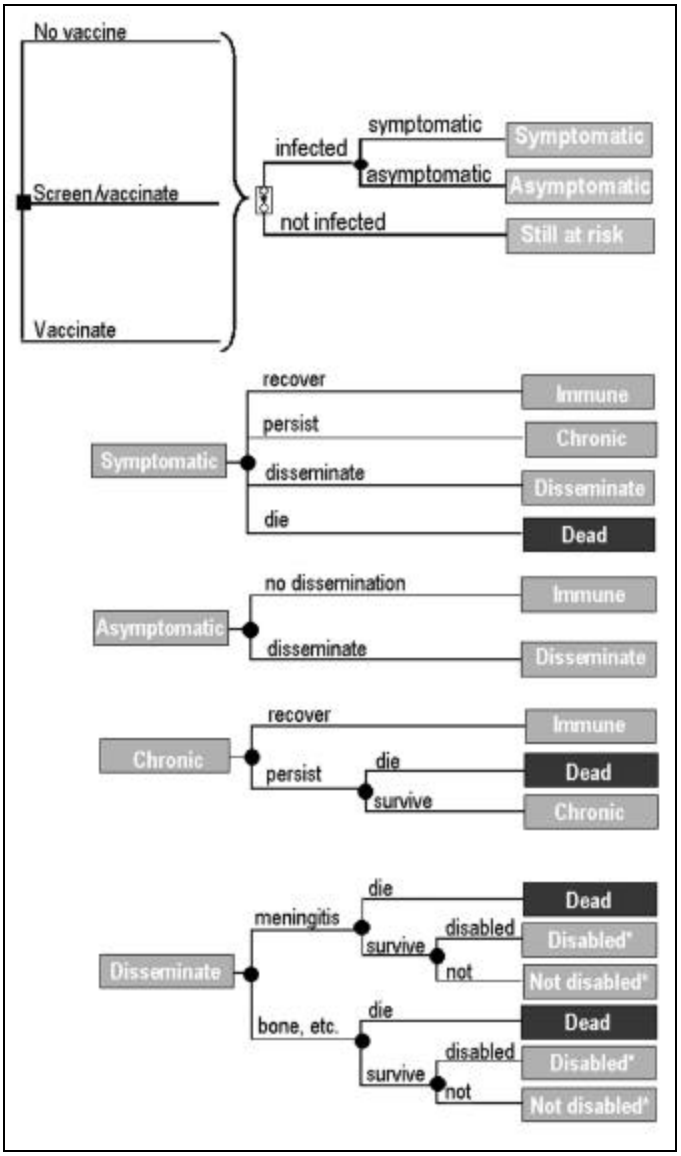

Figure 2. Schematic representation of the decision model and decision model subtrees. The square node represents a decision to use one of the three strategies: no vaccination, vaccination of susceptible persons identified through a screening skin test, or vaccination of all persons. Circles represent chance nodes. After a strategy is chosen, the patient enters a Markov tree (denoted by a rectangle containing circles connected by an arrow). The Markov tree represents clinical events that can occur during each 1-month period as a patient is followed until death. Subtrees show events that may occur to patients during a 1-month cycle. Dissemination subtree: The site of extrapulmonary dissemination can be to the brain (meningitis) or elsewhere (e.g., bone). The outcome of dissemination can be death from other causes, death from disseminated coccidioidomycosis, or survival. If the patient survives, he or she can survive in a disabled or nondisabled state. Each month thereafter, the patient is at risk for relapse. Chronic subtree: Each month a patient with chronic pulmonary coccidioidomycosis can die from other causes, remain infected, die from chronic pulmonary infection, or be cured and rendered immune. Immune subtree: On any given month, a patient who is immune to infection can die of other causes or remain alive and immune to coccidioidomycosis.
*At risk for dissemination relapse.

sons do not have simple self-limited disease and instead more serious illness develops, such as respiratory failure, chronic pneumonia, and extrapulmonary dissemination (20-23). We present more clinical detail in Appendix I, online, only (at URL:www.cdc.gov/eid/v7n5/barnato-appendix1.htm) and summarize probability estimates, costs, and utilities in the Appendix II Table.

Vaccine and Skin-Test Characteristics

Animal trials of the vaccine rely on intraperitoneal or intranasal challenge with large loads of *C. immitis*. The outcome measure is survival or death from disseminated disease. Because the mechanism of action of the putative vaccine is not known, we conservatively assumed that vaccination prevents extrapulmonary dissemination but not primary infection. We evaluated this assumption in a sensitivity analysis.

We assumed that all vaccinated persons comply with 3 doses in 1 year and all develop an antibody response. We assumed that the vaccine reduced the probability of dissemination by 75%, with lifelong duration, based on experience with hepatitis B vaccine, a widely used subunit vaccine. We examined the effects of compliance with each strategy and the effect of waning immunity.

Using data from hepatitis B vaccine as a proxy, we assumed that 25% of patients would experience mild side effects such as local arm pain, fever, and nausea and that 1 in 600,000 patients would experience nonfatal anaphylaxis requiring hospitalization (24).

For screening before vaccination, studies suggested a sensitivity of 70% and a specificity of 90% for the spherulin intradermal test (25-30).

Quality of Life

Our model included intermediate health states that may be associated with decrements in quality of life. To adjust for such decrements, we included quality adjustments in our model (Appendix II Table). Because there are no quality-of-life studies for coccidioidomycosis, we used proxy health state utilities in published studies or clinical judgment. All illness utilities were multiplied by age-specific "healthy" utilities to account for the age at which an illness was contracted. We assumed that primary pulmonary infection causes a substantial heath status decrement only in those who are sick enough to be diagnosed. Among those with primary pulmonary disease that goes undiagnosed, we assigned a health state utility equal to well for this illness episode, but accounted for lost time from work due to illness (see Costs, below). For the short-term vaccine side effects, we capture quality-of-life impact as a decrease in utility of one-tenth of a quality-adjusted day. The base case and ranges for these quality-of-life adjustments reflect our judgment about the impact of these episodes on patients. We evaluated the effects of these utility assumptions in sensitivity analyses.

Costs

Direct Medical Costs

All costs are presented in 2000 U.S. dollars (Appendix II Table). For costs of inpatient care, we used cost-adjusted charges from the 1996 Nationwide Inpatient Sample of the Healthcare Cost and Utilization Project (31). For outpatient services, we used the 2000 Medicare national physician fee schedule (32). For outpatient services not listed in the Medicare fee schedule, we used reimbursement received by Kern County service providers (R. Talbot, pers. comm.) We assumed that patients severely disabled by coccidioidal meningitis would require home support and used the average payment per Medicare home health beneficiary as a proxy for this cost (33).

Vaccine and Other Costs

In our base-case analysis, we assumed the cost of the vaccine was $180. We chose this value for the base case because it is the reimbursed fee for the three-shot hepatitis B series, an existing subunit vaccine. We examined a broad range of vaccine costs ($100 to $400) in sensitivity analyses.

For circumstances in which quality-of-life changes do not capture the inconvenience of illness or medical care, we included time costs, as noted in the online Appendix I.

Results

Prevention of Illness and Death

We calculated the number of cases of disseminated disease and deaths per 100,000 persons that would be prevented over the lifetime of each cohort if children and adults were immunized, assuming rates of vaccine coverage of 40%, 60%, 80%, and 100% (Table 1). For example, if 60% of children were vaccinated, 93 cases of dissemination, 64 cases of disability, and 7 deaths would be averted and $2 million saved per 100,000 population. If 60% of adults underwent screening followed by vaccination, 38 cases of dissemination, 24 cases of disability, and 2 deaths would be prevented at a cost of $5.4 million per 100,000 population.

Table 1. Lifetime cases of dissemination, disability, and death prevented and costs per 100,000 population if children are vaccinated and adults are screened, then vaccinated

	Dissemination prevented		Disability prevented		Deaths prevented		Net cost ($ millions)	
Compliance	Children	Adults	Children	Adults	Children	Adults	Children	Adults
100%	154	63	106	39	12	4	-3.3	9
80%	124	50	85	32	10	3	-2.6	7.2
60%	93	38	64	24	7	2	-2	5.4
40%	62	25	42	16	5	2	-1.3	3.6

Costs and Effectiveness

We calculated the effectiveness, costs, and cost-effectiveness of each strategy in each population, expressed in Table 2 as the per-person cost and health benefit. Among children, vaccination saved 1.9 quality-adjusted life days (QALD) and $33 per person. Among adults, screening followed by vaccination saved 0.5 QALD per person and cost $62,000 per QALY gained over no vaccination. For adults, the incremental gain from vaccinating all persons compared with screening followed by vaccination contributed an additional 0.05 QALD at a cost of $235,000 per QALY gained.

Sensitivity Analyses

We performed one-way sensitivity analyses over the ranges of all input variables listed in the table in Appendix II and on critical assumptions, such as the vaccine mechanism of action and target population. Among children, vaccination was no longer cost-saving at the lowest ranges of vaccine efficacy, infection rate, dissemination rate, long-term care cost for severe disability, and medical follow-up cost after nonmeningeal dissemination and chronic pulmonary disease; the vaccine also was not cost-saving in children when we used the highest ranges of vaccine cost, meningitis mortality, emigration, and discount rate among children. The counter-intuitive effect of meningitis deaths is due to the decrease in survivors subject to long-term care costs from post-meningitis disability. Among adults, sensitivity analyses that changed the cost-effectiveness ratio by $\geq$$40,000 per QALY gained included infection rate, vaccine effectiveness, discount rate, vaccine cost, dissemination rate, emigration rate, and office visit time. Only the vaccine cost changed the preferred strategy among adults. Below $106 per 3 doses, vaccination was preferred over screening/vaccination, with a cost-effectiveness ratio of $30,000 per QALY gained.

Vaccine Duration

Our base-case analysis assumed lifetime immunity after vaccination, but vaccine protection may wane. If vaccine protection waned to zero in 15 years, vaccination saved 0.82 QALD per child and cost $47,300 per QALY gained, and screening/vacccination saved 0.28 QALD per adult and cost $165,500 per QALY gained.

Vaccine Mechanism of Action

If the vaccine prevented primary infection rather than dissemination alone, vaccination saved 2.26 QALD and $46 per vaccinated child over no vaccination. Screening/vaccination saved 0.66 QALD per adult, costing $46,500 per QALY saved.

Three-Way Sensitivity Analysis of Infection Rate, Vaccine Effectiveness, and Cost

Our base case assumed an infection rate of 2% per year, vaccine effectiveness of 75%, and a vaccine cost of $180. However, the vaccine may have some use in areas of lower endemicity. Also, because the vaccine does not yet exist, its cost and effectiveness are unknown. We present the effects of varying costs and effectiveness of the vaccine under two conditions: 0.5% infection rate per year and 2% per year (our base case of highly endemic regions) (Figure 3). In our base case for children, the $180 vaccine remains cost-saving down to an effectiveness of 65% and costs <$50,000 per QALY until vaccine effectiveness drops below 30%, confirming that the vaccine need not be highly effective to be cost-effective in

Table 2. Health and economic outcomes of vaccination strategies[a]

Age	Strategy[b]	Life expectancy (years; days)	Incremental life expectancy[c] (days)	Quality-adjusted life expectancy (years; days)	Incremental quality-adjusted life expectancy[c] (days)	Cost ($)	Incremental cost[d] ($)	CE ratio ($/QALY)[e]
$\leq$17	No vaccine	28; 160.15	-	25; 172.98	-	35,196	-	-
	Screen/ vaccinate	28; 160.58	0.43	25; 174.66	1.68	35,187	-9	-
	Vaccinate	28; 160.62	0.04	25; 174.84	0.18	35,163	-24	Dominates
18-65	No vaccine	21; 272.68	-	18; 60.12	-	47,477	-	-
	Screen/ vaccinate	21; 272.82	0.14	18; 60.65	0.53	47,568	90	62,000
	Vaccinate	21; 272.84	0.02	18; 60.70	0.05	47,601	33	235,000

[a]Life expectancy and costs are discounted at 3% per year.

[b]Strategies are ranked by effectiveness, from the least to the most effective, for each age group.

[c]All incremental values compare an alternative with the next most effective strategy (e.g., cost [screen/vaccinate] – cost [no vaccine] = incremental cost [screen/vaccinate over no vaccine]).

[d]Negative values reflect cost savings compared to the next most effective strategy.

[e]Cost-effectiveness ratio: refer to online Appendix I for formula. A strategy dominates if it is both more effective and less expensive than all comparison strategies.

Screen/vaccinate = vaccination of susceptible persons identified through a screening skin test; vaccinate = vaccination of all persons

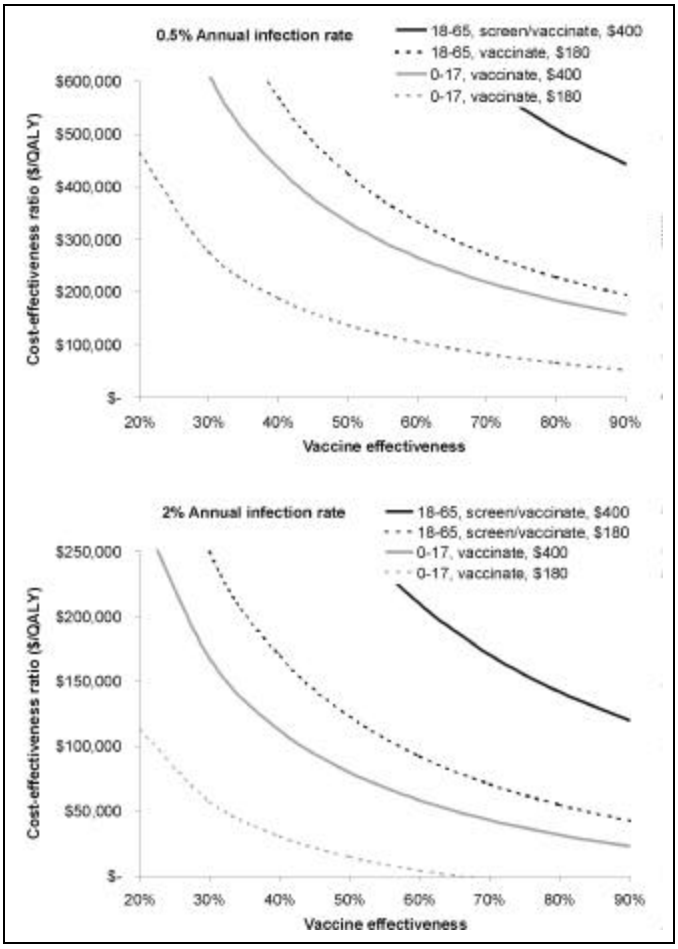

Figure 3. Sensitivity to infection rate, vaccine cost, and vaccine effectiveness. The vaccine is less cost-effective at higher vaccine cost, lower vaccine effectiveness and lower annual infection rates. When the line crosses the x-axis, the strategy is cost-saving. QALY = quality-adjusted life year; screen/vaccinate = vaccination of susceptible persons identified through a screening skin test; vaccinate = vaccination of all persons.

younger populations. A $400 vaccine costs approximately $50,000 per QALY gained among children at 65% effectiveness. A $400 vaccine would be economically unfavorable for the screening/vaccination strategy in adults at an annual infection rate of 2%, even at 90% vaccine effectiveness. At an infection rate of 0.5% per year, as might be seen in southern California (34), all strategies except $180 vaccination at 90% effectiveness in children are economically unfavorable.

Discount Rate

Among children, vaccination saved 5.95 QALD and $563 over no vaccination when costs and benefits were not discounted. At a discount rate of 5%, vaccination was no longer cost-saving at $27,600 per QALY gained. For adults, changing the discount rate from 0% to 5% changed the cost-effectiveness ratio of screening/vaccination compared with no vaccination from $30,800 to $155,000 per QALY gained.

Dissemination Rate

Our base-case analysis used a dissemination rate of 0.38% based on the assumption that only blacks and Asians had higher rates of dissemination than whites. We evaluated a range of 0.25% to 0.55% to determine the effect of assuming all non-whites (including Hispanics) had rates of dissemination equal to that of whites (0.25%) or that of blacks (3.4%). Among children, vaccination saved 2.71 QALD and $143 per child vaccinated at an overall dissemination rate of 0.55%, and 1.22 QALD at a cost of $15,300 per QALY gained at the 0.25% dissemination rate. Among adults, varying the dissemination rate from 0.25% to 0.55% changed the cost-effectiveness ratio of screening/vaccination over no vaccination from $25,400 to $125,000 per QALY gained.

New Residents

If one considers new residents separately, vaccination saved 2.18 QALD and $75 per immigrant child vaccinated, and saved 1.13 QALD per immigrant adult vaccinated, costing $13,500 per QALY gained.

We present best- and worst-case scenarios in Appendix I (online).

Discussion

We evaluated the usefulness of a potential vaccine against *C. immitis* in highly disease-endemic regions. Vaccination was cost-saving among children. A screening/vaccination strategy cost $62,000 per QALY gained among adults. Although the increase in quality-adjusted life expectancy from an immunization program is modest for one individual patient, this aggregates to an important number of illnesses and deaths prevented (Table 1). Furthermore, the life expectancy gains are comparable with gains from other immunizations. Vaccination for coccidioidomycosis (universally among children and after screening in adults) saved 0.14 to 0.47 life days and 0.53 to 1.86 QALD per person over no vaccination. In comparison, vaccination against pneumococcal bacteremia among elderly people saves 1.2 QALD per person vaccinated (35); infant vaccinations against measles, mumps, rubella, and pertussis each save 2.7, 3, 0.3, and 3.3 life days, respectively (36). To further place benefits in perspective, if the birth cohort in highly endemic counties of California and Arizona were immunized in 2001, 11 deaths would be averted and $3 million would be saved (in net present value) over the lifetime of these children.

The only previous analysis of the cost-effectiveness of a vaccine against coccidioidomycosis drew different conclusions. An IOM report considered the costs and benefits of research and development into a vaccine for *C. immitis* and found that the immunization of infants in endemic regions and immigrants of any age would cost >$100,000 per QALY (14). Our analysis evaluated a different question: If a vaccine were currently available, would it be cost-effective to immunize people in highly endemic regions? Although the IOM report acknowledged that the committee used rough estimates and a simplified model, the primary reason that it reached a different conclusion was that the cost of vaccine development was included in the IOM model. Vaccine development costs will influence the sale price of the vaccine. Project directors of the Valley Fever Vaccine Project estimate that research and development through phase III clinical trials, supported largely by public and private philanthropic sources, will cost $28 million (G. Rutherford, pers. comm.). This figure is 10 times lower than that used by the IOM in its analysis. To recoup $28 million over 5 years by immunizing 60% of the 90,000 annual birth cohort in highly endemic regions of California and Arizona, the vaccine would have to

be priced at $100 per vaccinated person. This is less than the $180 vaccine cost assumed in our base-case analysis.

Sensitivity analyses found that the vaccine would be cost-effective in children under assumptions of waning immunity and would be even more effective and cost-saving in children and cost-effective in adults if the vaccine prevented primary infection. In highly endemic regions, vaccination of children is cost-effective even at relatively low levels of vaccine effectiveness if the vaccine is priced at $180. A $180 vaccine had to be >85% effective for screening/vaccination to cost ≤$50,000 per QALY in adults. Paradoxically, an even higher annual infection rate would make a screening/vaccination strategy less favorable in adults because most of them would have already acquired natural immunity by age 40. At an annual infection rate one-fourth of that seen in the Central Valley of California, such as might be seen in southern California, the only cost-effective strategy would be to immunize children with an inexpensive and highly efficacious vaccine. If dissemination rates are higher than our base case, as might be seen in the elderly and those with chronic diseases, screening/vacccination becomes cost-effective for adults.

The vaccine is much more effective in persons without naturally acquired immunity. A vaccination strategy that targeted new residents in highly endemic regions would exploit easily obtainable risk factor information. However, such a policy might be unacceptable to members of the population excluded by such a strategy. Finally, because vaccination is a preventive intervention that can take years before accruing a health benefit (in contrast to acute health-care interventions), our results were highly sensitive to discount rate.

Our model has limitations. The ecology of endemic regions has changed substantially with urbanization since Smith's study in the 1940s (20). Furthermore, his studies documenting infection and dissemination were conducted in the military, which may not be representative of general population exposures. Our base-case analysis used the demographics in highly endemic areas to estimate a composite dissemination rate for a multiracial population. We did not model race independently, nor did we model exposure risk. Workers with high levels of dust exposure may have higher rates of infection and more to gain from early immunization.

Our model assumed that all age groups without prior immunity were at equal risk for adverse health outcomes and had identical health-care costs. However, illness severity and costs may be higher among the elderly. Reported cases of coccidioidomycosis during Arizona's 1991 to 1995 epidemic occurred disproportionately among older adults (11). The study did not discern whether this was due to reporting differences, immune-suppressing coexisting conditions, a preponderance of new immigrants with higher risk for infection, or an independent age-related phenomenon. The death rate from primary pulmonary infection is as high as 26.8% among persons over 65 (37), much higher than our composite base-case estimate of 0.5%. The Kern County data on which we based our cost assumptions revealed that hospitalization, the greatest health-care expenditure associated with acute illness, was 4.2 times more likely among people >50 years old. Thus, we may have underestimated the potential effectiveness and cost savings of the vaccine among older adults.

Finally, our analysis did not model the effects of an immunization program in immunocompromised patients. Persons with compromised cell-mediated immunity, including those with AIDS, malignancy, or therapeutic medical immunosuppression, have higher rates of serious illness. Many of these patients may have reactivation of previous infection; it is unclear whether vaccination would be useful in this clinical scenario. This is an area for future research as results from clinical trials become available.

An uncommon disease in a national context, coccidioidomycosis has substantial ramifications for several regions where epidemics have produced reported annual case rates as high as 15 per 100,000 and substantial economic impact. Diseases affecting <200,000 persons annually (orphan diseases) require more incentives for research and development. Our findings may contribute to the policy decisions for vaccine development and distribution for *C. immitis* (39). Our analysis suggests that a vaccine against *C. immitis* would have substantial public health benefit. An update of this cost-effectiveness analysis can be performed when the results of human vaccine trials become available.

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Dr. Barnato was a fellow in health care research and policy in the Department of Medicine at Stanford University School of Medicine when she completed this work. She is currently assistant professor of medicine at the University of Pittsburgh School of Medicine. Her research interests include quantitative assessment of preventive interventions and policy tools for quality improvement and cost control in health care.

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Appendix I. Clinical and Economic Background

Appendix I is online only; it contains the formula used to calculate the incremental cost-effectiveness of these strategies; URL: http://www.cdc.gov/ncid/eid/vol7/no5/barnato_appendix1.htm

Appendix II: Model Input Variables

Appendix II Table. Input variables, quality of data, and sources[a]

Input variable	Base-case estimate (range)	Quality of evidence[b]	Source
Epidemiology (%)			
Vaccine effectiveness	75 (20-90)	I	2
Skin-test sensitivity	70 (50-80)	II-2	3-5
Skin-test specificity	90 (70-97)	II-2	4,6,7
Annual infection rate	2 (0.25-3)	II-3	5,8-16
Annual emigration among vaccinees out of highly endemic region	0.5 (0-4.2)	II-2, III	[c]
Symptomatic primary pulmonary disease after infection	40	II-2	18
Diagnosed symptomatic primary pulmonary disease	10 (5-15)	III	d
Death from primary pulmonary disease, given diagnosis	0.5 (0-26)	II-2	19-22
Chronic pulmonary disease after diagnosed primary infection	5 (1-10)	III	23-26
Death from chronic pulmonary disease	5 (0-20)	III	24[e]
Dissemination after infection	0.38(0.25-0.55)	II-2	17
Meningitis, given dissemination	33 (23-44)	II-2	21,26
Death from meningeal dissemination	7 (5-40)	II-2, III	27[d,e]
Moderate disability after meningeal dissemination	50 (40-60)	III	27[d,e]
Severe disability after meningeal dissemination	17 (10-30)	III	27[d,e]
Annual meningeal dissemination relapse rate, on treatment	2 (0-5)	I, II-2	28-30
Death from nonmeningeal dissemination	2 (0-10)	III	[e]
Moderate disability after nonmeningeal dissemination	33 (20-50)	III	[d,e]
Annual nonmeningeal dissemination relapse rate			
On treatment	2 (0-5)	I, II-2, III	4[c]
Off treatment	50 (35-65)	I, II-2, III	31-34[c]
Mild vaccine side effects	25 (10-40)	II-2	35
Vaccine anaphylaxis, x 10^{-4}	1.67 (0.1-10)	II-2	35
Direct medical costs ($)			
Three doses of vaccine	180 (100-400)	III	36,37
Skin test	12 (9-15)	III	38
Home care, per month	2,450 (1,840-3,060)	II-2	39
Diagnosed pulmonary disease	2,090 (1,570-2,610)	II-2, III	40
Incident meningeal dissemination	9,510 (7,130-11,890)	II-2	40
Medication and follow-up after *Coccidioides immitis* meningitis,[f] per month	1,510 (1,130-1,890)	II-2	41[e,g]
Incident nonmeningeal dissemination	6,950 (5,210-8,690)	II-2	40
Medication and follow-up for chronic pulmonary infection and nonmeningeal dissemination,[f] per month	530 (290-790)	II-2	41[e,g]
Inpatient vaccine anaphylaxis treatment	2,180 (1,640-2,730)	II-2	40

Appendix II Table. Input variables, quality of data, and sources[a] (continued)

Input variable	Base-case estimate (range)	Quality of evidence[b]	Source
Time costs[h]			
Average wage ($ per hour)	12 (9-15)	II-2	[d]
Average clinic visit (hours)	1.25 (0.5-2)	III	Assumed
Lost work due to undiagnosed primary pulmonary disease (days)	5 (0-10)	III	Assumed
For parents of sick children (days)	3 (0-5)	III	Assumed
Utilities			
Well	0.94 to 0.70[i]	II-2	42
Diagnosed primary pulmonary infection	0.90 (0.85-0.95)	III	[d]
Chronic pulmonary infection (proxy, pulmonary tuberculosis)	0.57 (0.29-0.84)	II-2, III	42
Meningeal dissemination (proxy, paraplegia)	0.40 (0.21-0.52)	II-2, III	42
Nonmeningeal dissemination (proxy, orthopedic impairment)	0.59 (0.34-0.84)	II-2, III	42
Severe disability after meningitis (proxy, hemiplegia)	0.27 (0.10-0.38)	II-2, III	42
Moderate disability after meningitis (proxy, sciatica)	0.72 (0.52-0.92)	II-2, III	42
Moderate disability after nonmeningeal dissemination (proxy, arthritis)	0.69 (0.51-0.92)	II-2, III	42
Chronic azole treatment (proxy, warfarin treatment)	0.98 (0.92-1.0)	II-2, III	43
Dead	0	III	Assumed
Vaccine side effect quality-of-life decrement (days)	0.1 (0-0.2)	III	Assumed
Other variables (%)			
Discount rate	3 (0-5)	III	44

[a]The base-case estimate represents our best estimate for each value. All costs are in 2000 U.S. dollars.

[b]The quality rating is derived from the U.S. Preventive Services Task Force Guide to Clinical Preventive Services (1). Source of evidence: I: at least one properly randomized controlled trial; II-1: well-designed controlled trial without randomization; II-2: well-designed cohort or case-control analytic studies; II-3: multiple time series with or without intervention; III: opinions of respected authorities; descriptive studies and case reports; or reports of expert committees (1).

[c]Internal Revenue Service, unpub. data.

[d]John Galgiani, pers. comm.

[e]Hans Einstein, pers. comm.

[f]We assumed that meningitis patients were treated with 800 mg of daily fluconazole, and chronic pulmonary and nonmeningeal dissemination patients with either 400 mg fluconazole or 400 mg ketoconazole daily (Royce Johnson, pers. comm., 1999). A 50:50 distribution of fluconazole and ketoconazole use represents our base case; the upper end of the range assumes all nonmeningeal dissemination patients receive fluconazole in follow-up, whereas the lower end assumes they receive the less expensive ketoconazole.

[g]Ron Talbot, pers. comm.

[h]Based on a weighted adjusted gross income of $24,105 for taxpayers in the 10 highly endemic counties (Internal Revenue Service, unpub. data).

[i]Mean HALex scores for healthy persons, by age group (when men and women had differing mean scores, we chose the higher of the two scores): <5=0.94; 5-17=0.93; 18-24= 0.92; 25-34=0.91; 35-44=0.90; 45-54=0.87; 55-64=0.81; 65-74=0.78; >75=0.70 (43).

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First Isolation of *La Crosse Virus* from Naturally Infected *Aedes albopictus*

Reid R. Gerhardt,* Kristy L. Gottfried,† Charles S. Apperson,‡ Brent S. Davis,† Paul C. Erwin,§ A. Brent Smith,* Nicholas A. Panella,† Eugene E. Powell,‡ and Roger S. Nasci†

*University of Tennessee, Knoxville, Tennessee, USA; †Centers for Disease Control and Prevention, Fort Collins, Colorado, USA; ‡North Carolina State University, Raleigh, North Carolina, USA; and §State of Tennessee Department of Health, East Tennessee Region, Knoxville, Tennessee, USA

La Crosse virus (LAC), a California serogroup bunyavirus, is the leading cause of pediatric arboviral encephalitis in the United States and an emerging disease in Tennessee, West Virginia, and North Carolina. Human cases of LAC encephalitis in Tennessee and North Carolina have increased above endemic levels during 1997 to 1999 and may represent an expansion of a new southeastern endemic focus. This report describes the isolation of LAC virus from the exotic mosquito *Aedes albopictus.* The discovery of LAC virus in wild populations of *Ae. albopictus* , coupled with its expanding distribution in the southeastern United States, suggests that this mosquito may become an important accessory vector, potentially increasing the number of human cases in endemic foci or expanding the range of the disease.

Historically, *La Crosse virus* (LAC) encephalitis has been most common in the upper midwestern United States, primarily Illinois, Iowa, Indiana, Minnesota, Ohio, and Wisconsin (1), where the primary vector is *Ochlerotatus triseriatus*, a native mosquito that breeds in tree holes and artificial containers. In recent years, LAC encephalitis activity has increased in West Virginia, which has had more recorded cases than any other state, accounting for more than half of cases reported from 1996 to 1999 (2). A recent cluster of new cases in Tennessee is the first report of a possible extension of the West Virginia endemic focus and may represent a new southeastern endemic focus (1).

From 1963 to 1996, nine cases of pediatric LAC encephalitis were reported in Tennessee (1). From 1997 to 1999, however, the East Tennessee Regional Health Office in Knoxville confirmed 26 pediatric LAC encephalitis cases in Tennessee and southeastern Kentucky (10 confirmed cases in 1997 [1], 10 in 1998, and 6 in 1999 [3]) making LAC encephalitis the most common arboviral disease in Tennessee. During 1997 and 1999, counties reporting confirmed cases were Knox (9 cases); Anderson (3 cases); Cumberland (3 cases); Sevier and Claiborne (2 cases each); and Blount, Jefferson, Cocke, and Campbell (1 case each) in Tennessee; Bell County (1 case) in southeastern Kentucky; and 2 cases in the region with undetermined infection location (Erwin PC, unpub. data). The presumptive sites of transmission in Tennessee are in the Ridge and Valley Province, with the exception of three 1999 cases in Cumberland County, on the Cumberland Plateau. Despite the marked increase in human cases since 1996, LAC virus had never been isolated from any mosquito species in Tennessee before this finding. In a retrospective serologic study, the overall rate of seropositivity to LAC virus was 0.5% for human sera (n = 1,000) collected in 15 eastern Tennessee counties (3).

In contrast to Tennessee, approximately three cases per year of LAC encephalitis have been reported from western North Carolina from 1977 to 1995 (4,5). LAC virus has been isolated from *Oc. triseriatus* (6,7) collected from the homes of LAC encephalitis patients in North Carolina. As in Tennessee, LAC encephalitis cases have increased in North Carolina since 1996. Twenty-five cases were reported for the period 1996 to 1999, with 10 cases for 1999 and 5 cases for each of the other years (NC Department of Health and Human Services, unpub. data). In a retrospective serologic survey, the overall rate of seropositivity to LAC virus was 9.6% for human sera (n = 1,016) collected in 12 western North Carolina counties (5).

Ae. albopictus, a mosquito that breeds in tree holes and artificial containers, was discovered in Houston, Texas, in 1985. It was probably introduced from its natural range in Asia to the United States in imported used tire casings (8). Subsequently, this species has spread rapidly throughout much of the United States (8) and is now found in 928 counties and 30 states (Moore CG, pers. comm.), including all Tennessee (9) and North Carolina counties (Apperson CS, Harrison BA, unpub. data). In addition to being a nuisance, this species is known to transmit dengue, yellow fever, and a variety of other arboviruses. *Ae. albopictus* has been tested frequently for the presence of human arboviruses since its introduction into the United States (8). Eastern equine encephalitis virus, Cache Valley virus (8), and Jamestown Canyon virus (10) are the only human pathogens that have previously been isolated from naturally infected *Ae. albopictus* in the United States, although none of these isolations have been in association with an outbreak or known human cases. *Ae. albopictus* is a competent laboratory vector of LAC

Address for correspondence: Kristy L. Gottfried, Centers for Disease Control and Prevention, P.O. Box 2087, Fort Collins, CO 80522, USA; fax: 970-221-6476; e-mail: kig4@cdc.gov

virus (11) and can transmit the virus vertically by the transovarial route (12,13).

Active surveillance for human LAC encephalitis cases and virus-infected mosquitoes has been conducted in eastern Tennessee since 1997. In North Carolina, case surveillance remains passive; however, efforts to collect virus-infected mosquitoes on the home properties of some case patients were made from 1997 to 1999. We describe the results of surveillance programs designed to examine container-breeding mosquitoes near the homes of LAC encephalitis patients in an attempt to isolate LAC virus from its vector(s) in eastern Tennessee and western North Carolina.

Materials and Methods

Collection Sites and Mosquito Rearing

During 1997 to 1999 in Tennessee, surveillance of container-breeding mosquitoes was conducted weekly at the homes of six patients with confirmed LAC encephalitis. Collections were made from two sites in Knox County (Karns and Oak Ridge Highway) and Anderson County (Clinton and Holt Road) and one site each in Blount County (Townsend) and Cumberland County (Crab Orchard) (Figure 1). In North Carolina, mosquitoes were collected weekly at nine sites on the Cherokee Indian Reservation (six in Jackson and three in Swain counties) and 10 sites in Buncombe County (four in 1998 and six in 1999) (Figure 1). Except for one site in Buncombe County, all sites were the homes of LAC encephalitis patients. Standard oviposition traps (14) with seed germination paper as an oviposition substrate (15,16) were used to collect eggs of *Ae. albopictus* and *Oc. triseriatus*. In Tennessee, 30 oviposition traps were placed at Karns, 20 at Oak Ridge Highway, and 10 at each of the other sites. In North Carolina during 1998, four oviposition traps were placed at each site, and in 1999, eggs were collected from 10 oviposition traps per site.

Eggs on the oviposition strips were hatched by placing them in 2 L H_2O with 2.5 g of liver powder or a mixture of liver powder:brewers yeast (1:2 by weight) for 48 hours. Larvae were reared at 28°±2°C with constant light. Immediately after adult emergence, the mosquitoes were sorted by species and sex, placed in pools of $\leq$50 individuals, and stored at 70°C until tested for the presence of virus.

Virus Isolation and Identification

Pools of mosquitoes were placed in 12 x 75 mm (5 mL) Falcon tubes (Becton Dickinson Labware, Franklin Lakes, NJ) with 2 mL BA-1 diluent (1 x M199 with Hanks balanced salt solution, 0.05 M Tris buffer [pH 7.6], 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 g/L streptomycin, 100 units/mL penicillin, and 1 g/mL Fungizone [Apothecon, Princeton, NJ]). Pooled mosquitoes were ground by placing four 4.5-mm steel beads (BB caliber airgun shot) into the tube with the mosquitoes and diluent and vortexing for 20 to 30 seconds. The mosquito homogenate and steel beads were centrifuged to form a pellet.

Specimens were tested for the presence of live virus with a Vero cell culture plaque assay in six-well plates (17). Supernatant from the centrifuged mosquito homogenate (0.1 mL) was added to each of two wells, incubated at 37°C for 60 minutes, and covered with 3.0 mL of an agar overlay (1% Seakem LE agarose in M199, 0.2% sodium bicarbonate, 100 units/mL penicillin, 100 µg/mL streptomycin, 250 µg/mL gentamycin, and 4.5 µg/mL Fungizone). After 4 days of incubation (37°C, 4.7% CO_2), 3.0 mL of a second 1% agar overlay containing 0.004% neutral red was added to each well, and incubation was continued. Wells were examined for plaques daily for 10 days. Cells in virus-positive wells were harvested in 2 mL BA-1 diluent containing 20% fetal calf serum and frozen at -70°C. The positive original mosquito homogenates were reinoculated on Vero cells to confirm the presence of virus.

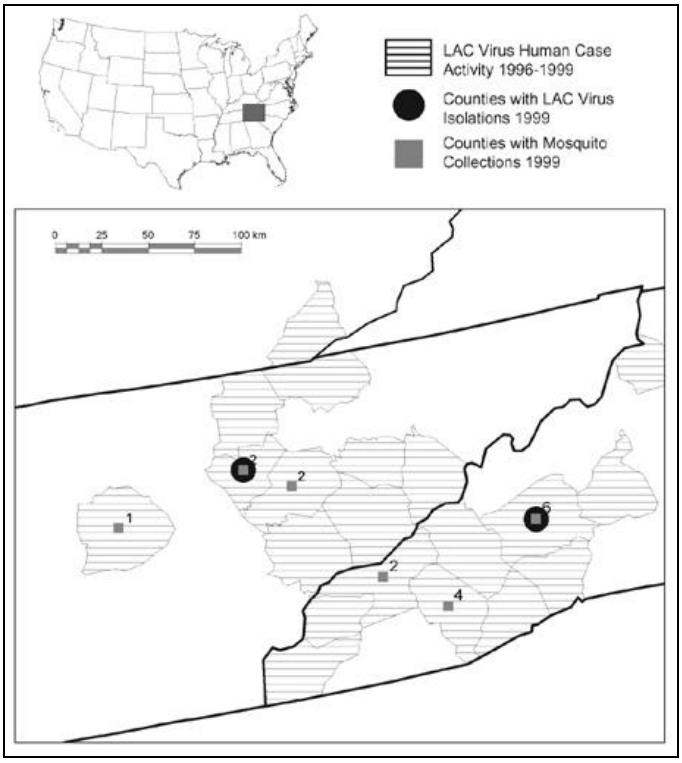

Figure 1. Regional map of eastern Tennessee, western North Carolina, and southeastern Kentucky, showing counties reporting human cases of La Crosse encephalitis from 1996 to 1999, counties with 1999 mosquito collection sites, and counties with 1999 *La Crosse virus* isolations from *Aedes albopictus*.

Virus isolates were identified by reverse transcription polymerase chain reaction (RT-PCR), followed by genomic sequencing performed on an ABI Prism 377 Sequencer (Applied Biosystems/PerkinElmer, Foster City, CA) according to the manufacturer's recommendations. Previously published S-segment (BCS82C, BCS332V) and L-segment (LCL80C, LCL199V) (18) and LAC M-segment primers (5'-CTTCATATTGACCACATG-3', 5'-CCATGCCTGTTTCAATCAGCATATGTC-3') were used to amplify viral RNA by RT-PCR for sequencing.

Molecular Identification of the Species in the Original Mosquito Pool

Genomic DNA was extracted from the original ground mosquito pool. The homogenate (50 µL) was incubated at 65°C for 30 minutes in a 1.7-mL microcentrifuge tube (Co-Star, Corning, NY). Potassium acetate (7 µL of 8 M) was added for a homogenate final concentration of 1 M, incubated on ice for 30 minutes, and centrifuged for 15 minutes at 14,000 rpm. The supernatant was transferred to a fresh 1.7-mL microcentrifuge tube. DNA was precipitated by add-

ing 140 µL of absolute ethanol, mixing by inverting the tube gently, and incubating at room temperature for 5 minutes. After centrifugation at 14,000 rpm for 15 minutes, the pellet was washed with cold 70% ethanol and resuspended in 10 µL of 10 mM tris buffer solution (pH 8.5).

Species-specific primers were designed to amplify the large subunit ribosomal DNA sequence of *Ae. albopictus* and *Oc. triseriatus*. These primers were used to confirm the species composition of the original mosquito pool. The oligonucleotides were designed from the complete rDNA sequences published for *Oc. triseriatus* (19) and *Ae. albopictus* (20). The 18-bp forward primer (5'-CGTGGATCGATGAAGACC-3'), located in the highly conserved 5.8s region of rDNA, was used for both species. The reverse primers for both species were unique for that species and located in the ITS2 region of rDNA. The reverse primer for *Ae. albopictus* was 5'-GACACCGCACCGCACAACTCACAC-3' and *Oc. triseriatus* was 5'-TATGCTATCCGTTCGAGAG-3'. The Expand Fidelity PCR System (Roche Diagnostics, Indianapolis, IN) was used to amplify the DNA product. Each 50-µL PCR reaction contained 5 µL 10X buffer #2, 2 µL (10 µM) of forward and reverse primer, 1 µL (10 mM) deoxynucleoside-triphosphate (dNTP) mix, 5 µL genomic mosquito pool DNA, and 34 µL of nuclease-free water. Primer reactions were amplified in a Perkin-Elmer model 9600 thermocycler (GeneAmp PCR System). The reaction was incubated at 94°C for 5 minutes, followed by 4°C after 1 µL of enzyme was added. This 50-µL reaction was amplified by PCR for 1 cycle of 94°C for 15 seconds, 50°C for 20 seconds, and 68°C for 1 minute; 9 cycles of 94°C for 15 seconds, 55°C for 20 seconds, and 68°C for 1 minute; and 20 cycles of 94°C for 15 seconds, 55°C for 20 seconds, and 68°C for 1 minute + 5 seconds/cycle. The amplification product was analyzed by electrophoresis of 5 µL of the reaction on a 2% agarose gel containing ethidium bromide and visualized by UV light. The sensitivity and specificity of the species-specific primers were evaluated by using pools of *Ae. albopictus*, *Oc. triseriatus*, and *Ae. albopictus*/*Oc. triseriatus* combinations (Figure 2).

Results

For Tennessee, 10,717 mosquitoes were reared to the adult stage and analyzed for virus (Table). At all collection sites, the *Ae. albopictus* population density was approximately fourfold greater than that of *Oc. triseriatus*. LAC virus was isolated from one pool of 14 female *Ae. albopictus* (TN00-2266) collected at the Clinton site in Anderson County on August 16, 1999. When base sequences of the 120-bp L-segment, 775-bp M-segment, and 251-bp S-segment amplicons were compared with published LAC virus genome sequences (GenBank U-12396, U-18980, AF025479), homology was 98%, 95%, and 100%, respectively, confirming the product is LAC virus. By PCR analysis, the species composition of the original mosquito pool was *Ae. albopictus* only, with no detectable traces of *Oc. triseriatus* tissue (Figure 2). The minimum field infection rate (21) for *Ae. albopictus* oviposited during the week the positive pool was collected is estimated to be 6.5 infected mosquitoes per 1,000 specimens. The ratio of *Ae. albopictus* to *Oc. triseriatus* was 153:1 for the weekly collection (August 16, 1999) containing the positive mosquito pool. To ensure that no isolates were missed in the initial Vero cell plaque assay screening, all *Ae. albopictus* and *Oc. triseriatus* mosquito pools collected from the Clinton site during August were tested by RT-PCR for the presence of LAC viral RNA and found to be negative.

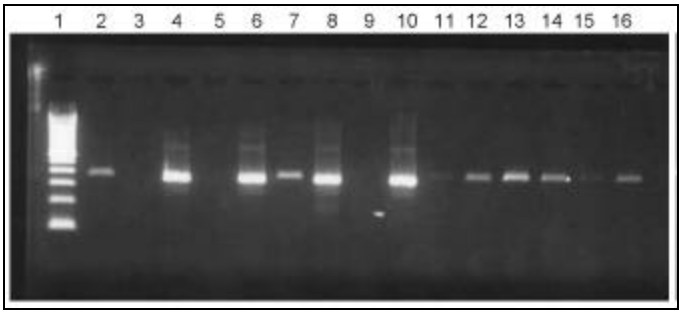

Figure 2. Species-specific gel electrophoresis of *La Crosse virus*-positive mosquito pools from Tennessee and North Carolina and *Aedes albopictus* pools with varying degrees of *Ochlerotatus triseriatus* contamination. Lane 1, 100-bp ladder; lane 2, *Oc. triseriatus* pool with *Oc. triseriatus* primers; lane 3, *Oc. triseriatus* pool with *Aedes albopictus* primers; lane 4, *Ae. albopictus* pool with *Ae. albopictus* primers; lane 5, *Ae. albopictus* pool with *Oc. triseriatus* primers; lane 6, *Ae. albopictus* pool contaminated by 1 *Oc. triseriatus* individual, with *Ae. albopictus* primers; lane 7, *Ae. albopictus* pool contaminated by 1 *Oc. triseriatus* individual, with *Oc. triseriatus* primers; lane 8, TN00-2266 *Ae. albopictus* pool with *Ae. albopictus* primers; lane 9, TN00-2266 *Ae. albopictus* pool with *Oc. triseriatus* primers; lane 10, NC00-1547 *Ae. albopictus* pool with *Ae. albopictus* primers; lane 11, NC00-1547 *Ae. albopictus* pool with *Oc. triseriatus* primers; lane 12, *Ae. albopictus* pool contaminated by 1 *Oc. triseriatus* head, with *Oc. triseriatus* primers; lane 13, *Ae. albopictus* pool contaminated by 1 *Oc. triseriatus* thorax, with *Oc. triseriatus* primers; lane 14, *Ae. albopictus* pool contaminated by 1 *Oc. triseriatus* abdomen, with *Oc. triseriatus* primers; lane 15, *Ae. albopictus* pool contaminated by 3 *Oc. triseriatus* legs, with *Oc. triseriatus* primers; and lane 16, *Ae. albopictus* pool contaminated by 6 *Oc. triseriatus* legs, with *Oc. triseriatus* primers.

In North Carolina, LAC virus was isolated from one pool of 44 female *Ae. albopictus* (NC00-1547) and one pool of 50 male *Oc. triseriatus* collected on September 6, 1999, from the same site in Buncombe County. When base sequences of the 120-bp L-segment, 775-bp M-segment, and 251-bp S-segment amplicon were compared with published LAC virus genome sequences (GenBank U-12396, U18980, AF025479), homology was 96%, 95%, and 100%, respectively, confirming the product is LAC virus. Minimum field infection rates (21) for the week of the LAC virus-positive oviposition trap collections were 4.7 and 3.8 infected mosquitoes per 1,000 specimens for *Ae. albopictus* and *Oc. triseriatus*, respectively.

PCR analysis to verify the species composition of the original mosquito pool suggested that the *Ae. albopictus* pool was contaminated with *Oc. triseriatus* tissue (Figure 2). The putative *Oc. triseriatus* amplicon could not be sequenced because of the small amount of product initially amplified. However, in a subsequent PCR test, amplification for an extended period of time provided sufficient material. Sequence analysis of the amplicon matched the *Oc. triseriatus* sequence (19), verifying that the *Ae. albopictus* pool contained *Oc. triseriatus* tissue.

The Clinton site where the positive mosquito pool from Tennessee was obtained is the home of a child who had onset of LAC encephalitis on June 23, 1998. The residence is well maintained, with few permanent or disposable containers; the property is partially wooded, with oak and hickory trees. The house and property are within 15 m of an oak and hickory forest. Numerous disposable containers were observed along the road (10 m distant) and at a nearby residence (40 m). The Buncombe County residence where infected mosquitoes were collected is the site where a child is presumed to

Table. Numbers of *Aedes albopictus* and *Ochlerotatus triseriatus* adults reared from eggs and pooled specimens tested for *La Crosse virus* from mosquitoes collected at La Crosse encephalitis case sites in eastern Tennessee and western North Carolina, 1999

		Ae. albopictus		*Oc. triseriatus*	
County-case site	Collection dates	No. individuals	No. pools	No. individuals	No. pools
Tennessee					
Knox-Karns	5/10-9/18	3,999	131	1,838	72
Knox-Oak Ridge Hwy	8/10-9/17	464	16	10	2
Anderson-Clinton[a]	6/6-9/13	2,733	92[a]	649	40
Anderson-Holt Road	8/10-9/17	309	11	36	2
Blount-Townsend	8/23-9/15	252	8	0	0
Cumberland-Crab Orchard	8/13-9/6	427	15	0	0
Total		8,184	181	2,533	116
North Carolina					
Buncombe-site 9[b]	5/20-10/4	1,427	86	1,944	87

[a]Virus isolated from one pool of *Ae. albopictus* (14 females).
[b]Virus isolated from one pool of *Ae. albopictus* (44 females) and three pools of *Oc. triseriatus* (4, 6, and 50 males).

have contracted LAC encephalitis on July 30, 1997. The residence is one of numerous mobile homes located 50 m from the Swannanoa River. *Ae. albopictus* and *Oc. triseriatus* larvae have been periodically collected from discarded containers in a trash dump behind the mobile home park adjacent to the river. Most of this area is heavily shaded by mature oaks and poplars, and the understory is made up of knee-high grasses and other herbaceous plants.

Conclusion

We report the first isolation of LAC virus from naturally infected *Ae. albopictus* mosquitoes (TN00-2266). The discovery of vertically infected *Ae. albopicus* in the field indicates that an adult female mosquito fed on a viremic host, became infected with the virus, and successfully transmitted the virus to offspring. However, the possibility that the adult female was infected by the venereal or transovarial route cannot be completely dismissed. In light of the ability of this species to orally transmit LAC virus (11), this finding indicates that *Ae. albopictus* may become an important accessory vector of LAC virus in enzootic foci and may facilitate expansion of the existing enzootic foci into new areas.

We believe that the North Carolina isolate from the *Ae. albopictus* pool resulted from a single virus-infected *Ae. albopictus*, rather than from contamination by a single virus-infected *Oc. triseriatus*. *Oc. triseriatus* tissue was present in the original mosquito pool that was amplified by PCR and visualized by gel electrophoresis (Figure 2). *Ae. albopictus* pools were made with known degrees of *Oc. triseriatus* tissue contamination (three and six legs, head, thorax, abdomen) (Figure 2). The original pool likely did not contain an intact *Oc. triseriatus* specimen because the amplification intensity was considerably lower than that produced by a whole mosquito (Figure 2). Instead, at least two *Oc. triseriatus* legs were likely present in the *Ae. albopictus* pool. However, the possibility of LAC virus contamination cannot be completely dismissed because an LAC virus isolate was obtained from *Oc. triseriatus* collected from the same site on the same date.

Ae. albopictus has been collected at every site examined (68 sites) in 13 Tennessee counties from 1997 to 1999 (Gerhardt RR, Gottfried KL, unpub. data). Although once considered rare in Tennessee, the species is now ubiquitous in the eastern part of the state. Likewise, in western North Carolina, *Ae. albopictus* is widely distributed throughout the mountains, where LAC virus transmission is endemic (6).

The relationship between the recent increase in LAC encephalitis cases (1997 to present) in the region and the expanding range of *Ae. albopictus* is one of association at this time. Additional research is needed to firmly establish *Ae. albopictus* as a vector of LAC virus in eastern Tennessee and western North Carolina. The remarkable disparity in seroprevalence rates of LAC antibodies in human sera between eastern Tennessee (0.5%) and western North Carolina (9.6%) provides indirect evidence that the disease is relatively new in the eastern Tennessee region. We can only speculate that the continued range expansion of *Ae. albopictus* will result in an increase in both incidence and distribution of LAC virus in Tennessee and North Carolina and throughout the southeastern United States.

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Dr. Gerhardt is a professor of entomology at the University of Tennessee, Knoxville. His research interests includes medical and veterinary entomology with emphasis in biology and ecology of mosquitoes, ticks, and biting flies.

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Factors Contributing to the Emergence of *Escherichia coli* O157 in Africa

Paul Effler,* Margaretha Isaäcson,† Lorraine Arntzen,† Rosemary Heenan,‡ Paul Canter,§ Timothy Barrett,* Lisa Lee,* Clifford Mambo,§ William Levine,* Akbar Zaidi,* and Patricia M. Griffin*

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †South African Institute for Medical Research, Johannesburg, South Africa; ‡GOAL, Dublin, Ireland; and §Ubombo Sugar Limited, Big Bend, Swaziland

In 1992, a large outbreak of bloody diarrhea caused by *Escherichia coli* O157 infections occurred in southern Africa. In Swaziland, 40,912 physician visits for diarrhea in persons ages ≥5 years were reported during October through November 1992. This was a sevenfold increase over the same period during 1990-91. The attack rate was 42% among 778 residents we surveyed. Female gender and consuming beef and untreated water were significant risks for illness. *E. coli* O157:NM was recovered from seven affected foci in Swaziland and South Africa; 27 of 31 patient and environmental isolates had indistinguishable pulsed-field gel electrophoresis patterns. Compared with previous years, a fivefold increase in cattle deaths occurred in October 1992. The first heavy rains fell that same month (36 mm), following 3 months of drought. Drought, carriage of *E. coli* O157 by cattle, and heavy rains with contamination of surface water appear to be important factors contributing to this outbreak.

Early in November 1992, physicians arriving for duty at a small hospital on a sugar plantation in Swaziland found >100 persons sprawled on the ground in front of the casualty department. Many had bloody diarrhea, and almost all were suffering severe abdominal pains. The next day the number of patients with the dysenteric illness nearly doubled, yet stool specimens sent to local laboratories did not yield common parasitic or bacterial pathogens, including *Shigella* spp. With the etiologic agent still unknown into the second week of the outbreak, specimens were forwarded to a reference laboratory in South Africa, where a surprising discovery was made: *Escherichia coli* O157 had emerged in Africa (1).

An outbreak of *E. coli* O157 infections was heretofore unheard of in Africa, or for that matter, anywhere in the developing world. *E. coli* O157 had been isolated only once before in southern Africa, from an elderly man undergoing surgery for lower gastrointestinal bleeding in Johannesburg in 1990 (2). We present a comprehensive account of a 1992 regional outbreak in Africa, perhaps the largest *E. coli* O157 outbreak ever reported.

The Kingdom of Swaziland occupies 17,360 km^2 in southern Africa; 62% of the surface area is pastureland used primarily for cattle. The estimated human population in 1992 was 850,000. The sugar plantation referred to in this report is located in the Lubombo District, a lowland area that shares borders with Mozambique and South Africa (Figure 1). The plantation irrigates its extensive cane fields with water drawn from the nearby Usutu River. Twenty villages dispersed across the plantation housed approximately 5,000 workers and dependents. Standpipes with treated potable water and other pipes carrying untreated surface water were located in most villages. Plantation workers were provided with a weekly ration of beef obtained from local abattoirs.

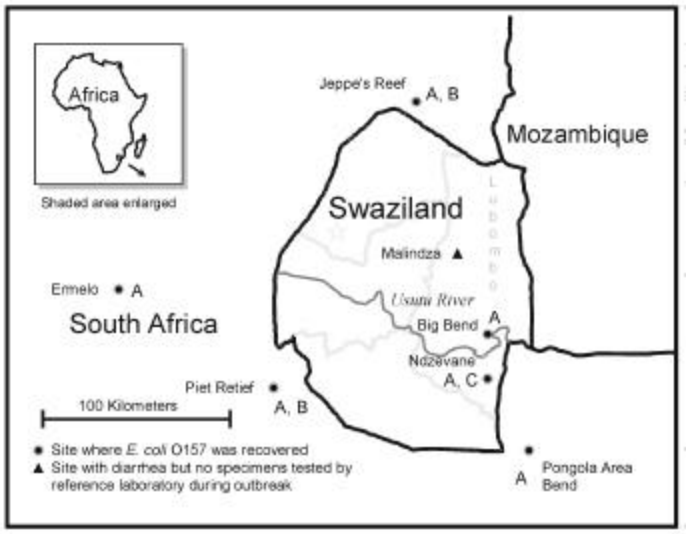

Figure 1. Map of the area affected by an outbreak of diarrheal illness in 1992.[a]
[a]Pulsed-field gel electrophoresis of 31 of the *Escherichia coli* O157:NM isolates from six locations identified three distinct patterns, designated A, B and C; the locations where these isolates were obtained are indicated on the map by the corresponding letter. Not shown on the map is Empangeni, South Africa, located approximately 200 km south of Swaziland border, where *E. coli* O157 was also recovered. The Usutu River is also called the Lusutfu River.

When the outbreak occurred, two refugee settlements, Ndzevane (population 7,700) and Malindza (population 11,900), were located 19 km and 60 km, respectively, from

Address for correspondence: Paul V. Effler, State of Hawaii Department of Health, 1250 Punchbowl Street, Room 444, Honolulu, Hawaii 96813, USA; fax: 808-586-8347; e-mail: pveffler@mail.health.state.hi.us

the plantation. Refugees and Swazi nationals residing locally received health care at settlement clinics. Jeppe's Reef, Pongola, Empangeni, Piet Retief, and Ermelo are towns and villages in nearby South Africa.

Methods

Diarrheal Disease Surveillance

The number of patients with diarrhea at clinics in the affected area was obtained by reviewing outpatient logbooks at Malindza, Ndzevane, and the plantation hospital. National and district figures for reported diarrheal illness by month and year were provided by the Statistics Section of the Ministry of Health, Swaziland. Data on deaths were unavailable.

Rainfall Measurements

Data on rainfall at the Big Bend Experimental Farm, located approximately 1 km from the plantation, were obtained for 1991-92 from the Swaziland National Meteorological Service, Ministry of Transport and Communication. A meteorologic station on the plantation site also provided precipitation figures for October 1992.

Surveillance of Cattle Deaths

Periodic dipping of cattle with insecticides used for the control of ticks was mandatory, and losses were closely monitored in this manner. Figures for cattle deaths from 1988 through 1992 were obtained from the Ministry of Agriculture, Swaziland.

Clinical and Environmental Laboratory Methods

Initial stool specimens were cultured at local laboratories in Swaziland, which did not test for *E. coli* O157. Subsequent specimens were submitted to the South African Institute of Medical Research (SAIMR), which routinely attempted to identify *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp., and *Yersinia enterocolitica* from all specimens; methods for isolating *E. coli* O157 were introduced in response to the outbreak. Human stool specimens and rectal swabs, cattle dung, and food samples were plated on alkaline peptone water, thiosulfate citrate bile salts sucrose (TCBS) agar, blood agar, selenite broth, desoxycholate citrate agar (DCA), and sorbitol/MacConkey agar and were incubated overnight. Water samples were vacuum filtered, and the filters were cut in half and incubated overnight in alkaline peptone water and selenite broth. These waters and broths were then subcultured onto TCBS, blood, and sorbitol/MacConkey agars; the selenite broth was also subcultured onto DCA and *Shigella-Salmonella* agars. Sucrose-fermenting colonies were further investigated biochemically as potential *V. cholerae*, and sorbitol-nonfermenting colonies as potential *E. coli* O157. Final identification was done by serotyping of *V. cholerae* with Wellcome polyvalent (Burroughs Wellcome Co., Research Triangle Park, NC), Inaba and Ogawa agglutinating sera, and of *E. coli* with the Prolex latex agglutination test kit. All other lactose-nonfermenting colonies were further investigated.

A sample of isolates identified as *E. coli* O157 by the Prolex kit underwent confirmatory testing at the Centers for Disease Control and Prevention (CDC), using standard methods (3). Isolates were also examined for the presence of genes encoding Shiga toxins 1 and 2, and the *uid*A allele specific for *E. coli* O157:H7 by polymerase chain reaction (PCR) (4,5). All isolates were characterized by macrorestriction analysis pulsed-field gel electrophoresis (PFGE) (6). The *fli*C gene from selected isolates was amplified and restriction digested as previously described to determine whether the isolates had the allele characteristic of *E. coli* O157:H7 (7).

Survey Methods

To characterize the diarrhea and identify risk factors for illness during the outbreak, a survey was conducted at the sugar plantation. The survey questionnaire solicited information on demographic characteristics, travel, and food and water consumption in the 2 weeks before the outbreak (October 15 to 31) and diarrheal illness from November 1 to December 31, 1992. All villages that had a village health worker available to assist on the day of the survey were included (16 of the 20 villages). Maps of each village were reviewed, and houses were randomly selected. All persons at home at the time of survey who had lived on the premises since October 1, 1992, were included.

Statistical analyses were conducted by using EpiInfo Version 6.4c. Confidence intervals (CI) for means and proportions were calculated according to the Fleiss-Quadratic method, taking stratified-cluster sampling framework into consideration. An exposure effect was considered to be statistically significant if the 95% CI for the summary risk ratio did not include 1.0.

Results

Surveillance of Cattle Deaths

Reports from Swaziland for the 5 years from 1988 to 1992 indicate that an increase in cattle deaths began in August 1992 (Figure 2). In Lubombo District, 12,685 cattle died in September and October 1992; this represented a sevenfold increase over the monthly average of cattle deaths in Lubombo for the previous 8 months (data not shown).

Rainfall Measurements

The Big Bend Agricultural Experimental Farm reported 1.5 mm of precipitation during the 3 months preceding October 1992 (Figure 2), compared with 40 mm of precipitation for the same period in 1991 (data not shown). Of the 36 mm

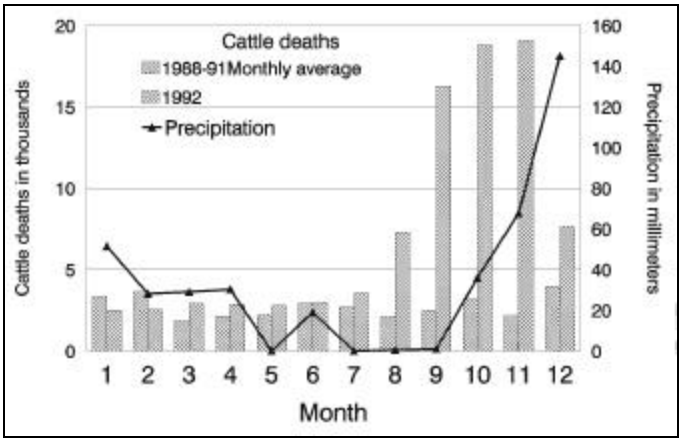

Figure 2. Reported cattle deaths in Swaziland (1988-1992) and monthly precipitation at the Big Bend Agricultural Experimental Farm, Swaziland, 1992.

of rain that fell on Big Bend during October 1992, 27 mm (75%) fell on the 29th and 6 mm (17%) the next day. At the plantation, 34 mm of rain fell on October 29, accounting for 72% of the monthly total.

Diarrheal Disease Surveillance

The number of visits for diarrhea at the plantation hospital from October 15 through December 5, 1992, is shown in Figure 3. Three days after the heavy rainfall, the number of patients with diarrhea seen at the plantation hospital began to increase rapidly. A total of 2,868 persons were treated for diarrhea in November; 1,884 (66%) of these presented in the first 2 weeks of the outbreak.

When the plantation outbreak occurred, nearby refugee settlements in Swaziland and townships in proximal areas of South Africa were also reporting increases in diarrheal illness. Clinician accounts indicated that patients seen at the refugee settlements in early November had abdominal pain and bloody diarrhea and were frequently locally residing Swazi nationals, not refugees. By the end of the month, however, the clinical picture had shifted to profuse watery diarrhea, characteristic of cholera, initially observed among the refugees. These impressions are supported by data from the refugee settlement clinics (Figure 4). Ndzevane clinic staff routinely recorded whether the patient was a local Swazi resident or refugee. Examining the visits of local residents and refugees separately indicates that there were two waves of diarrheal illness at Ndzevane: the first, predominantly among local residents, peaked in the second week of November; the second, primarily among refugees, peaked in early December 1992. Figures for the Malindza clinic confirm that a large outbreak occurred there as well, also peaking in the second week of November, followed by a second, subtler wave of diarrheal illness that peaked in the latter half of December. The Malindza clinic staff classified cases of diarrhea as dysentery if blood was visible in the stool. Although this diagnosis was usually made infrequently, for the week ending November 4, 1992, 74 (29%) of the 255 diarrheal illnesses treated were recorded as dysentery.

Swaziland's national diarrheal disease surveillance figures for 1990 through 1992 for persons ≥5 years of age are shown in Figure 5. A substantial increase in diarrheal illness in Swaziland began in October 1992, and 40,912 consultations for diarrheal illness in persons aged ≥5 years were reported during October and November, representing a sevenfold increase over the mean number of visits for diarrhea reported for the comparable period during 1990 and 1991. This increase was also observed among children <5 years of age, who had 15,312 consultations for acute diarrheal illness reported in November 1992, a figure more than double the monthly average for the preceding 34 months. All areas of Swaziland had increases in reported diarrheal illness in October and November 1992; this increase was most pronounced in Lubombo District (data not shown).

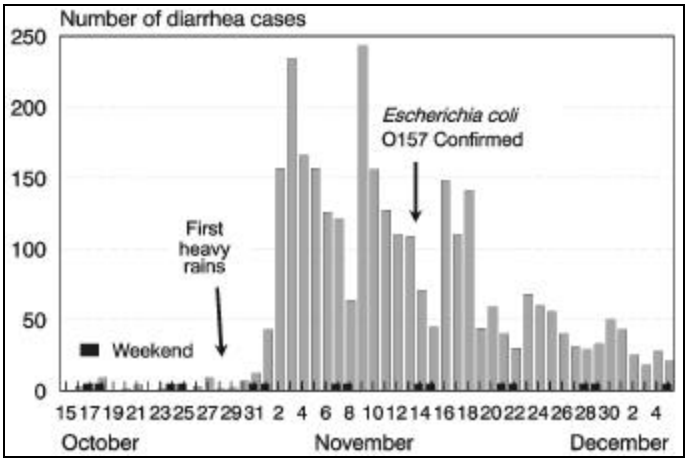

Figure 3. Number of persons with diarrhea visiting a plantation hospital in Lubombo, Swaziland, October 15 through December 5, 1992. [a]

[a]Date of onset of the first heavy rains (October 29, 1992) established by precipitation data from two sites in Lubombo.

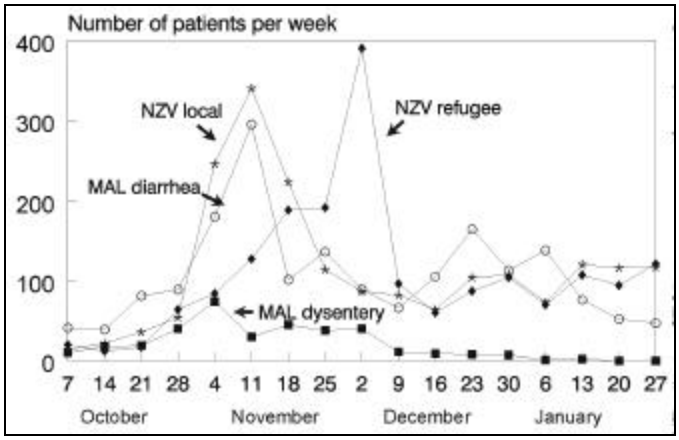

Figure 4. Number of persons with diarrhea visiting the Ndzevane (NZV) and Malindza (MAL) settlement clinics, Lubombo, Swaziland, October 7, 1992, through January 17, 1993. Data were obtained by retrospectively reviewing the clinic logbooks at each site. "NZV local" indicates Swazi nationals residing locally who sought care at the Ndzevane clinic. "NZV refugee" indicates refugees resident in the settlement treated at the clinic. "MAL dysentery" indicates all persons seen at the Malindza clinic with bloody diarrhea. "MAL diarrhea" indicates all persons seen at the Malindza clinic with diarrhea that was not specified as bloody.

Laboratory Results

Clinical Specimens

Specimens submitted to local laboratories in Swaziland during the first 10 days of the outbreak did not yield a pathogen. At SAIMR, four of five rectal swabs collected on November 13, 1992, from patients treated at the plantation hospital yielded *E. coli* O157; no other pathogens were identified. From November 21 to December 3, 1992, 51 stool specimens were collected at the Ndzevane clinic; 7 yielded only *E. coli* O157, 18 yielded only *V. cholerae* El Tor, serotype Ogawa, and 8 yielded both pathogens.

In neighboring areas of South Africa, stool specimens obtained during nearly simultaneous outbreaks of diarrhea also yielded *E. coli* O157, including 3 of 20 specimens received on December 2 and 3, 1992, from Ermelo; 1 of 8 specimens received on December 2, 1992, from Empangeni; and 4 of 40 specimens collected on December 17, 1992, in Peit Retief. None of these stool specimens yielded *V. cholerae*, although nontyphoid *Salmonella* spp. were isolated from two stool specimens from Piet Retief.

Environmental Specimens

Between November 21 and December 4, 1992, *E. coli* O157 was isolated from 16 of 81 water samples from Swazi-

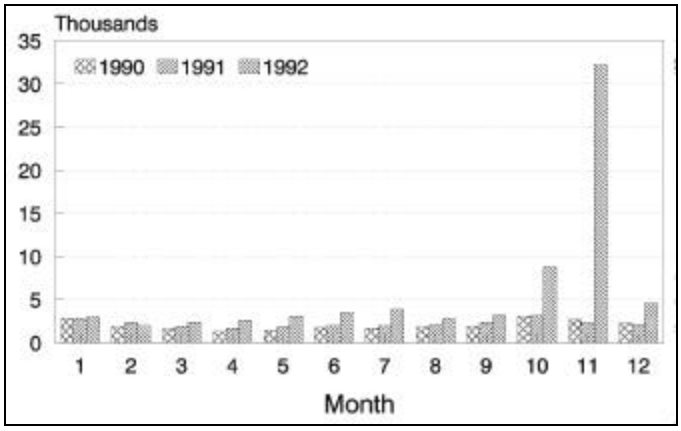

Figure 5. Clinic visits for diarrheal illness among persons ≥5 years of age in Swaziland, by month, 1990 through 1992. Data were obtained from Ministry of Health, Government of Swaziland, February 1993.

land and South Africa. Eight of the positive samples came from surface water on the sugar plantation (including the Usutu River), four were obtained from various sites in Ndzevane (including a borehole, a water distribution standpipe, and water stored in the home of a patient), and four were collected near Jeppe's Reef during a diarrhea outbreak (including samples from the Magadu and Sithlangu rivers). *V. cholerae* was not isolated from any water sample.

E. coli O157 was isolated from 3 (20%) of 15 cattle dung specimens collected at the sugar plantation on November 21, and from 3 (11%) of 27 cattle dung specimens collected near Jeppe's Reef on December 4, 1992. A single sample of day-old cooked maize collected December 3, 1992, in the Ndzevane refugee camp also yielded *E. coli* O157, as did two of two sewage specimens received December 2, 1992, from the Pongola area.

Plantation Survey

A total of 778 (92%) of 850 survey questionnaires distributed to residents of the 16 participating villages on the plantation were completed. Four hundred (53%) of the 752 respondents were male. The median age was 26 years.

A total of 327 (42%; 95% CI 38% to 46%) of the 778 plantation residents surveyed reported having diarrheal illness during November or December 1992; 128 (60%) of 212 persons who recalled the time of onset indicated that the illness began in the first 2 weeks of November. Of those ill, 90 (28%) reported bloody diarrhea, 285 (87%) abdominal pain, and 145 (44%) vomiting. The mean duration of illness was 4.2 days (95% CI: 3.6 to 4.8). A total of 257 (79%) of ill persons sought care at the plantation hospital, and 52% indicated they were the first person in their household to have diarrhea. All ages were affected; diarrheal attack rates were 25% to 56% for all age groups when aggregated by 5-year intervals; the attack rate varied widely among villages, from 13% to 75%.

Univariate analyses identified consumption of beef or untreated water in various forms, as well as female gender, as factors significantly associated with diarrheal illness (Table). These associations remained significant even when the case definition was restricted to those who became ill during the first 2 weeks of the outbreak or to those who stated they had been the first ill person in their household. Defining cases as only bloody diarrhea generally increased the relative risk for each of the dietary exposures. The respondents' job duties on the plantation, a history of travel away from the plantation during the last 2 weeks of October, location of their homestead in Swaziland, and household size were not associated with diarrheal illness.

Stratified analyses were conducted to assess possible confounding or interaction between the exposures identified as significant on univariate analyses. All exposure variables except the consumption of beef not purchased from the butcher remained statistically associated with self-reported diarrheal illness when the variables were stratified by each of the other exposures individually.

Molecular Analysis and PFGE Typing

Thirty-one suspected *E. coli* O157 isolates obtained from clinical and environmental specimens were referred to CDC, and all were confirmed as *E. coli* O157:NM. All isolates had the *uid*A allele specific for *E. coli* O157:H7 and the genes encoding Shiga toxins 1 and 2. All eight isolates characterized by restriction digestion of the PCR-amplified *fli*C gene showed a pattern characteristic of the H7 allele found in *E. coli* O157:H7 isolates. Twenty-seven (87%) of the 31 isolates had indistinguishable PFGE patterns (Pattern A in Figure 1), indicating that the outbreak was largely caused by a single predominant clone. This clone was isolated from various specimens, including human stools, cattle dung, surface water, sewage water, and maize, and was recovered from all six sites where specimens were obtained and tested by PFGE (isolates from a seventh site, Empangeni, were not evaluated by PFGE). Two other PFGE patterns were identified among the four remaining isolates.

Discussion

This report provides comprehensive data on the first outbreak of *E. coli* O157 infections from the developing world. Drought, carriage of *E. coli* O157 by cattle, and contamination of surface water appear to have been important contributing factors. Given the extent of the outbreak, the multiple modes of transmission, and the likely recurrence of predisposing factors, the emergence of *E. coli* O157 has major implications for diarrheal disease control strategies in Africa.

Although the simultaneous occurrence of cholera in some areas where *E. coli* O157 infections were detected makes it difficult to definitively determine the magnitude of the *E. coli* O157 outbreak, national disease surveillance figures for Swaziland leave little doubt that a massive outbreak of diarrheal illness occurred there in 1992, and several lines of evidence suggest *E. coli* O157 played a major role.

First, the clinical presentation of illnesses at the plantation and of the initial patients at the refugee settlements is consistent with *E. coli* O157 infection. Symptomatic cholera infection almost always manifests as profuse watery diarrhea. Severe abdominal pain and bloody stools are very uncharacteristic for cholera but are the hallmark of hemorrhagic colitis caused by *E. coli* O157 (8-10). *E. coli* O157 can also present as nonbloody diarrhea (11,12). In a household survey performed during a waterborne outbreak of *E. coli* O157 infections in the United States, bloody stools were reported by only 35% of those with diarrhea (13). This figure is similar to the proportion of ill persons reporting bloody

Table. Risk factors for diarrheal illness among sugar cane plantation residents during an outbreak of *Escherichia coli* O157 infections in Swaziland, 1992[a]

	Respondents with risk or exposure			Respondents without risk or exposure				
Risk or exposure	Total	No. with diarrhea[a] (%)	No. with bloody diarrhea (%)	Total	No. with diarrhea* (%)	No. with bloody diarrhea (%)	RR for diarrhea (95% CI)	RR for bloody diarrhea (95% CI)
Untreated water consumption								
From a standpipe	270	152 (56)	49 (18)	467	157 (34)	36 (8)	1.7 (1.4-2.0)	2.4 (1.5-3.7)
Stream or river	391	204 (52)	65 (17)	354	113 (32)	22 (6)	1.6 (1.3-2.0)	2.7 (1.7-4.3)
Irrigation trench	250	139 (56)	50 (20)	507	178 (35)	38 (7)	1.6 (1.3-1.9)	2.2 (1.8-4.1)
On fruits and vegetables	182	102 (56)	36 (20)	533	199 (37)	49 (9)	1.5 (1.2-1.8)	2.2 (1.3-3.4)
Beef consumption								
In the weekly ration	707	311 (44)	87 (12)	63	14 (22)	3 (5)	2.0 (1.2-3.3)	2.6 (0.8-8.2)
From a cow found dead	180	106 (59)	31 (17)	476	172 (36)	47 (10)	1.6 (1.3-2.0)	1.7 (1.2-2.7)
Not purchased from a butcher	488	226 (46)	67 (14)	255	89 (35)	20 (8)	1.3 (1.1-1.7)	1.8 (1.1-2.9)
Female gender	352	173 (49)	48 (14)	400	143 (36)	41 (10)	1.4 (1.2-1.7)	1.3 (0.9-2.0)

[a] Total responses for any given risk factor or exposure may vary slightly and may not equal the total number of survey participants because indeterminate responses to any exposure question (e.g. "Don't remember" or missing value) were not included in the analyses. RR = relative risk; 95% CI = 95% confidence interval.

stools in the plantation survey (28%) and to that observed among the patients seen initially at the Malindza clinic (29%). It seems reasonable, therefore, to infer that some of the cases of nonbloody diarrhea, which were temporally and geographically associated with cases of bloody diarrhea caused by *E. coli* O157, represent milder manifestations of illness caused by this pathogen.

Second, medical personnel clearly recalled that there were two waves of diarrhea, dysentery followed by watery diarrhea, with each surge initially affecting different populations. (Although the term dysentery technically refers to the presence of blood and pus in stools, it is used here to mean nonwatery diarrhea, which may be bloody, accompanied by severe abdominal pain.) In a report filed November 27, 1992, following an assessment of the situation at Ndzevane, the World Health Organization (WHO) representative in Swaziland documented this impression, stating "the type of diarrhea [seen at Ndzevane among the refugees] appeared to be different from that noticed in the neighboring areas," i.e., among the Swazi nationals residing outside the camp (14). Although we suspect that cholera was the likely cause of much of the diarrhea during the second wave of illnesses at the refugee camps, we do not believe the initial dysentery outbreak among the local Swazi population can be attributed to this pathogen.

Third, *E. coli* O157 was isolated from specimens collected from seven different diarrhea-affected foci that spanned a distance of several hundred kilometers. The actual area involved may have been even greater because in the months immediately following this outbreak, investigators in Malawi and Angola reported outbreaks of diarrheal illness in which nucleic acid amplification tests of stool specimens suggested *E. coli* O157 as the cause (15,16).

Estimates of the magnitude of the *E. coli* O157 outbreak ultimately depend on how one apportions the 64,699 consultations for diarrhea (all ages) in Swaziland during October and November 1992, after counterbalancing the identification of cholera in late November with widespread reports of dysentery in the local population and confirmation of *E. coli* O157 over an extended area. Even if *E. coli* O157 accounted for only a small proportion of visits, this would represent thousands, perhaps tens of thousands, of infections and one of the largest outbreaks of this agent in history.

Understanding the complex interaction of environmental and behavioral factors that enabled *E. coli* O157 to emerge so intensely in Africa is important for future diarrheal disease control efforts. Cattle are the major reservoir of *E. coli* O157 and shed this organism more frequently when stressed (17). This region had been experiencing several years of drought, a situation that worsened from July to Sep-

tember 1992. As the drought continued, cattle began to graze close to the remaining sources of surface water and vegetation. The marked increase in cattle deaths beginning in August 1992 indicates that available pastures and water sources were often insufficient.

Although the number of cattle decreased, demand for beef apparently did not. Nearly one quarter of persons surveyed on the plantation indicated that they had eaten beef from cattle known to have been found dead. Commercial beef slaughter and distribution practices were also affected by the shortage of cattle. Earlier in the year, cattle imports from South Africa to Swaziland were banned, a decision said to be supported by some ranchers because it enabled them to sell off dying animals (18). A senior health inspector noted that a single large abattoir was supplying meat to many butchers in the country since the drought and that "preventive health is not taken as a matter of priority by the look of things" at the abbatoir (19). Plantation staff also indicated, for the first time in their memory, that just before the outbreak some of the weekly beef rations were spoiled. Although adequate cooking temperatures will kill *E. coli* O157, much of this beef would have been grilled, leaving the possibility that portions remained undercooked.

How *E. coli* O157 originally found its way to cattle in southern Africa is not known, but once there it is not difficult to speculate on how it spread. Recovery of *E. coli* O157 from surface water in Swaziland and South Africa plausibly explains how this pathogen might be rapidly dispersed over vast stretches of land. According to local residents and our own observations, during the drought, cattle often frequented the trickling streambeds, defecating and dying there (Figure 6). With the onset of heavy rains, water contaminated with cattle feces (and presumably *E. coli* O157) would have become readily available to both livestock and residents in villages downstream. Such a scenario is consistent with the epidemic curve from the sugar plantation outbreak; *E. coli* O157 has a median incubation period of 3 to 4 days, and the outbreak exploded 3 days after the first heavy rains in 4 months (10). PFGE results indicate that a single clone was responsible for much of the outbreak, suggesting that either there was an exceptionally narrow range of diversity among *E. coli* O157 isolates in south African cattle or that a single clone was able to expeditiously spread over large distances, perhaps via surface waterways revitalized by heavy rains.

By early 1993 we no longer detected *E. coli* O157 in patient stools or environmental specimens obtained from the affected area, intimating that the outbreak had ended almost as abruptly as it started. Since that time, however, two other outbreaks of dysentery have been attributed to *E. coli* O157 elsewhere in Africa, the first in Central African Republic in 1996, and the second in Cameroon beginning in 1997 (20,21). Both these reports have some constraints, however; in the Central African Republic, *E. coli* O157 was not isolated at the outbreak site but only implicated from molecular tests. In the Cameroon outbreak, *E. coli* O157 and *Shigella* were each isolated in about half of the specimens tested.

Although reported outbreaks of *E. coli* O157 in Africa have been few to date, available information indicates that the pathogen has wide geographic distribution. Since the 1992 outbreak, culture-proven *E. coli* O157 diarrheal illness has been reported from multiple locations, including Kenya, Nigeria, Côte d'Ivoire, and Central Africa Republic (22-25). In Egypt, 6 (5%) of 125 meat specimens obtained from slaughterhouses yielded *E. coli* O157 (26). Because *E. coli* O157 is not detected by the usual methods used to isolate and identify traditional enteric bacterial pathogens and microbiology laboratories in many countries in Africa do not routinely test for this pathogen, *E. coli* O157 infections may go unrecognized (27,28). Reports on African dysentery outbreaks attributed to *Shigella* sometimes indicate that specimens were not tested until several months into the outbreak or do not describe laboratory methods that are suitable for detecting *E. coli* O157 (29,30). This is unfortunate because the spectrum of clinical illness resulting from *Shigella* infection overlaps considerably with that of *E. coli* O157 and mixed outbreaks have been reported (20).

WHO has stated that *Shigella dysenteriae* type 1 "is the *only* cause of large-scale, regional outbreaks of dysentery" (emphasis WHO), but data presented here suggest that this assertion may be too restrictive (27). We are concerned that if another large outbreak of severe bloody diarrhea caused by *E. coli* O157 occurred today in Africa, it might be assumed to be due to *Shigella* spp. and *E. coli* O157 would not be sought.

Determining the extent to which *E. coli* O157 is responsible for dysentery in Africa is of more than academic con-

Figure 6. Cattle grazing in a trickling stream bed, Lubombo, Swaziland, 1992. The nearly ubiquitous hoof prints, visible in the foreground, indicate cattle often frequented the stream beds. Photo credit: Paul Effler.

cern because syndromic management protocols for dysentery in areas where the etiologic agent cannot be readily confirmed often include empiric treatment with antibiotics. Antibiotics have not been clearly shown to ameliorate *E. coli* O157 infection, and recent data indicate that treating these infections with antibiotics may predispose the patient to developing serious complications, including hemolytic uremic syndrome (HUS) (10,31). HUS is estimated to occur in 8% of children and an unknown proportion of adults with *E. coli* O157 and, without dialysis, can be fatal (10).

More work is needed to better define the incidence of *E. coli* O157-associated dysentery in Africa so that optimal recommendations for empiric antibiotic therapy can be provided. At present, we recommend that in outbreaks of dysentery in Africa, stools from ill patients be tested for *E. coli* O157 in addition to *Shigella* spp. If the etiologic agent is not identified, consideration should be given to examining stools for other Shiga toxin-producing *E. coli*. Regional studies of *E. coli* O157 carriage rates among livestock in various parts of Africa might also be useful in assessing the potential for future outbreaks.

In this investigation, *E. coli* O157 was recovered from multiple water sources, including a borehole, a standpipe, and water stored in the home. Thus, ensuring the purity of potable water through practical, low-cost strategies might have reduced the risk for infection (32-34).

Several limitations of this investigation should be noted. First, the survey at the plantation did not adjust the period of exposure inquired about to account for differences in date of illness onset for each patient. Instead we asked all persons, both ill and well, about exposures during the 2 weeks preceding the outbreak.

Second, with conditional logistic regression analyses we were unable to identify significant independent predictors of illness among the risk exposures reported by the plantation residents. Many household clusters had at least one indeterminate response to an exposure question (e.g., replied, "Don't remember" or no response) and therefore the entire cluster had to be excluded from such analyses, resulting in conditional regression models that were unstable.

A third limitation is our inability to assess the impact of the *E. coli* O157 outbreak on human deaths. While visiting area hospitals, we heard anecdotal accounts of patients who became edematous and died without known cause. We suspect that these cases represented end-stage manifestations of HUS, but quantifiable data were not available.

Despite widespread recognition of the importance of environmental factors in facilitating the emergence of infectious agents, the information available in the scientific literature remains relatively limited (35-38). This manuscript combines environmental and veterinary data together with disease incidence, environmental testing, and microbial "fingerprinting" to develop one of the more complete examples currently available. The data presented suggest that carriage of *E. coli* O157 by cattle, cattle deaths secondary to drought, and heavy rains that resulted in contamination of surface water were important factors contributing to the emergence of *E. coli* O157 in Africa. Given that drought and heavy rains will likely recur in Africa, the possibility that *E. coli* O157 will once again emerge to cause a major regional outbreak cannot be excluded. Clinicians need to be aware of this so that delayed diagnosis and inappropriate treatment resulting in loss of lives can be avoided.

Acknowledgments

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Dr. Effler is State Epidemiologist for the Hawaii Department of Health. His current responsibilities encompass disease surveillance and outbreak control, oversight of the state's immunization program, and bioterrorism preparedness.

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Clinical Consequences and Cost of Limiting Use of Vancomycin for Perioperative Prophylaxis: Example of Coronary Artery Bypass Surgery

Giorgio Zanetti,*† Sue J. Goldie,‡ and Richard Platt*§

*Channing Laboratory, Brigham and Women's Hospital, and Eastern Massachusetts CDC Prevention Epicenter, Boston, Massachusetts, USA; †University Hospital, Lausanne, Switzerland; ‡Harvard School of Public Health, Boston, Massachusetts, USA; and §Harvard Medical School and Harvard Pilgrim Health Care, Boston, Massachusetts, USA

Routine use of vancomycin for perioperative prophylaxis is discouraged, principally to minimize microbial resistance to it. However, outcomes and costs of this recommendation have not been assessed. We used decision-analytic models to compare clinical results and cost-effectiveness of no prophylaxis, cefazolin, and vancomycin, in coronary artery bypass graft surgery. In the base case, vancomycin resulted in 7% fewer surgical site infections and 1% lower all-cause mortality and saved $117 per procedure, compared with cefazolin. Cefazolin, in turn, resulted in substantially fewer infections and deaths and lower costs than no prophylaxis. We conclude that perioperative antibiotic prophylaxis with vancomycin is usually more effective and less expensive than cefazolin. Data on vancomycin's impact on resistance are needed to quantify the trade-off between individual patients' improved clinical outcomes and lower costs and the future long-term consequences to society.

The emergence of vancomycin-resistant enterococci has opened a new era of hardly treatable bacterial infections, and there is now evidence that more virulent common pathogens such as *Staphylococcus aureus* can also develop resistance to vancomycin (1,2). The use of vancomycin is hypothesized to promote the development or transmission of this resistance (3,4). Restrictive guidelines have therefore been disseminated for the use of vancomycin or teicoplanin, another glycopeptide agent (5). These guidelines include a recommendation against the routine use of vancomycin as perioperative antibiotic prophylaxis for surgical site infections.

However, vancomycin is preferred for preventing infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) or methicillin-resistant coagulase-negative staphylococci. This is the rationale for recommending vancomycin prophylaxis when the risk for infection from methicillin-resistant pathogens is high (6-11), although no guideline has made a clear statement on when to use this alternative. Since antibiotics are commonly used for prophylaxis, liberal interpretation of the prophylaxis guidelines will clearly jeopardize efforts to limit the use of vancomycin. Vancomycin is also more expensive to purchase and administer than cephalosporins.

To inform both the clinical and public policy debate with respect to the optimal prophylaxis regimen, we conducted a cost-effectiveness analysis to compare the short- and long-term consequences of using vancomycin and cefazolin as first-line perioperative prophylaxis. We focused on patients who underwent coronary artery bypass graft surgery (CABG) because this is a large, relatively homogeneous population with substantial risk for serious surgical site infection (12,13).

Address for correspondence: Giorgio Zanetti, Division of Infectious Diseases, University Hospital, 1011 Lausanne, Switzerland; fax: 4121-314-1018; e-mail: Giorgio.Zanetti@chuv.hospvd.ch

Methods

Cost-Effectiveness Analysis

We developed a decision-analytic model (Figure 1) to calculate the clinical benefits and costs associated with alternative strategies for antibiotic prophylaxis in a hypothetical cohort of 10,000 patients undergoing CAGB surgery. The three strategies evaluated were 1) no prophylaxis; 2) routine cefazolin, reserving vancomycin for those with a history of allergic reaction to beta-lactam antibiotics; and 3) routine vancomycin. In the base-case analysis, we adopted a payer perspective and included clinical outcomes and direct medical costs in the 3 months after surgery. Clinical outcomes included deep and superficial surgical site infections, as well as hospital deaths.

We also conducted a reference case analysis, as recommended by the Panel on Cost-Effectiveness in Health and Medicine (14), which assumed a societal perspective and relied on a longer time horizon. The reference case was a 65-year-old man undergoing CABG surgery for stable multivessel coronary heart disease. A state-transition model incorporated the lifetime probability of death, myocardial infarction, angina, or asymptomatic coronary artery disease following CABG surgery (15,16) to estimate life expectancy, quality-adjusted life expectancy, and total lifetime costs. Future costs and benefits were discounted at an annual rate of 3%.

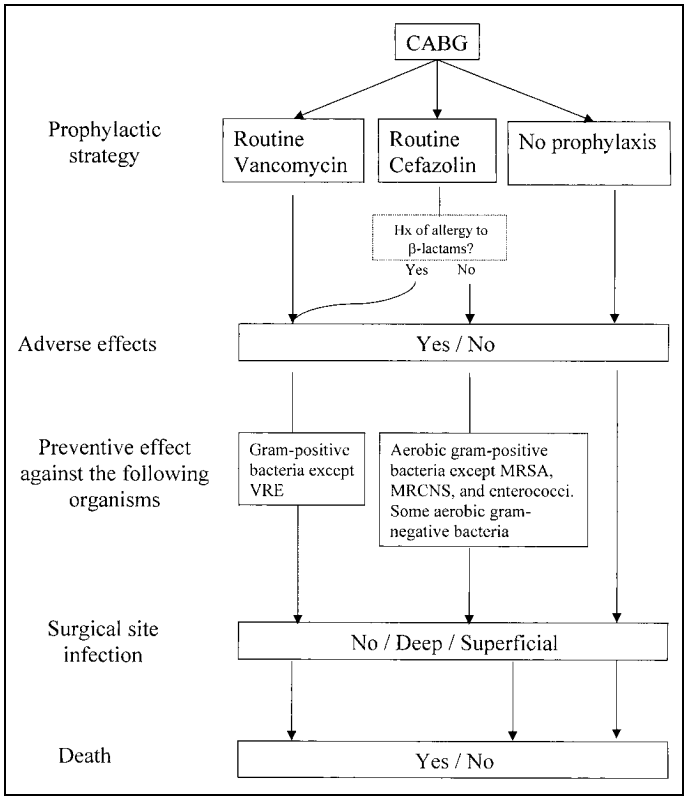

Figure 1. Model of the decision tree.*
*CABG = coronary artery bypass graft surgery; Hx = history of; MRSA = methicillin-resistant *Staphylococcus aureus;* MRCNS = methicillin-resistant coagulase-negative staphylococci; VRE = vancomycin-resistant enterococci.

To conduct the cost-effectiveness analysis, the three strategies were ranked by increasing effectiveness; those that cost more but were less effective than an alternative strategy were eliminated by strong dominance. When one strategy was more effective but more costly, an incremental cost-effectiveness ratio was calculated by dividing the additional cost of this specific strategy by its additional clinical benefit, compared with the next least expensive strategy. We conducted uni- and multivariate sensitivity analyses to assess the stability of the results in the face of plausible variation in the underlying parameter estimates. All analyses were performed by using DATA 3.5 (TreeAge Software, Inc, Williamstown, MA).

Clinical Data

Table 1 summarizes the parameter estimates and their plausible ranges. We assumed that antibiotics used to prevent surgical site infection were partially protective only against infections caused by susceptible bacteria. Vancomycin-susceptible bacteria included all gram-positive organisms except vancomycin-resistant enterococci. Cefazolin-susceptible bacteria included aerobic gram-positive organisms (except enterococci, MRSA, and methicillin-resistant coagulase-negative staphylococci) as well as some aerobic gram-negative bacteria. We based the proportion of surgical site infections attributed to specific causative organisms on microbiologic data from two published studies of patients undergoing CABG surgery, most of whom received a first-generation cephalosporin for prophylaxis (12,13).

The efficacy of antibiotic prophylaxis in patients undergoing CABG surgery is difficult to quantify directly since the only available placebo-controlled studies were terminated early because significantly better outcomes occurred in the patients assigned to prophylaxis compared with controls (34,35). Therefore, we assumed a relative risk of 0.4 for a surgical site infection in patients who received antibiotic prophylaxis compared with those who did not, which corresponds to the highest effectiveness of antibiotic prophylaxis within the range of results from completed placebo-controlled studies in clean surgical procedures other than CABG surgery (18-23). We used data on the incidence of surgical site infections among patients receiving cefazolin from five surveillance programs in university-affiliated hospitals in Boston (17). By assuming that patients who received either cefazolin or vancomycin shared the same relative risk of 0.4 of developing a surgical site infection due to a susceptible organism, compared with patients who did not receive prophylaxis, we were able to estimate the incidence of surgical site infections for each strategy. We used data from the National Nosocomial Infection Surveillance System (26) to estimate the proportion of surgical site infections caused by antibiotic-resistant organisms. We recognize that the incidence of surgical site infections caused by antibiotic-resistant organisms varies from one institution to another, and therefore varied the resistance pattern over a wide range in sensitivity analysis.

We assumed that 10% of patients had a history of allergy to beta-lactam antibiotics (27). The incidence of adverse events secondary to vancomycin was based on data from a prospective study in which vancomycin was used for 10 days (24). We adjusted these estimates to reflect the probability of toxicity with a 2-day prophylactic regimen by assuming a linear relationship between the incidence of adverse events and the duration of therapy. We assumed that the incidence of adverse events was the same for cefazolin and vancomycin, since several comparative prophylactic studies have reported the toxicity profiles to be similar (25,36,37). Death rates secondary to deep surgical site infection (12), and anaphylactic reaction to cefazolin (28) were obtained from published studies. We then derived the deaths associated with the surgical procedure and its noninfectious complications by using data on all-cause hospital deaths following CABG surgery reported for the state of Massachusetts (29).

We estimated quality-adjusted life expectancy by applying quality weights to the health states representing death, myocardial infarction, angina, or asymptomatic coronary artery disease. These quality weights were obtained from the Beaver Dam Health Outcomes Study, in which time trade-off techniques were used to elicit utilities (38). We explored a wide range of quality-weights for the temporary health states reflecting a superficial or deep surgical site infection.

Costs

To estimate the costs associated with surgical site infections and hospital deaths, we relied on published estimates (30-33) and adjusted these to 1998 U.S. dollars by using the medical care component of the consumer price indexes published by the Bureau of Labor Statistics (39). These studies used costs from a cost accounting system (30,32) or charges

Table 1. Model variables[a]

Variables	Base case	Plausible range	References
Incidence of SSI			
Superficial	0.08	0.02 – 0.12	(12,17)
Deep	0.04	0.01 – 0.06	(12,17)
Causative organisms			(12,13)
Staphylococcus aureus	0.25	0.20 – 0.35	
Coagulase-negative *Staphylococci*	0.25	0.20 – 0.35	
Enterococci	0.05	0.02 – 0.15	
Gram-negative bacteria	0.30	0.15 – 0.50	
Relative risk of SSI caused by susceptible organisms			
Vancomycin vs. no prophylaxis	0.4	0.20 – 0.80	(18-23)
Cefazolin vs. no prophylaxis	0.4	0.20 – 0.80	(18-23)
Incidence of antibiotic-related adverse events			
Vancomycin	0.08	0.01 – 0.20	(24)
Cefazolin	0.08	0.01 – 0.20	(24,25)
Incidence of SSI due to resistant organisms			(26)
MRSA (% of all SSI due to *S. aureus)*	0.012 (0.40)	0 – 0.03	
Methicillin-resistant CNS (% of all SSI due to CNS)	0.024 (0.80)	0 – 0.03	
VRE (% of all SSI due to enterococci)	0.003 (0.15)	0 – 0.006	
Incidence of SSI caused by cefazolin-susceptible gram-negative bacteria (% of all SSI due to gram-negative bacteria)	0.01 (0.28)	0 – 0.036	
History of allergy to beta-lactams	0.1	0.05 – 0.15	(27)
Probability of hospital death			
Deep surgical site infection	0.082	0.01 – 0.10	(12)
Antibiotic allergic reaction	0.00002		(28)
Coronary artery bypass graft surgery-related events	0.036	0.01 – 0.1	(29)
Costs per case (US$)			
Vancomycin	80	60 – 250	(24)
Cefazolin	24	10 – 50	(24)
Superficial SSI	8,000	3000 – 15,000	(30,31)
Deep SSI	36,800	10,000 – 50,000	(30,31)
Vancomycin-related adverse event	107	20 – 1,000	(24)
Cefazolin-related adverse event	107	20 – 1,000	(24)
Death	5,900	0 – 10,000	(32)
Multiplication factor for infections due to methicillin-resistant organisms	1.13	0.9 – 2	(33)
Multiplication factor for infections due to VRE	1.5	0.9 – 3	[b]

[a]SSI = surgical site infection; MRSA = methicillin-resistant *Staphylococcus aureus*; CNS = coagulase-negative staphylococci; VRE: vancomycin-resistant enterococci.
[b]We assumed that the cost of an infection caused by a vancomycin-resistant enterococcus was 50% greater than the cost of a comparable infection caused by a susceptible strain.

converted to costs (31,33) as a proxy for direct medical costs. We based the costs of prophylaxis-related adverse events on a study of vancomycin (24) and assumed identical costs for cefazolin-related adverse events. We assumed that both vancomycin and cefazolin would be used for 48 hours, which implies a total of 5 doses of 1 g of vancomycin or 6 doses of 1 g of cefazolin. Antibiotic costs were based on hospital pharmacy acquisition costs, although we added the cost associated with perfusion for both antibiotics (24). A preparation cost was added for vancomycin only, since cefazolin is available in bags ready for infusion.

We estimated the costs of follow-up care by extrapolating the 5-year follow-up medical care cost after CABG surgery among patients included in the Bypass Angioplasty Revascularization Investigation (40).

Results

Base Case

Table 2 shows the intermediate health outcomes and costs associated with the three prophylactic strategies for a hypothetical cohort of 10,000 patients undergoing CABG surgery. With no prophylaxis, the model predicted 570 deep surgical site infections, 1,141 superficial surgical site infections, and 405 all-cause hospital deaths. Prophylaxis using routine cefazolin, reserving vancomycin for patients with a history of beta-lactam allergy, resulted in 173 fewer deep surgical site infections, 347 fewer superficial surgical site infections, and 14 fewer deaths compared with no prophylaxis. Prophylaxis using routine vancomycin resulted in 29 fewer deep surgical site infections, 58 fewer superficial surgical site infections, and 3 fewer deaths, compared with routine cefazolin. Routine vancomycin use was also associated with the lowest direct medical costs for a 3-month period and cost $1,170,000 less than routine cefazolin strategy per 10,000 patients. Since the routine vancomycin strategy was more effective and less costly, the strategy of routine cefazolin was eliminated by strong dominance.

Sensitivity Analysis

A strategy of no prophylaxis was always less effective and more costly than using prophylaxis. Ranking of the routine vancomycin and cefazolin strategies, both in terms of costs and clinical outcomes, was not affected when the following parameters were changed over the plausible range described in Table 1: deaths from all causes and surgical site infection-related deaths; incidence of deep or superficial surgical site infection; distribution of causative organisms; incidence of prophylaxis-related adverse events; proportion of patients with allergy to beta-lactam antibiotics; costs of cefazolin, deep or superficial surgical site infections, death, or prophylaxis-related adverse events.

Results were most sensitive to changes in the cost of vancomycin, efficacy of cefazolin and vancomycin in preventing surgical site infections, and prevalence of bacterial resistance to antibiotics. If the acquisition and administration cost of 5 doses of vancomycin exceeded a threshold of $215, cefazolin was no longer dominated by vancomycin since routine vancomycin became more costly. Similarly, routine vancomycin became more costly than routine cefazolin if vancomycin prevented 18% fewer infections caused by susceptible organisms compared with cefazolin; if this difference exceeded 25%, the routine vancomycin strategy was less effective and more costly and was thus dominated by the cefazolin strategy.

We explored the impact of different antibiotic susceptibility profiles, as might be observed in different hospitals, on these results. Routine vancomycin remained the most effective and the least costly strategy independent of the prevalence of vancomycin-resistance in enterococci. Figure 2 shows a three-way sensitivity analysis of the incidence of surgical site infection caused by each of the following pathogens: an MRSA; a methicillin-resistant coagulase-negative staphylococcus; and a cefazolin-susceptible gram-negative bacteria. For a given incidence of surgical site infection caused by MRSA, each line represents the threshold combinations of methicillin resistance in coagulase-negative staphylococci and cefazolin susceptibility in gram-negative bacteria for routine vancomycin to be more cost-effective than routine cefazolin. For example, in the base case, we assumed a 2.4 per 100 risk for infection caused by methicillin-resistant coagulase-negative staphylococci and a 1.0 per 100 risk for surgical site infection caused by cefazolin-susceptible gram-negative bacteria. Routine cefazolin was more cost-effective than vanco-

Table 2. Base-case analysis: clinical outcomes and costs for a hypothetical cohort of 10,000 patients undergoing coronary artery bypass graft surgery

Strategy	Deep SSI	Increm. deep SSI[a]	Superficial SSI	Increm. superficial SSI[a]	Hospital deaths	Increm. hospital deaths[a]	Deaths, SSI, or both	Increm. deaths, SSI, or both	Costs (x $1,000)	Increm. costs (x $1,000)[a]
No prophylaxis	570	-	1141	-	405	-	2,008	-	33,410	-
Routine cefazolin	397	- 173[b]	794	- 347[b]	391	- 14[b]	1,506	- 502[b]	24,530	- 8,880[b]
Routine vancomycin	368	- 29[b]	736	- 58[b]	388	- 3[b]	1,423	- 83[b]	23,360	- 1,170[b]

SSI = surgical site infection; increm = incremental.

[a]Routine cefazolin compared with no prophylaxis; routine vancomycin compared with routine cefazolin.

[b]Negative incremental numbers of infections or deaths represent the numbers of infections or deaths averted; negative incremental costs represent costs saving.

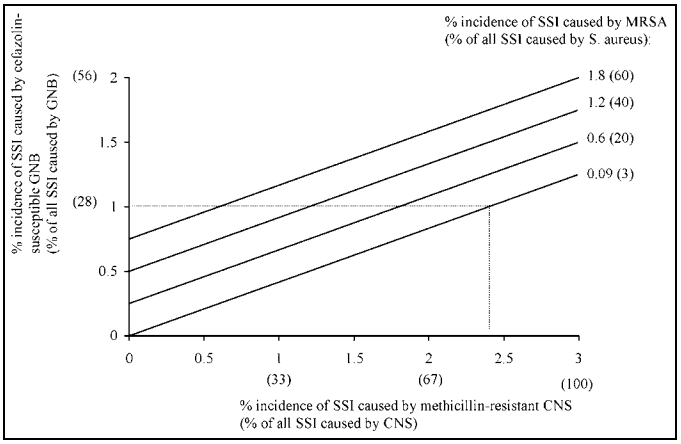

Figure 2. Three-way sensitivity analysis of the incidence of surgical site infection (SSI) caused by methicillin-resistant *Staphylococcus aureus* (MRSA); methicillin-resistant coagulase-negative staphylococci; or cefazolin-susceptible gram-negative bacteria. The lines show the incidence of infection caused by methicillin-resistant *Staphylococcus aureus* necessary for routine cefazolin prophylaxis to be more cost-effective than routine vancomycin (0.09%, 0.6%, 1.2%, and 1.8%). For a particular line, points to the lower right indicate that routine vancomycin is more cost-effective; points to the upper left indicate that routine cefazolin is more cost-effective. The dotted line represents the example cited in text. SSI = surgical site infection; MRSA = methicillin-resistant *Staphylococcus aureus;* CNS = coagulase-negative staphylococci; GNB = gram-negative bacteria.

mycin only when the incidence of surgical site infections caused by MRSA was lower than 0.09 per 100, which represents 3% of the infections caused by *S. aureus*.

The impact of different patterns of antimicrobial resistance on the incremental cost-effectiveness ratio associated with routine vancomycin compared with routine cefazolin is shown in Figure 3. This ratio represents the added cost of using vancomycin divided by the additional clinical benefits provided by vancomycin (i.e., additional death or surgical site infection averted), compared with the next least expensive strategy. For example, in a hypothetical hospital where MRSA caused surgical site infections in 1% of the patients undergoing CABG surgery, methicillin-resistant coagulase-negative staphylococci in 2.5%, and cefazolin-susceptible bacteria in 1.5%, the incremental cost-effectiveness ratio for routine vancomycin was $10,237 per additional infections or death averted, compared with routine cefazolin.

Reference Case

In a hypothetical cohort of 65-year-old men undergoing CABG surgery, quality-adjusted life expectancy was 8.312 quality-adjusted life-years, and per person lifetime costs were $62,892 in the absence of prophylaxis (Table 3). A strategy of prophylaxis with routine cefazolin was $876 less costly and provided an additional 0.023 quality-adjusted life years compared with no prophylaxis. The most effective strategy, prophylaxis with routine vancomycin, saved an additional $103 compared with cefazolin and therefore dominated a strategy of routine cefazolin.

Similar to the results for the base case, our reference case results were most sensitive to estimates for the acquisition and administration cost of vancomycin; the efficacy of vancomycin and cefazolin in preventing surgical site infections; and the prevalence of bacterial resistance to antibiotics.

Discussion

In the base-case analysis, a strategy of routine vancomycin prophylaxis in the overall population of CABG patients was more effective than a strategy of routine cefazolin, since it prevented more surgical site infections or deaths caused by methicillin-resistant staphylococci or enterococci. Routine vancomycin was also less costly than cefazolin, an advantage that was offset neither by higher acquisition and administration costs nor by the absence of protection against gram-negative bacteria. Although these results were dependent on the prevalence of antibiotic resistance in the causative organisms, the range of circumstances in which a strategy of routine cefazolin was either more effective or less costly than vancomycin was narrow, restricted to situations in which MRSA represented no more than 3% of all *S. aureus*.

In the reference case analysis, a strategy of routine vancomycin was also more effective and less costly than a strategy of routine cefazolin. Because of a lack of available data, we were not able to quantify the precise contribution of routine vancomycin use to the development of vancomycin-resistance in gram-positive organisms. However, we did conduct sensitivity analyses to explore the impact of a decrease in efficacy of vancomycin on our results. Estimating the future economic consequences that might be associated with the development of vancomycin resistance is more difficult. Antibiotic resistance, described in economic terminology as an externality associated with the use of antimicrobials (41), is an effect of antimicrobial use that is unlikely to be felt by either the patient or the provider. We know from experience with vancomycin-resistant enterococci that surveillance programs, isolation of colonized patients, and treatment of infections that resist most existing antibiotics increase costs.

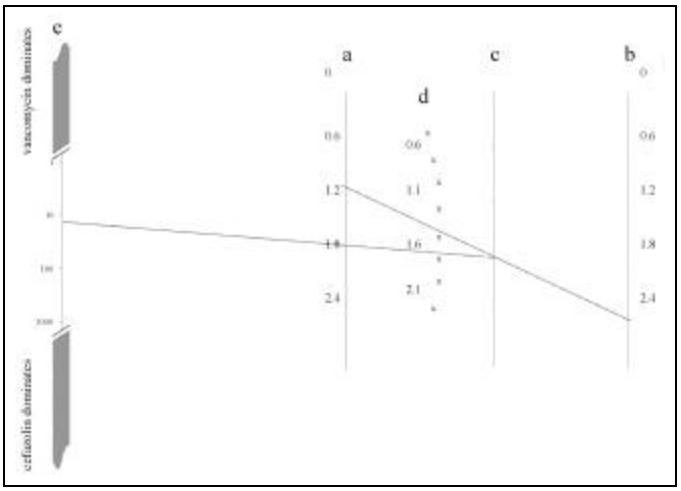

Figure 3. Determination of the incremental cost-effectiveness ratio of the routine vancomycin strategy relative to the routine cefazolin strategy, according to bacterial resistance pattern. To determine the incremental cost-effectiveness ratio: 1) Place percent incidence of surgical site infection caused by methicillin-resistant *Staphylococcus aureus* on the a axis; 2) Place percent incidence of surgical site infection caused by methicillin-resistant coagulase-negative staphylococci on the b axis; 3) Draw a line between these 2 points. This line crosses the c axis at a point x; 4) Place percent incidence of surgical site infection caused by cefazolin-susceptible gram-negative bacteria on the scale d; 5) Draw a line passing through this point and the point x on the c axis. If this line crosses the e axis between the two zones of dominance, the incremental cost-effectiveness ratio can be red (in thousands of dollars per additional death or surgical site infection averted). The dotted lines represent the example cited in text.

Table 3. Reference case analysis: quality-adjusted life expectancy and lifetime costs for a 65-year-old man undergoing coronary artery bypass graft surgery

Strategy	Total costs ($)	Incremental costs[a] ($)	QALYs	Incremental QALYs[a]	Incremental cost-effectiveness ratio[a]
No prophylaxis	62,892	-	8.312	-	-
Routine cefazolin	62,016	- 876[b]	8.335	0.023	Dominated[c]
Routine vancomycin	61,913	- 103[b]	8.339	0.004	Cost saving

QALYs = quality-adjusted life years.

[a]Routine cefazolin compared with no prophylaxis; routine vancomycin compared with routine cefazolin.

[b]Negative incremental costs represent cost savings.

[c]A dominated strategy is one that costs more and is less effective than an alternative strategy.

Similarly, the spread of vancomycin resistance in highly pathogenic species such as *S. aureus* could have substantial clinical and economic consequences. When data become available to document and quantify the relationship between the routine use of vancomycin and vancomycin resistance, we will be able to better describe the trade-off between the short-term benefits to individual patients and the long-term consequences to society at large. However, there are similar uncertainties with respect to the consequences of routine cefazolin, which selects for MRSA and cefazolin-resistant gram-negative organisms and is a recognized risk factor for the acquisition of infection caused by vancomycin-resistant enterococci (4).

We believe our decision not to model the relationship between antibiotic prophylaxis and resistance had a limited impact on our results. For instance, routine vancomycin would still be more effective and less costly than routine cefazolin if all enterococci were resistant to vancomycin because of the small proportion of surgical site infections caused by enterococci after CABG surgery. The spread of vancomycin resistance in staphylococci, however, would impact the model more substantially. We therefore simulated a hypothetical scenario in which prevalence of vancomycin resistance in enterococci would continue to increase by about 2% per year, as reported in U.S. hospitals from 1989 through 1997 (4), and in which the same trend would be observed in staphylococci (data not shown). We arbitrarily assumed that vancomycin prophylaxis, but not cefazolin prophylaxis, would accelerate this trend by 50%. Under these circumstances, routine vancomycin would no longer be less costly than routine cefazolin after 6 years and would also become less effective after 13 years. Although such a simulation addresses the issue of future resistance crudely and incompletely, it illustrates how speculative it would be to model this evolution, given the current lack of knowledge about glycopeptide resistance in staphylococci.

The conclusions of this analysis were not meaningfully influenced by uncertainty around the model parameters, according to sensitivity analyses conducted over a wide range of plausible values. Other than the influence of susceptibility patterns discussed above, the results were sensitive only to large variations of the price of vancomycin and to the relative effectiveness of the two prophylactic drugs. This last aspect is a limitation of the study due to insufficient data. The effectiveness of cefazolin may have been underestimated if cefazolin has some effect against methicillin-resistant staphylococci when the inoculum is small, as may be the case during surgery. We can also speculate that a large proportion of methicillin-resistant organisms are acquired after surgery; perioperative antibiotic prophylaxis may not prevent infections caused by these organisms. We are not aware of data supporting this hypothesis, however. We assumed that both vancomycin and cefazolin had the same preventive efficacy against susceptible organisms. However, vancomycin would no longer be the best option if it were 18% less effective than cefazolin in preventing infections caused by susceptible organisms. Beside uncertainty regarding the relative intrinsic effectiveness of the two drugs, this effectiveness may be differently affected by suboptimal use such as wrong timing or wrong dose, because of distinct pharmacokinetic characteristics. In general, vancomycin's longer half-life in serum would be expected to make it more tolerant than cefazolin if delays occur between administration of prophylaxis and initiation of surgery. Finally, we did not include patient time costs in the reference case analysis. However, their inclusion would only have increased the estimated benefit associated with preventing infections.

These results underscore the importance of using a perioperative prophylaxis regimen with reasonable activity against gram-positive organisms. Failure to provide patients undergoing CABG with an acceptable perioperative prophylaxis regimen is an example of a medical error; in the aggregate, these errors have been recognized to negatively affect clinical outcomes and to impose a large burden on health resources (42). Because the circumstances in which perioperative prophylaxis must be administered are highly structured, development of systems to achieve near-perfect compliance could be feasible for the health- care delivery system. The motivation for making this a priority is particularly strong because prophylaxis was estimated to save at least $900 per person. The strategy of no prophylaxis was always associated with both higher costs and a greater number of deaths and surgical site infections than either of the two alternative prophylactic strategies. In fact, the cost attributed to failure to provide prophylaxis may have been underestimated, since we assumed that the risk reduction was similar to that observed in other clean surgical procedures (18-23).

Approximately 366,000 CABG operations are performed yearly in the United States (43). Using the best data currently available and considering clinical outcomes to individual patients, our model predicts that routine vancomycin

would prevent 110 deaths and 3,184 surgical site infections compared with routine cefazolin. Under conditions similar to those in our base case, the routine vancomycin strategy would also save $43 million nationwide. Similar conclusions are to be expected from the analysis of clean surgical procedures other than CABG, since most surgical site infections are caused by staphylococci in these settings. Because data are insufficient to provide information about the potential downstream societal consequences of routine vancomycin use on the development of vancomycin resistance, we are reluctant to recommend the universal routine use of vancomycin. However, the incremental clinical benefits and cost savings associated with routine vancomycin compared with routine cefazolin for perioperative prophylaxis in CABG surgery provide an estimate of the magnitude of benefits that would need to result, at least for society at large, from slowing the emergence of vancomycin resistance by restricting its use. We recommend that immediate priority be given to studies that will inform the impact of vancomycin use on the development of resistance in gram-positive organisms.

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Dr. Zanetti is an associate hospital epidemiologist and attending physician in infectious diseases at the Lausanne University Hospital, Lausanne, Switzerland. He is also a visiting scientist at the Channing Laboratory, Brigham and Women's Hospital, Boston, MA. His main fields of research are optimization of antibiotics use, surgical site infection, and infection in cancer and intensive-care unit patients.

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Intraoperative Redosing of Cefazolin and Risk for Surgical Site Infection in Cardiac Surgery

Giorgio Zanetti,*† Richard Giardina,* and Richard Platt*‡
*Brigham and Women's Hospital, Harvard Medical School, and the CDC Eastern Massachusetts Prevention Epicenter, Boston, Massachusetts, USA; †University Hospital, Lausanne, Switzerland; and ‡Harvard Medical School and Harvard Pilgrim Health Care, Boston, Massachusetts, USA

Intraoperative redosing of prophylactic antibiotics is recommended for prolonged surgical procedures, although its efficacy has not been assessed. We retrospectively compared the risk of surgical site infections in 1,548 patients who underwent cardiac surgery lasting >240 min after preoperative administration of cefazolin prophylaxis. The overall risk of surgical site infection was similar among patients with (43 [9.4%] of 459) and without (101 [9.3%] of 1,089) intraoperative redosing (odds ratio [OR] 1.01, 95% confidence interval [CI] 0.70-1.47). However, redosing was beneficial in procedures lasting >400 min: infection occurred in 14 (7.7%) of 182 patients with redosing and in 32 (16.0%) of 200 patients without (adjusted OR 0.44, 95% CI 0.23-0.86). Intraoperative redosing of cefazolin was associated with a 16% reduction in the overall risk for surgical site infection after cardiac surgery, including procedures lasting <240 min.

Surgical site infections are important causes of illness and resource utilization (1,2). Perioperative antibiotic prophylaxis is widely used to reduce their incidence. On the basis of pharmacokinetic considerations, most published guidelines recommend intraoperative redosing of the prophylactic antibiotic for procedures of prolonged duration to maintain effective antibiotic concentrations (1,3-6).

Support for intraoperative redosing of antibiotics has been inferred from observational studies in which increased duration of surgery was associated with increased risk for surgical site infection, as well as loss of the protective effect of prophylaxis over time (7-9). However, the actual clinical benefit of intraoperative antibiotic redosing has not been confirmed or quantified in either clinical trials or observational studies.

We therefore carried out a retrospective cohort study to assess the effect of intraoperative redosing of prophylaxis on the occurrence of surgical site infection after prolonged cardiac procedures. Cardiac surgery was chosen for the study because its duration is typically long enough to meet the threshold for redosing in most published guidelines and because it carries a substantial risk for surgical site infection (10,11).

Methods

This retrospective cohort study involved patients operated on in the Division of Cardiac Surgery at Brigham and Women's Hospital, Boston, Massachusetts, from April 1, 1998, to September 30, 1999. The study population was restricted to patients who received a first preoperative 1-g dose of cefazolin beginning ≤90 minutes before incision of the skin and whose procedures lasted ≥240 minutes after that dose. This minimum duration was chosen because it was the redosing interval recommended by the hospital's guidelines during the study period. No antibiotic redosing was given for any procedures of shorter duration; they would therefore not have contributed to our study. Patients were excluded if they received therapeutic antibiotics at the time of surgery. Patients were included only once in the analysis.

Intraoperative redosing was defined as the administration of a second dose of cefazolin at any time before surgical closure. In addition to the preoperative dose and an intraoperative redose when applicable, patients typically had at least six additional 1-g doses of cefazolin prescribed during the postoperative period.

For each eligible operation, the following data were abstracted from the anesthesiologist's report: age and sex of patient, date and type of surgery, surgeon, and the time of all antibiotic administrations. In addition, data on reoperations during the same hospital stay (except if reoperation followed a diagnosis of a surgical site infection) were collected from the hospital information system. To avoid comparisons between small subgroups, surgeries were categorized as to whether they included coronary artery bypass grafting.

Surgical site infections were prospectively identified by modified National Nosocomial Infection Surveillance methods and criteria (1) by an infection control practitioner who did not know whether the patient had received an intraoperative redose of antibiotic. This method encompassed both inpatient components and postdischarge information from the surgeons' offices.

To compare the patients who had received intraoperative redosing of cefazolin with those who had not, we used the 2-sided Wilcoxon rank sum test for continuous variables and the chi-square test for proportions. The significance level was 0.05 in all tests. Significant univariate predictors

Address for correspondence: Giorgio Zanetti, Division of Infectious Diseases, University Hospital, 1011 Lausanne, Switzerland; fax: 41-21-314-1018; e-mail: Giorgio.Zanetti@chuv.hospvd.ch

of surgical site infection were then candidates for inclusion in a logistic regression model that was built through a forward selection process (12). The absence or presence of intraoperative antibiotic redosing was always forced in the model, as was an interaction term of procedure duration and intraoperative redosing, as described below. The model was then tested for confounding by each of the excluded covariates. The Wald test was used to report the significance level of the predictors in the final model (13). The odds of surgical site infection were also compared for redosing, categorized as either absent, given after 240 min, or given within 240 min, with the likelihood ratio test used to assess deviance from linearity.

To investigate whether intraoperative redosing had different effects on the risk for infection across different procedure durations, we created an interaction term with duration (categorized as <300 min, 300 to 400 min, or >400 min) and intraoperative redosing. These thresholds were chosen before the analysis began. The likelihood ratio test was used to assess the significance of this interaction term. Statistical analyses were performed with SAS statistical software (SAS Inc., Cary, NC).

Results

Among 2,751 cardiac operations performed from April 1998 through September 1999, 1,886 (69%) lasted >240 min from the time of preoperative administration of antibiotic prophylaxis. We excluded 214 procedures (11%) because the patients received antibiotics other than cefazolin for prophylaxis, 44 (2%) because cefazolin had been administered either after surgical incision or >90 min before; 17 (1%) because of ongoing antibiotic therapy; and 8 (0.4%) because the patients had already been included in the analysis. Data were available for 1,548 (97%) of the 1,603 eligible patients.

Intraoperative redosing of cefazolin was administered to 459 (30%) of the patients, including 276 (18%) who received it within 240 min. These patients were compared with those who did not receive redosing (Table 1). The mean duration of surgery, measured from the administration of the preoperative dose of antibiotic, was significantly longer (p = 0.0001) in patients who were redosed. The distribution of surgeons also differed between the two groups. Intraoperative redosing was not associated with any of the available covariates, i.e., age, sex, type of surgery, need for reoperation, or calendar date.

Surgical site infection was diagnosed in 144 (9.3%) patients. One third of these infections were deep. There was no statistically significant difference among surgeon-specific infection rates (range 7.7% to 11.3%). In the whole study population, the overall risk for infection was similar in patients who received intraoperative redosing of cefazolin (43 [9.4%] of 459) and in those who did not (101 [9.3%] of 1,089) (OR 1.01, 95% CI 0.70 - 1.47). Multivariate analysis showed that the risk for surgical site infection increased with patient age and procedure duration and was also higher in coronary artery bypass grafting surgery. The latter finding was expected because of the additional incision for vein graft harvesting. There was also a significant interaction between surgery duration and intraoperative redosing (p = 0.015); redosing was associated with a lower infection rate in the longer procedures but not shorter ones.

Table 1. Characteristics of patients undergoing cardiac surgery

Variable	Intraoperative antibiotic redosing: Yes (n = 459)	Intraoperative antibiotic redosing: No (n = 1,089)	p
Mean age (range, years)	65.2 (17–91)	65.7 (20–92)	NS[a]
Mean duration of surgery[b]	393 (241-900)	345 (241–700)	0.0001
Male sex (%)	328 (71.5)	744 (68.3)	NS
Type of surgery			
CABG	317 (69.1)	784 (72.0)	NS
Others	142 (30.9)	305 (28.0)	
Reoperation[c]	53 (11.6)	105 (9.6)	NS

[a]NS = not significant; CABG = coronary artery bypass graft.
[b]Minutes elapsed between administration of preoperative antibiotics and skin closure.
[c]Reoperation within the same hospital stay is included, except for surgery following a diagnosis of surgical site infection.

Independent predictors of surgical site infection were analyzed for two categories of procedure duration (Table 2). Intraoperative redosing had a significant protective effect only in procedures lasting >400 min, for which it was associated with a 0.44 odds ratio (OR; 95% confidence interval [CI] 0.23 to 0.86) of surgical site infection. This corresponds approximately to a 56% reduction in risk for infection among these procedures, resulting in a risk that does not differ significantly from that observed in surgery that lasted 240 to 400 min.

Table 2. Independent predictors of surgical site infections after cardiac surgery

Predictor	Adjusted odds ratio for SSI[a]	95% confidence interval	p[b]
Procedures lasting ≤400 min			
Age[c]	1.2	1.00-1.45	0.049
CABG surgery	1.84	1.05-3.20	0.032
Duration of surgery[d]	1.38	1.00-1.82	0.032
Intraoperative redosing of antibiotics	1.27	0.80-2.02	0.319
Procedures lasting >400 min			
CABG surgery	2.2	1.05-4.61	0.036
Intraoperative redosing of antibiotics	0.44	0.23-0.86	0.016

[a]SSI: surgical site infection; CABG: coronary artery bypass graft.
[b]Wald test.
[c]Odds ratio for every additional decade of age.
[d]Odds ratio for every additional hour of surgery.

We also explored different redosing schedules during procedures of >400 min. There was a significant trend toward a lower risk for infection when redose was either not given, given after 240 min, or given within 240 min (p = 0.001).

Of the patients who received prophylaxis with cefazolin, 20% had a procedure that lasted >400 min. For all patients, including those with a procedure lasting <240 min, we estimate that the infection rate in the absence of any redosing would have been 9.4%. If every patient whose procedure lasted >240 min had been redosed, the expected infection rate would have been 7.9%, representing a 16% reduction in the overall risk of postoperative surgical site infection attributable to redosing. The distribution of this expected risk in operations more or less than 400 min in duration, with or without redosing, is shown in the Figure.

Since procedures had been arbitrarily categorized before inspection of the data according to a duration of more or less than 400 min, we analyzed the impact of intraoperative redosing with different thresholds of duration. The benefit of intraoperative redosing was significant for a boundary between 385 and 415 min. However, there was a general trend toward greater benefit for higher thresholds.

Discussion

This retrospective study demonstrates that intraoperative redosing of cefazolin provided additional protection against surgical site infection among patients undergoing cardiac surgery lasting longer than approximately 6.5 to 7 h. Although this group includes only a minority of procedures, we estimate from our data that a strategy of redosing in all procedures >240 min long results in a 16% reduction in the overall infection rate in cardiac surgery. This rate in our study population was comparable with that reported by others (10,11,14,15). The benefit from redosing had been assumed but had not been proven, and the minimum duration at which redosing is beneficial had been derived from theoretical considerations. Redosing provided similar protection from both deep and superficial infections (data not shown).

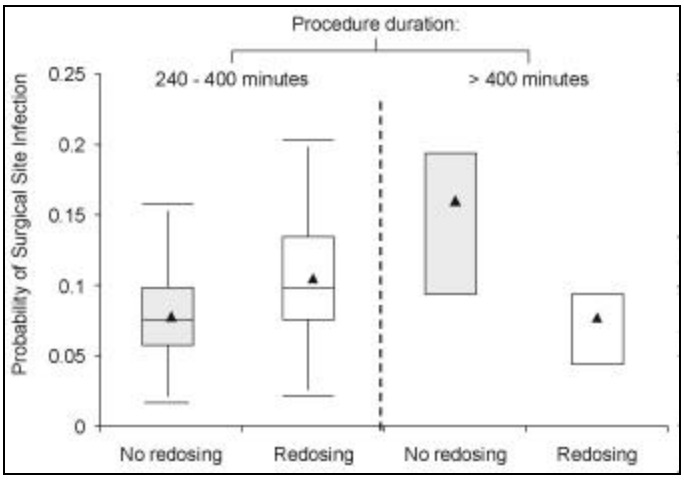

Figure. Effect of intraoperative redosing of cefazolin on the probability of surgical site infection. Box-and-whisker plots represent the probabilities of surgical site infection in 1,548 patients undergoing cardiac surgery, stratified by procedure duration, with or without intraoperative redosing of cefazolin. The probabilities for each member of the cohort were computed on the basis of redosing of antibiotic prophylaxis, the patient's age, and the type and duration of the procedure. The mean is represented by a triangle and the median by a bar within the boxes. There is no bar for procedures >400 min in duration because all the probabilities were clustered at the extremities of the boxes.

The positive association between duration of surgery and risk for surgical site infection has been reported (7-9). Our data show that this association persists even when antibiotic is redosed. This observation suggests that the risk related to duration not only reflects a diminution of antibiotic concentration over time but also may be a proxy for risk factors independent of antibiotic use, such as the technical difficulty of the procedure.

Guidelines usually recommend redosing intervals of 3 to 4 h for cefazolin (1,3-6). In a study on hysterectomy, for instance, a protective effect of prophylaxis was no longer observed when the operation lasted >3.3 h (8). The benefit extended beyond this threshold in our study, a finding that may reflect the markedly prolonged serum half-life of cefazolin during cardiopulmonary bypass. Although the half-life of cefazolin is 1.8 h in healthy persons (3), several studies have shown a slower elimination during cardiopulmonary bypass (14,16,17). Therefore, any benefit of redosing in noncardiac surgery may be observed for shorter procedures than in cardiac surgery.

This study has several limitations. Because of its retrospective design, the results were adjusted for a limited number of risk factors for surgical site infection, including surgery duration, age of the patient, and need for reoperation. Certain coexisting conditions, such as diabetes mellitus or obesity, smoking status, length of previous hospital stay, and a violation of asepsis during surgery, are among the predictors of surgical site infection that might confound our results, should they be related to the probability of an intraoperative antibiotic redose. Since the most plausible effect of a high-risk profile is to increase the likelihood of intraoperative redosing, adjustment for this profile would increase the apparent benefit. If patients undergoing more complicated (and therefore more infection-prone) procedures were less likely to be redosed for any reason, the effect of redosing would be overestimated. However, in that case, we would expect to see an effect for all procedures, not only longer ones. Our study also provides no information about the utility of additional doses of prophylaxis after surgery. Finally, our sample size limits the precision of our estimates, especially the ability to identify a precise threshold beyond which redosing is beneficial. Thus, we do not know whether similar protection could be obtained by redosing cefazolin only beyond the 400-min threshold. In an exploratory analysis of timing of the redose, there was a significant trend toward higher benefit when a redose was given within 240 min. Therefore, our results should not be used to support an extension of the 3- to 4-h redosing interval recommended by most guidelines (1,3-6).

We conclude that redosing of cefazolin prophylaxis for most cardiac procedures can prevent a substantial fraction of surgical site infections. It will be worthwhile to examine the effects of intraoperative redosing in other procedures.

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Dr. Zanetti is an associate hospital epidemiologist and infectious diseases attending physician at Lausanne University Hospital,

Lausanne, Switzerland. He is also a visiting scientist at Channing Laboratory, Brigham and Women's Hospital, Boston. His research focus includes optimization of antibiotic use, surgical site infection, and infection in cancer and intensive care unit patients.

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Pneumococcal Surface Protein A of Invasive *Streptococcus pneumoniae* Isolates from Colombian Children

María Claudia Vela Coral,* Nacxiry Fonseca,* Elizabeth Castañeda,*
José Luis Di Fabio,† Susan K. Hollingshead,‡ and David E. Briles‡
*Instituto Nacional de Salud, Bogotá, Colombia; †Pan American Health Organization, Washington, DC, USA; and ‡University of Alabama, Birmingham, Alabama, USA

Pneumococcal surface protein A (PspA) elicits protection in mice against fatal bacteremia and sepsis caused by genetically diverse pneumococci and protects against carriage and lung infection. We determined the PspA families of invasive isolates of *Streptococcus pneumoniae* recovered from Colombian children <5 years of age. That 97.5% of Colombian isolates belong to PspA families 1 and 2 supports the hypothesis that a human PspA vaccine covering a few PspA families could be broadly effective.

Streptococcus pneumoniae is a major respiratory pathogen that also causes meningitis, otitis media, and bacteremia (1). In adults, capsular polysaccharides of *S. pneumoniae* can elicit protective antibodies against pneumococcal infection (2). However, in children <2 years of age polysaccharide vaccines do not effectively elicit a protective response (3,4), and children can have repeated infections with strains of the same or different capsular serotype (5). Therefore, protein-polysaccharide conjugates and pneumococcal proteins, including pneumolysin, neuraminidase, pneumococcal surface adhesin A, and pneumococcal surface protein A (PspA), have been considered as alternative means to induce protective immunity in infants and children. The increased frequency of isolation of multidrug-resistant strains of *S. pneumoniae* accentuates the need for an effective vaccine (6).

PspA, a surface protein and virulence factor found on all isolates of *S. pneumoniae* (7), is highly immunogenic (6-8). PspAs share many cross-reactive epitopes, and immunization with a single PspA is cross-protective in mice against fatal infection with strains of the mouse virulent capsular types (6,9,10). Mucosal immunization with PspA can also elicit immunity to carriage (11,12).

Information about the basic protein structural domains of PspA came from the DNA sequences of the *pspA*/Rx1 and the *pspA*/EF5668 genes (13,14). The five domains include 1) a signal peptide, 2) an alpha-helical charged domain (amino acids 1-288), 3) a proline-rich region (amino acids 289-370), 4) a choline-binding domain consisting of 9 to 10 twenty-amino-acid repeats (amino acid 371-571), and 5) a C-terminal 17-amino-acid tail (amino acids 572-589). These amino-acid positions are based on the *pspA*/Rx1 sequence (14).

A portion of *pspA*/Rx1 (amino acids 192-260) has been identified that elicits cross-protective antibody responses (15). Many PspA molecules have been examined to aid in the development of PspA as a protein-based vaccine (16). From the alignment of PspA sequences of 24 strains, the sequence differences in a centrally located clade-defining region were used to group PspA proteins into six clades (16). Within the clade-defining region, sequences in the same clade share at least 80% amino-acid identity. The clade-defining region is roughly the same as that shown to elicit cross-protective responses (15,16). The six clades have also been grouped into three families. Sequences share at least 50% sequence identity in each family (16).

During the 1990s, the Colombian Pneumococcal Study Group investigated the capsular type distribution and antimicrobial susceptibility of invasive isolates from children <5 years of age (17). The data obtained may guide selection of polysaccharides to include in vaccines for use in Latin America. In Colombia and other Latin American countries, the prevalence of strains of capsular serotypes 1 and 5 is higher than in North America (18,19). The study demonstrated that vaccine formulations based only on North American data might not be as effective in Latin America because of the differing distributions of capsular types. Surveillance of isolates has continued to monitor any shifts in the antigenic types of pneumococci (20).

Our study was intended to expand our knowledge of vaccine coverage for a potential protein-based vaccine in Colombian isolates. We determined the frequency of family 1 and family 2 PspAs among *S. pneumoniae* isolates from Colombia that express one of the seven most common capsular types. Our results will be coordinated with those from laboratories in other Latin American countries to learn the diversity of PspA over the entire region. At present, however, only the Colombian isolates have been investigated completely.

Materials and Methods

Forty *S. pneumoniae* invasive isolates, representing each year from 1994 through 1998, were selected from the seven most common capsular types in Colombia. All isolates were confirmed to be *S. pneumoniae* by standard procedures (alpha-hemolysis, Gram stain, optochin test, and bile solubil-

Address for correspondence: María Claudia Vela Coral,Grupo Microbiología, Instituto Nacional de Salud, Avenida Calle 26 No. 50-60, Zona 6 CAN Bogotá, Colombia; fax: 571-222 0194/3055; e-mail: mvela@hemagogus.ins.gov.co

ity) (21). Antimicrobial susceptibility patterns were not considered in the selection of isolates.

Pneumococci were cultured for 6 hr in 10 mL Todd-Hewitt broth (Difco, Detroit, MI) supplemented with 1% yeast extract, 1% glucose, and 22 µg/mL glutamic acid. After centrifugation, the cells were resuspended in 0.1 mL TE (10 mM Tris HCL, 1mM EDTA, pH 8.0), and lysozyme (10 µL at 50 mg/mL) was added. The cells were incubated for 30 min at 37ºC, and 0.5 mL GES (5 M guanidine thiocyanate, 0.1 M EDTA, pH 8.0, and 0.5% sarkosyl) was added. After incubation at room temperature for 10 min, 0.25 mL (7.5 M) ammonium acetate was added, and the cells were mixed and placed on ice for 10 min. Chloroform/isoamyl alcohol (24:1) was added and mixed, the phases were separated by centrifugation, and 0.7 mL of aqueous phase was recovered. The DNA was precipitated with ethanol and resuspended in 50-100 µL of TE (22).

Polymerase chain reaction (PCR) was carried out on genomic DNA. The oligonucleotide primers for family 1 were LSM12, 5'CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT3' (23) and SKH63, 5'TTTCTGGCTCAT(C and T)AACTGCTTTC3' (at position 12 C and T in a 1:1 ratio) and for family 2 were LSM12, 5'CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT3' and SKH52, 5' TGGGGGTGGAGTTTCTTCTTCATCT3'. The following PCR conditions were used: an initial 95ºC (3 min), 30 cycles of 95ºC (1 min), 62ºC (1 min) and 72ºC (3 min), followed by 72ºC (10 min) (15) in a PTC 150 Thermocycler (MJ Research, Watertown, MA). PCR products were initially run at an annealing temperature of 62ºC. Any isolates yielding no product at 62ºC were repeated at annealing temperatures of 65ºC or 58ºC and then 55ºC to account for potential sequence divergence in the primer region. The amplified PCR products were approximately 1,000 bp for family 1 and 1,200 bp for family 2. The PCR product was run on an agarose gel at 80 volts for 1.5 hr, and the gel was stained with 0.5 µg/mL ethidium bromide. Molecular weight standard for gel electrophoresis was the 1.0-kb ladder DNA (Promega, Madison, WI). Strains BG9739 (clade 1) and AC122 (clade 3) were used as controls for family 1 and 2 tests, respectively (16). The PspA family of these strains has been confirmed by DNA sequence of their *pspA* genes (16).

A pool of immune sera for typing PspA families came from two rabbits, one immunized with rPspA/L82016 (clade 1) and the other with rPspA/Rx1 (clade 2). The antisera for typing PspA family 2 came from a pool of serum from two rabbits immunized with either PspA/V-024 (clade 3) or PspA/V-032 (clade 4). Recombinant PspA/Rx1 was added to this pool to reduce cross-reactivity with family 1 (clade 1 and 2) PspAs.

PspAs were recombinant products from *Escherichia coli* strains bearing plasmids with cloned *pspA* genes. The region of PspA in the cloned fragment includes the entire alpha-helical region of the protein and in some cases some of the proline-rich region, which is C-terminal to the alpha-helical region. The gene fragments were cloned into pET-20b vector from Novagen (Madison, WI) between the *Nco*I and the *Xho*I cloning sites. This procedure results in an additional 10 amino acids on the C-terminus of the recombinant protein, which includes a polyhistidine for purification. Recombinant proteins were produced in *E. coli* strain BL21 (DE3) from Novagen and purified by nickel-affinity chromatography, as recommended by the manufacturer. Rabbits were immunized subcutaneously with 10 µg of protein with complete Freund's adjuvant, followed by a second 10-µg injection 1 month later with incomplete Freund's adjuvant and a final 10-µg booster after another month. Rabbits were bled 2 weeks after the last booster.

Pneumococcal isolates were cultured for 6 hr in 10 mL Todd-Hewitt broth (Difco) supplemented as described. After centrifugation, cells were resuspended in 3 mL sterile phosphate-buffered saline (PBS), and 500 µL lysis buffer (0.1% sodium deoxycholate, 0.01% sodium dodecyl sulfate, and 0.15M sodium citrate in 100 µL deionized water) was added. The mixture was incubated at room temperature for 30 min.

Protein in lysates was measured by using bicinchoninic acid as described by Smith et al. (24) with some modifications. The lysate samples were diluted 1:3.5 in sterile PBS. Then, 10 µL of each sample was loaded into a microplate well (U bottom, Becton-Dickinson, Cockeysville, MD) and 200 µL of a 50:1 mixture of reagent A (bicinchoninic acid [BCA-SIGMA B9643, Sigma, St. Louis, MO]) and reagent B (copper [II] sulfate pentahydrate 4% [SIGMA C2284, Sigma]) was added. The microplate was incubated for 30 min and absorbance at 562 nm (BIO-RAD ELISA reader model 3550, Hercules, CA) was compared with a protein bovine serum albumin (BSA) standard curve (1 mg/mL).

After the protein concentration of each lysate was adjusted to 60 µg/mL, 1 µL of lysate and 1 µL each from dilutions 1:5 (12 µg/mL), 1:25 (2.4 µg/mL), and $\leq$1:125 (0.48 µg/mL) were spotted onto two nitrocellulose membranes (Millipore, Bedford, MA) for dot-blot analysis. Each membrane was immersed in 1% BSA/PBST blocking buffer (0.05% Tween 20, 1 mM EDTA pH 8.5, 1% BSA in sterile PBS, incubated at room temperature for 1 hr, and washed three times with sterile PBS. Membranes were then immersed in a dilution of anti-PspA rabbit polyclonal antibodies (1:5,000 in blocking buffer) and incubated at room temperature for 1 hr. Rabbit antisera for families 1 and 2 were processed on separate dot blots. The membranes were then washed three times with sterile PBS and incubated with a biotinylated goat anti-rabbit antibody diluted 1:3,000. The final incubation was with streptavidin-conjugated alkaline phosphatase diluted 1:3,000 and incubated for 1 hr, followed by another washing step. Alkaline phosphatase staining was developed with NBT solution (2 mg nitroblue tetrazolium, 10 mg BCIP [5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt], 200 µL dimethyl sulfoxide in 20 mL Tris HCl, pH 8.8) with constant shaking until control dots appeared purple. Assignment of the serologic dot-blot results was based on the highest titer of each lysate that reacted with the dilution of the anti-family 1 and anti-family 2 antiserum. BG9739 (clade 1) and EF10197 (clade 2) were used as reference strains for serologic family 1 typing. AC122 (clade 3), BG11703 (clade 4) and ATCC6303 (clade 5) were used as reference strains for typing family 2 (16).

Results

PCR products ranged from 960 to 1,000 bp for family 1, and 1,200 to 1,400 bp for family 2 (Figure). Although all lysates of family 1 strains reacted with the family 1 antiserum at 1:5,000 dilution, the antiserum cross-reacted weakly with family 2 lysates, which could generally be detected at a $\leq$1:25 dilution but not at higher dilutions. The family 2 antiserum reacted only with the PspAs of family 2 strains, regardless of the dilution. Nevertheless, the combination of

the two techniques could reliably detect the PspA family of all strains. The PspA PCR and the PspA serologic dot-blot techniques correlated in 100% of cases.

Of the 40 isolates studied, 25 (62.5%) were family 1 PspA (Table). Family 1 PspAs were found among isolates with capsular types 14, 6B, 23F, 5, 19F, 1, 8, and 35. All invasive isolates capsular type 6B, 5, and 19F were family 1. Fourteen (35%) isolates were family 2, including strains of capsular types 14, 23F, 9, 3, 8, and 35. The relative distribution of family 1 isolates to family 2 isolates did not fluctuate substantially over the 4-year period. Isolate Co-29, which was capsular type 1, was the only one (2.5%) of the 40 that was neither family 1 or family 2. The remaining four isolates of capsular type 1 were family 1.

Table. *Streptococcus pneumoniae* PspA families 1 and 2 from 40 Colombian isolates

Isolates (Code INS[a])	Date	Source	Capsular type	PCR & dot blot
Co-1(2)	17/12/93	Blood	14	Family 1
Co-2 (125)	12/12/94	CSF	14	Family 2
Co-3 (146)	25/01/95	Blood	14	Family 2
Co-4 (309)	17/01/96	PLF	14	Family 1
Co-5 (E-212)	16/06/98	CSF	14	Family 2
Co-6 (95)	17/06/93	CSF	6B	Family 1
Co-7 (20)	19/06/94	Blood	6B	Family 1
Co-8 (149)	04/02/95	CSF	6B	Family 1
Co-9 (322)	10/02/96	CSF	6B	Family 1
Co-10 (E-10)	03/05/96	Blood	6B	Family 1
Co-11(14)	20/04/94	PLF	23F	Family 2
Co-12 (23)	22/06/94	CSF	23F	Family 2
Co-13 (152)	15/02/95	CSF	23F	Family 1
Co-14 (E-8)	03/05/96	CSF	23F	Family 1
Co-15 (E-99)	19/03/97	CSF	23F	Family 2
Co-16 (7)	25/03/94	PLF	5	Family 1
Co-17 (106)	10/11/94	Blood	5	Family 1
Co-18 (177)	06/04/95	Blood	5	Family 1
Co-19 (316)	24/01/96	Blood	5	Family 1
Co-20 E-220)	24/07/98	CSF	5	Family 1
Co-21(10)	25/04/94	CSF	19F	Family 1
Co-22 (179)	08/04/95	CSF	19F	Family 1
Co-23 (318)	27/01/96	Blood	19F	Family 1
Co-24 (E-124)	04/07/97	CSF	19F	Family 1
Co-25 (E-159)	23/10/97	CSF	19F	Family 1
Co-26 (5)	19/03/94	Blood	1	Family 1
Co-27 (99)	04/11/93	CSF	1	Family 1
Co-28 (165)	01/03/95	Blood	1	Family 1
Co-29 (315)	26/01/96	PLF	1	0[b]
Co-30 (E-107)	16/04/97	Blood	1	Family 1
Co-31(51)	04/09/94	PLF	9V	Family 2
Co-32 (E-24)	14/06/96	CSF	9N	Family 2
Co-33 (119)	25/11/94	Blood	3	Family 2
Co-34 (E-140)	02/09/97	CSF	3	Family 2
Co-35 (216)	06/06/95	Blood	8	Family 2
Co-36 (E-135)	13/08/97	CSF	8	Family 1
Co-37 (E-66)	07/11/96	CSF	4	Family 2
Co-38 (300)	18/12/95	Blood	35B	Family 1
Co-39 (E-25)	14/06/96	CSF	35B	Family 2
Co-40 (320)	06/02/96	CSF	35F	Family 2

[a]INS = Instituto Nacional de Salud; PCR = polymerase chain reaction; CSF = cerebrospinal fluid; PLF = pleural fluid.

[b]Co-29 did not amplify or react with either family.

Figure. Polymerase chain reaction for PspA families 1 and 2: lanes 1 and 2 were controls for families 1 and 2, respectively. Lanes 3 to 7 and 9 (Co-24, Co-25, Co-26, Co-27, Co-28, Co-29, Co-30 isolates) were family 1. Lane 8 (Co-29) did not amplify with either family. The molecular weight was 1-kb ladder DNA (Promega).

Conclusion

A vaccine composed of PspA is hypothesized to protect against invasive disease and also eliminate the carriage state. Yamamoto et al. (25) reported that intranasal administration of PspA in mice together with a nontoxic adjuvant (mCT S61F) is an effective mucosal vaccine against pneumococcal infection. In another study, active immunization with PspA reduced the signs of purulent otitis media in rats, although the challenge strain contained a PspA that differed from the immunogen (26). More recently, immune sera from human volunteers immunized with PspA protected against fatal pneumococcal infection in mice (27).

All the Colombian isolates were invasive, and all but one belonged to PspA families 1 or 2. This finding is relevant to efforts to develop PspA into a human vaccine component. The distribution of the Colombian isolates between PspA families 1 and 2 did not differ substantially from that observed for isolates from North America and Europe (16). Therefore, a vaccine formulation including these two families might cover isolates from both North and South America with equal effectiveness. Latin America has a varied distribution of capsular serotypes (17-19), which lessens the potential for effectiveness of the heptavalent conjugate vaccine recently approved in the United States (28).

We have obtained information about pulsed-field gel electrophoresis and penicillin-binding protein patterns and capsular types of Colombian strains with diminished susceptibility to penicillin (20,29,30). Characterizing PspA families of these penicillin-resistant strains and examining representatives of different multiresistant international clones will be of interest for future studies.

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Ms. Vela is a scientific investigator in the Microbiology Group at Instituto Nacional de Salud in Bogotá, Colombia. Her research interests focus on resistant and multidrug-resistant *Streptococcus pneumoniae* recovered from children <5 years of age.

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Epidemiology of and Surveillance for Postpartum Infections

Deborah S. Yokoe,* Cindy L. Christiansen,† Ruth Johnson,‡ Kenneth E. Sands,§ James Livingston,* Ernest S. Shtatland,† and Richard Platt*†

*Channing Laboratory and Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA; †Harvard Pilgrim Health Care, Boston, MA, USA; ‡Harvard Vanguard Medical Associates, Boston, MA, USA; and §Beth Israel Deaconess Medical Center, Boston, MA, USA

We screened automated ambulatory medical records, hospital and emergency room claims, and pharmacy records of 2,826 health maintenance organization (HMO) members who gave birth over a 30-month period. Full-text ambulatory records were reviewed for the 30-day postpartum period to confirm infection status for a weighted sample of cases. The overall postpartum infection rate was 6.0%, with rates of 7.4% following cesarean section and 5.5% following vaginal delivery. Rehospitalization; cesarean delivery; antistaphylococcal antibiotics; diagnosis codes for mastitis, endometritis, and wound infection; and ambulatory blood or wound cultures were important predictors of infection. Use of automated information routinely collected by HMOs and insurers allows efficient identification of postpartum infections not detected by conventional surveillance.

The epidemiology of postpartum infections has not been well characterized. In part this is because of the limitations of surveillance systems, which usually monitor infections that are recognized during hospitalization. Most postpartum and nonobstetrical postsurgical infections, however, occur after hospital discharge (1-3). Decreasing lengths of hospital stay may further compromise detection of these infections.

Several methods for postdischarge surveillance of postpartum infections have been evaluated. Hulton et al. (1) used physician questionnaires for postdischarge surveillance of patients undergoing cesarean section. With only inpatient surveillance, 59% of postpartum infections they ultimately detected would not have been identified. The overall infection rate after postdischarge surveillance was implemented was fourfold higher than the previous rate (6.3% vs. 1.6%). Holbrook et al. (2) used patient self-administered questionnaires to conduct large-scale, routine postdischarge surveillance following vaginal delivery or cesarean section. Despite a modest return of questionnaires, self-reported questionnaire results identified twice as many apparent postpartum infections (4% infection rate) as did concurrent prospective in-hospital surveillance. Only 48% of reported maternal infections, however, were confirmed by questionnaires to the patients' physicians. Sands et al. (3) evaluated the use of automated ambulatory diagnosis, testing, and pharmacy code screening combined with discharge diagnoses to identify surgical site infections in nonobstetric patients undergoing surgery. They found that ambulatory code screening was a sensitive method for detecting patients with surgical site infections and that 84% occurred after hospital discharge. Of the postdischarge surgical site infections, most (63%) were diagnosed and treated entirely in the ambulatory setting. In addition, patient and surgeon questionnaires had low sensitivities (28% and 15%, respectively) for identifying postdischarge infections.

Routine surveillance for nosocomial infections is recommended by the Centers for Disease Control and Prevention and required by the Joint Commission on Accreditation of Healthcare Organizations, with the goal of using this information to compare infection rates over time and between institutions and to guide the allocation of resources towards improvements most likely to result in reduced infection rates.

In this study, we used the inpatient and outpatient data collected by a health maintenance organization (HMO) to identify postpartum infections and describe the epidemiology of these infections.

Methods

The study population consisted of all women who had a vaginal delivery or cesarean section at Brigham and Women's Hospital from January 1, 1993, to June 30, 1995, and who received postpartum care at Harvard Pilgrim Health Care (HPHC)/Harvard Vanguard Medical Associates (HVMA) centers with automated full-text ambulatory medical records. HPHC is a multimodel health maintenance organization that included a staff model division (now a multispecialty group practice, HVMA) with approximately 300,000 members in the greater Boston area at the time of the study. Brigham and Women's Hospital is the most active obstetrical facility for these members.

HMO data included three sources: an extensively automated ambulatory record, pharmacy dispensing data, and administrative claims for hospital, emergency room, and other care delivered outside the health center. The automated ambulatory medical record system (4) used standard-

Address for correspondence: Deborah S. Yokoe, 181 Longwood Ave., Boston, MA 02115; fax: 617-731-1541; e-mail: deborah.yokoe@channing.harvard.edu

ized forms that were completed for every patient encounter at HPHC/HVMA centers, including telephone calls, office visits (scheduled or unscheduled), urgent care visits, and hospitalizations. Information was recorded on forms that are customized for the type of encounter. The provider either wrote in or selected from a list of all coded diagnoses, tests, procedures, and prescriptions relevant to that encounter and enters additional comments as free text. All information, including free text, is entered into an automated medical encounter record. The results of diagnostic tests are entered directly into the automated record linked to the patient encounter during which they were ordered. Information about hospitalizations and emergency room visits appears in both encounter records and separate administrative records. HPHC/HVMA pharmacies are also computerized and linked to the automated medical record. Ninety percent of HPHC members had prepaid coverage for pharmaceuticals and so are likely to use HPHC/HVMA pharmacies.

Identification of Postpartum Infections

Automated medical records, pharmacy dispensing records, and hospital and emergency room claims were screened by a computerized search of HPHC records for the 30 days following delivery for the presence of any of 32 diagnostic, testing, or pharmacy dispensing codes indicative of postpartum infections (Table 1), as described (3).

Full-text ambulatory medical records and relevant hospital records were reviewed for the 30-day postpartum period for a random sample of 100 patients with at least one of the ambulatory screening codes. Surgical site infections (including endometritis), episiotomy site infections, mastitis, and urinary tract infections were confirmed by the Centers for Disease Control and Prevention's National Nosocomial Infection Surveillance system definitions (5,6).

Infection rates for the entire study population of postpartum women were extrapolated by standard methods from the estimated infection rates for the sample of individual medical records reviewed (7).

Two previously described surveillance screening methods were used to assess the completeness of postpartum infection detection. In one method, patient self-reported questionnaires were mailed to all women approximately 6 weeks after their infants were discharged, by using previously described instruments (2). These self-administered questionnaires asked whether the mother had specific infections, received an antibiotic, or was rehospitalized for an infection. All study patients with questionnaire results suggestive of postpartum infection were identified. In the second method, prospective inpatient surveillance was conducted by infection control practitioners during the entire period, as described (8).

Full-text ambulatory medical records and relevant hospital records were reviewed for the 30-day postpartum period for all patients identified through inpatient surveillance or self-reported questionnaire results as described.

Resource use associated with infections during the 30-day postpartum period was evaluated through review of ambulatory records for patients with confirmed postpartum infection. All free-text notes were reviewed, and encounters for which the principal focus was the postpartum infection were identified.

Table 1. Ambulatory record codes used to screen postpartum medical encounters for infections

Screening code data source	Description
Hospital and emergency room claims	
ICD-9 diagnosis codes[a]	
670.02	Major puerperal infection, with postpartum complication
670.04	Major puerperal infection, postpartum condition or complication
599.0	Urinary tract infection
674.34	Other obstetrical complication
675.14	Postpartum breast abscess
675.24	Postpartum nonpurulent mastitis
998.5	Postoperative infection
Ambulatory medical records	
COSTAR diagnosis codes	
DA140	Fever of unknown origin
DC150	Cellulitis
DC408	Abscess
DH140	Mastitis
DL101	Urinary tract infection
DM153	Endometritis
DR180	Wound infection
COSTAR therapy or test codes	
RR240	Incision and drainage
RT223	Bacterial culture taken
RY828	Fever control instruction
TB555	Blood culture
TB800	Wound culture
	Amoxacillin/clavulanate
	Amoxicillin
	Ampicillin
	Cefuroxime
	Cephalexin
	Cephradine
	Ciprofloxacin
	Clindamycin
	Dicloxacillin
	Doxycycline
	Erythromycin
	Metronidazole
	Trimethoprim-sulfamethoxazole

[a]Hospital claims from delivery admission or any readmission within 30 days or emergency department claims. ICD-9 = International Classification of Diseases, 9th revision, clinical modification, 3rd edition.

Predictors of Infection

Univariate analysis and logistic regression were used to select predictors of infection by using data from the sample of records with full-text ambulatory medical record review. One thousand bootstrap samples of two-thirds of the data were then used to simulate the model's performance in a new setting. The models were tested with the remaining data, and measures of sensitivity, specificity, and predictive value positive were extrapolated to the entire cohort (9). Selection of the final model was based on predictive performance and stability of the regression coefficient estimates. A separate model was constructed by the same methods to specifically predict surgical site infections, including endometritis, among women who delivered by cesarean section.

Results

The study population consisted of 2,746 HPHC/HVMA members who underwent 2,301 vaginal deliveries and 525 cesarean sections. Ninety-five confirmed infections were identified among the random sample of 100 women who had at least one screening code and whose ambulatory medical records were reviewed, plus the additional 210 women identified by patient questionnaire results or inpatient surveillance. Extrapolation of the reviewed sample to the entire source population predicted a total of 169 infections, for an overall infection rate of 6.0% (95% confidence interval [CI] 5.1%, 6.9%). The extrapolated postpartum infection rates were 7.4% after cesarean section (95% CI 5.3%, 10.0%) and 5.5% (95% CI 4.6%, 6.5%) after vaginal delivery.

Among women undergoing cesarean section, the site-specific infection rates (number of infections/100 deliveries) were mastitis 1.7% (0.8%, 3.2%), urinary tract infection 1.1% (0.4%, 2.5%), surgical site infection (excluding endometritis) 3.4% (2.0%, 5.4%), and endometritis 0.8% (0.2%, 1.9%). Following vaginal delivery, the infection rates were mastitis 3.0% (2.4%, 3.8%), urinary tract infection 2.0% (1.4%, 2.6%), episiotomy site infection 0.3% (0.2%, 1.9%), and endometritis 0.2% (0.1%, 0.5%) (Figure 1).

Approximately 94% of these infections were detected after hospital discharge. For these post-discharge infections, 74% of patients did not return to the hospital where they delivered for evaluation or treatment.

Completeness of Surveillance

Four hundred ten (15%) of the 2,826 deliveries were associated with at least one automated screening code for postpartum infection. Screening codes identified 65 of the 71 patients who had verified postpartum infections identified by either inpatient surveillance or self-reported questionnaire results. Using for comparison the extrapolated number of postpartum infections among patients identified by automated code screening, plus all verified infections identified by either prospective inpatient surveillance or self-reported questionnaire screening, we determined the sensitivity of ambulatory code screening for identifying patients with postpartum infections to be 96% (95% CI 92%, 99%), specificity to be 99% (95% CI 99%, 100%%), and predictive value positive to be 40% (95% CI 35%, 45%%).

In comparison, both inpatient surveillance and self-reported questionnaires missed most postpartum infections, with sensitivities of 21% (95% CI 15%, 28%%) and 25% (95% CI 19%, 32%), respectively. The sensitivity of inpatient surveillance for detecting infections diagnosed during the initial hospitalization or requiring readmission to the hospital, however, was 100%.

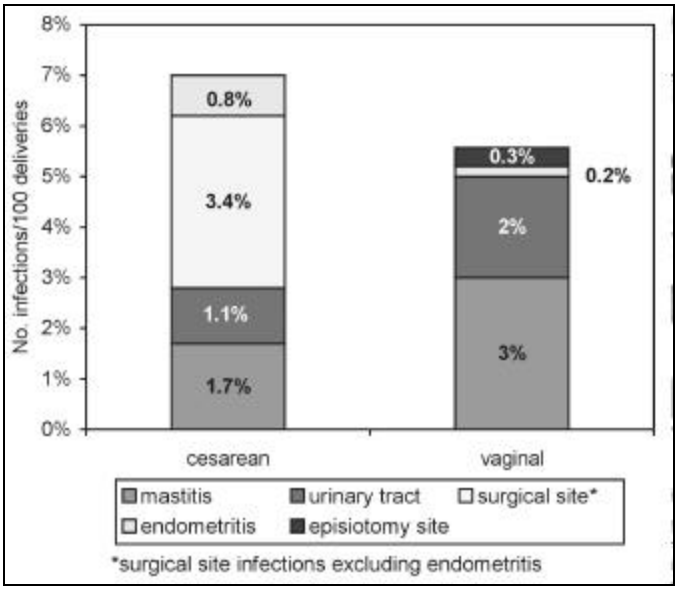

Figure 1. Extrapolated site-specific infection rates following vaginal and cesarean delivery.
*Surgical site infections, excluding endometritis

During the 30-day postpartum period, the 63 vaginal deliveries complicated by postpartum infection among women whose full-text ambulatory records were reviewed were associated with 14 emergency department visits, 106 scheduled visits, and 36 urgent-care visits. Of these ambulatory encounters, 9 (64%) emergency department visits, 44 (42%) scheduled visits, and 21 (58%) urgent-care visits could be verified as directly attributable to the postpartum infection. In addition, these infections resulted in 12 readmissions to the hospital and 85 nonappointment encounters, such as telephone calls or visits for laboratory tests. The 32 cesarean deliveries complicated by infection were associated with 10 emergency department visits, 102 scheduled visits, and 14 urgent-care visits. Of these ambulatory encounters, 8 (80%) emergency department visits, 74 (73%) scheduled visits and 12 (86%) urgent-care visits were directly attributable to the postpartum infection. These infections following cesarean section resulted in 8 readmissions to the hospital and 40 nonappointment encounters. The 74 postpartum infections that did not result in rehospitalization or emergency room visits were associated with 68% of postpartum infection-related ambulatory encounters.

Predictors of Infection

Important predictors of postpartum infection included rehospitalization within 30 days of delivery; cesarean versus vaginal delivery; dispensing of antistaphylococcal antibiotics (cephalexin, dicloxacillin, or both); diagnosis codes for mastitis, endometritis, and wound infection; and test codes for blood and wound microbiology cultures (Table 2). Cesarean section, although not statistically significant in this model, is included because it is a significant predictor of surgical site infection and endometritis. A cutoff probability of infection of $\geq$0.20 yielded an expected sensitivity of 87% (95% CI 72%, 94%), specificity of 97% (95% CI 96%, 98%), and predictive value

Table 2. Predictors of postpartum infection used in the logistic regression models

Variable	Odds ratio	95% confidence interval
Model 1: Urinary tract infection, mastitis, surgical site infection or episiotomy site infection following cesarean or vaginal delivery		
Cesarean section	1.21	0.59-2.47
Antistaphylococcal antibiotics	1.89	1.02-3.53
Rehospitalization within 30 days of delivery	3.23	1.32-7.91
Ambulatory diagnosis code for mastitis, urinary tract infection, or endometritis	5.70	2.97-10.95
Ambulatory blood or wound culture	5.85	1.97-17.84
Hospital or emergency department diagnosis code for mastitis, urinary tract infection, or other obstetrical complications	*	
Model 2. Surgical site infections (including endometritis) following cesarean section		
Ambulatory blood or wound culture	9.17	2.44-34.41
Ambulatory diagnosis code for endometritis or wound infection	*	

*In the prediction model, any woman with one or more of these codes was given an automatic probability of infection of 1.0 to maintain stability of the model during bootstrap sampling.

positive of 55% (95% CI 41%, 68%). A cutoff probability of infection of ≥0.40 yielded an expected sensitivity of 73% (95% CI 59%, 84%), specificity of 98% (95% CI 98%, 99%), and predictive value positive of 64% (95% CI 52%, 77%) (Figure 2).

A separate model was constructed to predict surgical site infections among women who delivered by cesarean section. This model included as important predictors of surgical site infection ambulatory medical record test codes for blood and wound culture and diagnosis codes for endometritis and wound infection (Table 2). A cutoff probability of infection of ≥0.25 yielded an expected sensitivity of 78% (95% CI 60%, 100%), specificity of 88% (95% CI 0%, 97%), and predictive value positive of 75% (95% CI 23%, 92%). The stability of this model in the simulation analysis was limited by the small number of surgical site infections (22) among women who had full-text ambulatory record review and who delivered by cesarean section.

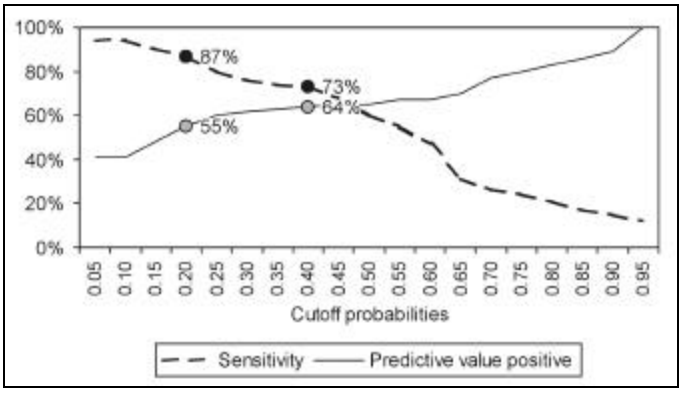

Figure 2. Sensitivities and predictive value positives of differing cutoff probabilities of infection based on 1,000 bootstrap samples. A cutoff probability of infection of ≥0.20 yielded a sensitivity of 87% and a predictive value positive of 55%. A cutoff probability of ≥0.40 yielded a sensitivity of 73% and a predictive value positive of 64%.

Conclusion

Accurate assessment of the epidemiology of postpartum infections has been hampered by the limitations of surveillance systems for identifying these infections, particularly infections detected after hospital discharge. In our study population, use of inpatient and ambulatory surveillance methods revealed that postpartum infections requiring medical attention were common following both vaginal delivery (5.5%) and cesarean section (7.4%). Mastitis and urinary tract infections accounted for >80% of these infections. The proportion of these infections directly attributable to healthcare practices cannot be determined from the information available. Our study also does not address whether these infections were associated with modifiable (and therefore potentially avoidable) risk factors, for example, suboptimal administration of perioperative prophylaxis during cesarean section or bladder catheterization.

Nearly all postpartum infections became manifest after hospital discharge (94%). Furthermore, most (74%) of these postdischarge infections were diagnosed and treated entirely in the ambulatory setting without the patients' returning to the hospital where they delivered for evaluation or treatment, emphasizing the need for postdischarge surveillance methods that are not dependent on hospital-based data.

Automated screening of ambulatory records was a sensitive method for identifying postpartum infections. Inpatient surveillance missed most infections that were diagnosed and treated in the ambulatory setting. Patient self-reported questionnaire results had limited sensitivity for detecting infections, explained in part by the large number of nonresponders. The questionnaire method was also more resource-intensive than automated ambulatory code screening. It is unclear whether questionnaire responders were representative of the entire postpartum patient population.

Although surveillance based on automated screening of ambulatory records depends on availability of ambulatory

diagnoses, tests, and pharmacy information, an increasing number of patients receive their health care through managed care organizations that routinely collect this information for administrative purposes. The specific diagnosis and test screening codes used for this study were based on a coding system unique to this HMO; however, similar information could be obtained by using an ICD-9-based outpatient claims database (International Classification of Diseases, 9th revision, clinical modification, 3rd edition). In principle, this method or a modification of it should be applicable for most of the U.S. population who have health insurance that includes pharmacy benefits. An additional limitation of this study is that the accuracy of the extrapolated infection rates depends upon the assumption that very few infections occur among postpartum women with none of the screening codes. This assumption is supported, however, by the finding that even among women identified as potentially infected through patient questionnaire results and inpatient surveillance, very few infections were confirmed through medical record review without at least one screening code.

In conclusion, our results indicate that postpartum infections requiring medical attention are common and that most postpartum infections occur after hospital discharge, so that use of routine inpatient surveillance methods alone will lead to underestimation of postpartum infection rates. Use of automated information routinely collected by HMOs and insurers allows efficient identification of women who are very likely to have postpartum infections that are not detected by conventional surveillance. Information resulting from more complete surveillance could be used to identify settings with unusually high or low infection rates to identify practices associated with lower infection rates. This information could then be used to focus, motivate, and assess the effectiveness of practice changes aimed at improving infection rates in all settings. Additional research is needed to evaluate the generalizability of this surveillance methodology to other health-care provider and insurer systems especially those that are entirely claims based, and to assess resource utilization associated with these infections.

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Dr. Yokoe is an associate physician and associate hospital epidemiologist at Brigham and Women's Hospital, hospital epidemiologist at the Dana-Farber Cancer Institute, and an assistant professor of medicine at Harvard Medical School. Her research interests focus on the epidemiology of nosocomial infections and use of automated data for surveillance.

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Molecular Identification of Streptomycin Monoresistant *Mycobacterium tuberculosis* Related to Multidrug-Resistant W Strain

Pablo Bifani,*† Barun Mathema,* Martha Campo,* Soraya Moghazeh,* Beth Nivin,‡ Elena Shashkina,* Jeffrey Driscoll,§ Sonal S. Munsiff,‡ Richard Frothingham,¶ and Barry N. Kreiswirth*

*Public Health Research Institute Tuberculosis Center, New York, NY, USA; †New York University School of Medicine, New York, New York, USA; ‡New York City Department of Health, New York, New York, USA; §New York State Department of Health, Albany, New York, USA; ¶Durham Veterans Affairs Medical Center, Durham, North Carolina

A distinct branch of the *Mycobacterium tuberculosis* W phylogenetic lineage (W14 group) has been identified and characterized by various genotyping techniques. The W14 group comprises three strain variants: W14, W23, and W26, which accounted for 26 clinical isolates from the New York City metropolitan area. The W14 group shares a unique IS*6110* hybridizing banding motif as well as distinct polymorphic GC-rich repetitive sequence and variable number tandem repeat patterns. All W14 group members have high levels of streptomycin resistance. When the streptomycin resistance *rps*L target gene was sequenced, all members of this strain family had an identical mutation in codon 43. Patients infected with the W14 group were primarily of non-Hispanic black origin (77%); all were US-born. Including HIV positivity, 84% of the patients had at least one known risk factor for tuberculosis.

With the advent of molecular techniques, tuberculosis (TB) investigators have a powerful tool to further the understanding of the transmission and phylogenetic properties of *Mycobacterium tuberculosis*. Molecular techniques have been used to discriminate exogenous versus endogenous disease (1-3), investigate suspected outbreaks (2,4-6) and cases of laboratory cross-contamination (7-9), study transmission within a defined geographic setting (10,11), and demonstrate the occurrence of exogenous superinfection in immunocompetent and immunocompromised patients (12,13).

Genotyping has facilitated identification and characterization of the W strain, a multidrug-resistant (MDR) clone associated primarily with nosocomial transmission in hospitals and detention facilities in New York City (NYC) in the early 1990s (14-17). In such studies, molecular markers were used to confirm and characterize the W strain outbreak and to elucidate a plausible evolutionary scenario for the sequential acquisition of multidrug resistance (14,17,18). In a recent study, when molecular techniques were applied in a population-based setting, genotyping identified a drug-susceptible group of isolates (W4) that represents a distinct branch of the W phylogenetic lineage. Members of this W4 group define a previously unidentified cluster of cases in a community in northern New Jersey; the cluster likely resulted from both historical and recent transmission (19). Although the W-MDR strain from NYC is clearly distinct from the drug-susceptible W4 group, both groups of isolates evolved from a common ancestor (20).

Strains that define the W family have several distinguishing genotypes in common: 1) they belong to principal genetic group 1 (17,21); 2) they have similar spoligotype patterns, characterized by a deletion of spacers 1-34 and the corresponding repeat in the direct repeats (DR) region (19, 22-24), an alteration that defines spoligopattern S00034 and closely related spoligotypes; 3) they contain a unique insertion in the origin of replication (19,20) and in the NTF locus (20,25). Strains grouped in the W or Beijing family are prevalent in China, Southeast Asia (26,27), Russia, and other former Soviet regions (unpub. data) and have recently been reported in South Africa (23).

By multiple genetic techniques, we investigated a cluster of NYC *M. tuberculosis* isolates (W14 group) that are resistant to at least streptomycin and share identical or closely related DNA fingerprint patterns. Results from multiple typing methods were used to show the relatedness of the three IS*6110* fingerprint patterns, W14, W23, and W26 and their evolution.

Materials and Methods

Mycobacterial Isolates and Patients

A total of 26 isolates from 26 patients were identified and grouped based on their IS*6110* fingerprint patterns. The IS*6110* fingerprint archive at the Public Health Research Institute Tuberculosis Center (PHRI TB Center) includes fingerprint patterns from >13,000 *M. tuberculosis* clinical

*Address for correspondence: Barry N. Kreiswirth, Public Health Research Institute Tuberculosis Center, New York, New York 10016; USA; fax: 212-578-0853; e-mail: barry@phri.org

specimens characterized since 1992. More than 80% of the isolates were cultured from NYC and New Jersey cases; remaining samples are from seven other states and international sources.

The W14 group isolates are part of a NYC convenience sample, which represents 44% (n=6,655) of the total number of culture- positive TB cases reported in 1992-1999 (~15,000). Isolates were collected for numerous outbreak, surveillance, and research studies (14,17,28-31). Basic clinical, demographic, and routine contact-tracing information was obtained for 22 of the 26 patients; data for 21 cases were obtained from the NYC Tuberculosis Control Program surveillance database; the one exception was from the New Jersey Department of Health and Senior Services. Isolates originated from 16 institutions, including NYC hospitals and correctional facilities. Laboratory cross-contamination was ruled out since none of the clinical specimens were cultured or processed during the same time period, and all patients had well-documented TB.

IS*6110* DNA Fingerprinting and Pattern Interpretation

M. tuberculosis isolates were cultured on Lowenstein-Jensen slants and grown at 37°C for 3 to 5 weeks. IS*6110* DNA fingerprint analyses were performed according to a standard method using both the 5' and 3' fragments of the IS*6110* genetic element (32). Hybridization patterns were compared on a Sun Sparc5 Workstation by using the BioImage Whole Band Analyzer software version 3.4 (Genomic Solutions, Ann Arbor, MI). The Jaccard matching method and an unweighted pair group method that used arithmetic averages and average linkage clustering identified related patterns, in accordance with the protocol of the National Tuberculosis Genotyping and Surveillance Network, Centers for Disease Control and Prevention (CDC). Nomenclature of the DNA fingerprint patterns was as follows: Isolates with identical banding patterns were assigned the same arbitrary letter code (e.g., W, J, AF). IS*6110* patterns that resembled but were not identical to one of these patterns were denoted by addition of a number (e.g., W14, W23).

Other Southern Blot Hybridization Probes: Polymorphic GC-Rich Repetitive Sequence and Direct Repeat

Chromosomal DNA was restricted with *Alu* I and hybridized with the polymorphic GC-rich repetitive sequence (PGRS) probe (GenBank accession number M95490) (33). Direct-repeat restriction fragment-length polymorphism (RFLP) primers DRa and DRb were used to generate a DR probe with H37Rv (strain ATCC 35177) as a template. The DR amplicon was used as a probe to re-hybridize both membranes (*Pvu* II and *Alu* I) previously generated for IS*6110* and PGRS genotyping, respectively (34).

Spacer Oligonucleotide Genotyping (Spoligotyping)

Spoligotyping was performed according to the protocol described by Kamerbeek et al. (34). The spoligotype of the 26 samples belonging to the W14 group was compared against a spoligotype database maintained by the Wadsworth Center, New York State Department of Health, comprising >2,500 clinical specimens.

Variable Number of Tandem Repeats

Tandem repeat loci ETR-A to ETR-E were amplified by polymerase chain reaction (PCR) and analyzed by gel electrophoresis to generate variable number of tandem-repeat (VNTR) allele profiles, as described by Frothingham and Meeker-O'Connell (35). Each digit of the allele profile represents the number of tandem-repeat copies at a particular locus. The patterns were compared against the PHRI TB Center VNTR database (~500 isolates) and profiles at the Durham Veterans Affairs Medical Centers database (745 isolates, 85 in the W phylogenetic lineage) (19).

Streptomycin/Isoniazid/Rifampin/Ethambutol and Ethionamide Susceptibility Testing

Primary drug resistance to streptomycin/isoniazid (INH)/rifampin/ethambutol (SIRE) was determined by using TB susceptibility Quad Plate I and II (Remel No. 3501). *M. tuberculosis* cultures were determined to be resistant to antimicrobial agents at concentrations $\geq$1 µg/mL for INH and rifampin, 7.5 µg/mL for ethambutol, and 10 µg/mL for streptomycin. The MIC for streptomycin was determined for representative samples according to standard methodology by using the agar diffusion assay (36). Isolates were subcultured onto 7H10/ADC medium (Difco, Detroit, MI) containing 2 to 500 µg/mL of streptomycin. The NYC Bureau of Laboratories performed pyrazinamide (PZA) testing (37).

DNA Sequencing Streptomycin (*rps*L) and INH (kat G) Resistance Genes

Analyses of the *rpsL* and *katG* genes were performed on a MicroGene Clipper 2 Dye Automated DNA Sequencer (Visible Genetics, Toronto, Ontario, Canada). Sequencing in the 5' and 3' directions was carried out simultaneously by using a two-dye system (Cy 5 and Cy 5.5; two-dye filter subsystem) to confirm mutations. The primers used to generate amplicons for sequencing were provided by Visible Genetics.

W-Strain Family Genotype

The principal genetic group was determined for each isolate as described (21). In addition, specific IS*6110* insertion site mapping probes were used to determine the presence of insertions in the origin of replication and the NTF chromosomal region (20,25). The region flanking the deletion in the DR locus commonly associated with the W family was analyzed by PCR amplification and comparative hybridization (23).

Results

The W Family Strain Collection

The W14 fingerprint pattern was identified in a database of >13,000 clinical specimens representing approximately 9,000 patients and 3,563 distinct IS*6110* fingerprint patterns genotyped at the PHRI TB Center. From the total IS*6110* fingerprint patterns archived (N=3,563), 357 similar yet distinct patterns representing 1,498 isolates were grouped into the W family database by using standard matching criteria. Twenty isolates had an identical hybridization pattern (W14); 6 other isolates shared closely related IS*6110* fingerprint patterns (3 W23 and 3 W26). These 26 isolates with three IS*6110* patterns (W14, W23, and W26)

form the W14 group, a subgroup within the W phylogenetic lineage.

Molecular Features of W14 Group Isolates

The W14 group was originally defined on the basis of IS*6110* patterns. The group includes three closely related fingerprints (W14, W23, and W26), with two characteristic common motifs (denoted by brackets in Figure 1A). Motifs A and B are unique to the W14 group. Southern blot hybridization of the *Pvu* II membrane with the 5'-IS*6110* probe confirmed that the difference in IS*6110* pattern between W23 and W26 was the product of two independent IS*6110* insertions, rather than the outcome of a single RFLP event (Figure 1B).

PGRS probing showed a distinct hybridization pattern (P00026) for all 26 isolates in this group (Figure 1C). The P00026 pattern was used to segregate the W14 group isolates from all other clinical samples, including other W family groups in a PGRS database of >600 isolates.

Results from five chromosomal loci (ETR-A to ETR-E) were combined as a VNTR allele profile. All isolates in the W14 group had the VNTR allele profile 42445. This allele profile was unique to the W14 group when compared with the isolates from the PHRI TB Center VNTR database (n=>500) and an independent collection archived in Durham VA Medical Center database (n=745). Together, these databases include 85 isolates of the W family, including isolates from Asia, Africa, the former Soviet Union, and the United States. However, the allele profile 42445 was closely related to other patterns found in members of the W family. The most common profile for the W family is 42435; all profiles identified to date within the W family differed from this profile by a single allele. These VNTR profiles include 32435, 42436, 42437, 4253, and the W14 group, which is defined by 42445 (19). Unlike other members of the W phylogenetic lineage, the W14 group has a unique spoligotype arbitrarily designated as S00069 (Figure 2). Spoligopattern S00069 differed from S00034 associated with other reported W family members by the absence of spacer number 40. Patterns obtained by rehybridizing the same membranes used for IS*6110* and PGRS fingerprinting with the DRab probe suggested that the disruption of spacer 40 was not due to an IS*6110* insertion (Figure 3). An IS*6110* insertion within spacer 40 would have generated two DR hybridizing bands, including one of a higher molecular weight, as found in strain CDC1551 (Figure 3) (38). Pattern S00069 was unique to the W14 group when compared with the Wadsworth spoligotype database of >2,500 isolates. The disruption of spacer 40 did not affect either chromosomal flanking region. Hence, in agreement with the S00034 spoligotype that groups all W family isolates analyzed, the region upstream from spacer 35 was deleted in isolates with S00069.

All members of the W14 group have high-level resistance (MIC>500 µg/mL) to streptomycin (STR^R). In addition, one W14 and one W23 isolate were resistant to INH (INH^R), one W14 was INH^R and ethambutol (EMB^R) resistant, two W14 isolates were ethionamide (ETH^R) resistant, and one W14 was ETH^R and pyrazinamide (PZA^R) resistant (Figure 4). Except for the 26 isolates in the W14 group, no W family isolate from a U.S. case in our collection was streptomycin monoresistant.

DNA sequencing of the gene commonly associated with streptomycin resistance, the *rpsL* gene, identified a single mutation (codon 43: AAG→AGG; Lys→Arg) in all the 26 W14 group isolates in this study. Three isolates were resis-

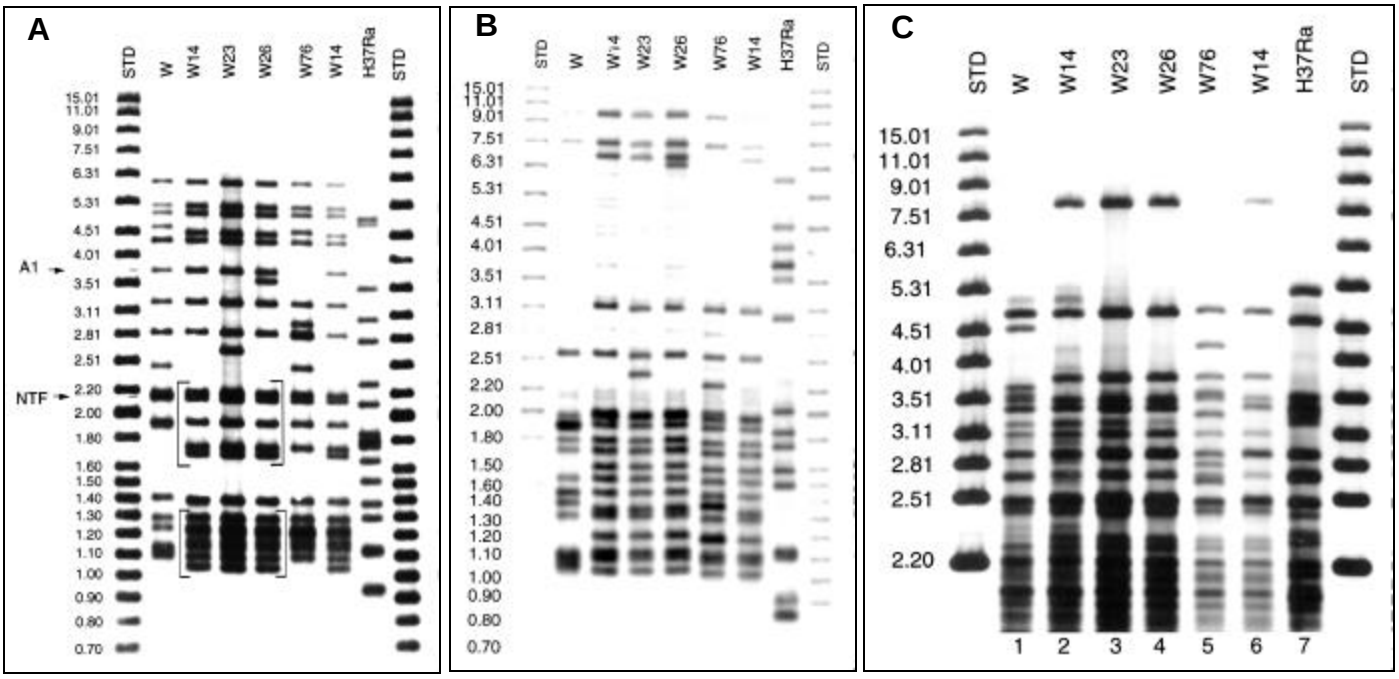

Figure 1. Southern blot hybridization of *Mycobacterium tuberculosis* isolates. A) IS*6110*-3' was used as a hybridization probe. The bracketed pattern motives regions are characteristic of the W14 family. A1 and NTF denote the bands corresponding to the IS*6110* insertions in the *dnaA-dnaN* region and the NTF locus, respectively. Lanes 1 and 9 are standard markers; lane 2: W-MDR from New York City (W index strain); lane 3, 4, 5 and 7: members of the W14 family; lane 6: W76 and lane 8: laboratory control strain H37Ra. B) Southern blot hybridization with IS*6110*-5' probe. W23 and W26 each have one additional band from W14, when hybridized with either the 3' or 5' IS*6110* probe. C) The polymorphic GC-rich repetitive sequence was used as a probe. The W14 group (W14; W23; W26) has a distinctive pattern when compared to all other isolates typed by polymorphic GC-rich repetitive sequence probe.

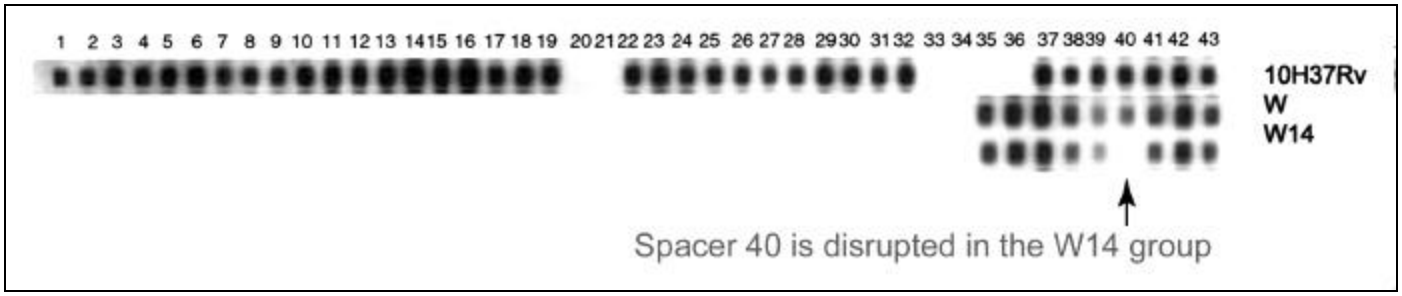

Figure 2. Spacer oligonucleotide typing (spoligotyping) of *Mycobacterium tuberculosis.* Positive hybridization with the 43 different spacer probes is denoted by black squares. W14 group has a distinctive disruption in spacer 40. Row 1: H37Ra, spoligotype S00001; 2: W-MDR, New York City isolate, spoligotype S00034; and 3: W14, spoligotype S000069.

tant to INH. Sequence analysis of the *katG* gene in these isolates revealed that the one INH^R-W23 isolate had a genetic alteration at codon 315 (AGC⟶ACC; Ser⟶Thr), while the two INH^R-W14 isolates had the same single nucleotide insertion generating a frameshift mutation at codon 283 (CTG⟶*A*TG), which results in a termination upstream at codon 310, previously unreported (Figure 4).

All 26 isolates in the W14 group were linked to the W family based on all the secondary typing methods used. All had *kat*G codon 463 sequence (CTG; Leu) and *gyrA* codon 95 sequence (ACC; Thr), placing them in genetic group 1 in the broad evolutionary framework outlined by Sreevatsan et al. (20,21). IS*6110* insertion site mapping showed that all 26 isolates had the A1 insertion in the origin of replication and a single IS*6110* copy in the NTF chromosomal locus (20,25).

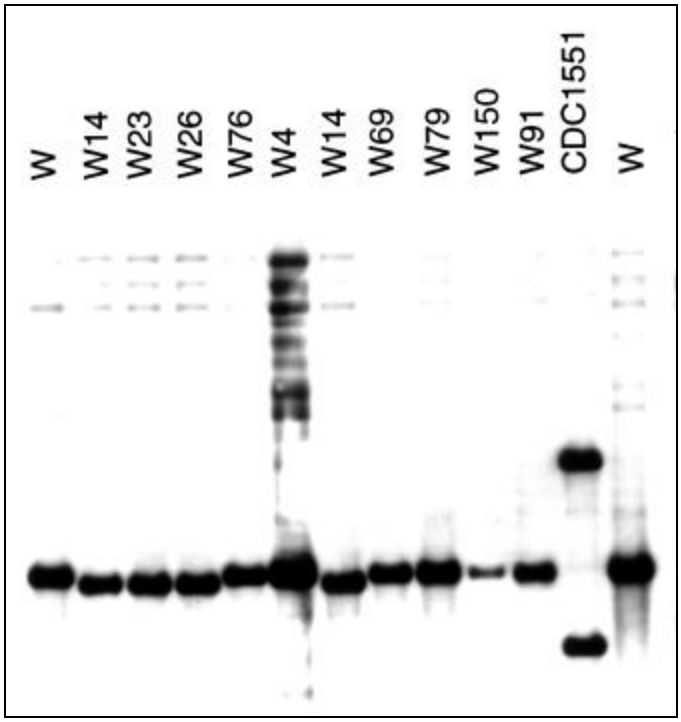

Figure 3. Southern blot hybridization with the DRab probe. The DRab probe was used to hybridize against the *Pvu*II-digested chromosomal DNA previously used for IS*6110* fingerprinting. Members of the W14 group had a single hybridizing band of a slight lower molecular weight than that of other W variants. In contrast, strain CDC1551, which is known to have an insertion within the direct-repeat locus, yielded two hybridizing bands, including one of higher molecular weight. Both hybridizing bands in strain CDC1551 can be superimposed with the IS*6110* patterns of this isolate.

Demographic Features of the W14 Cases

For the 22 patients in the W14 group, the mean age was 40 (range: 1 to 83 yrs) (Table). All were US-born, and 17 (77%) were of non-Hispanic black origin. In patients for whom HIV serology data were available, 74% (14 of 19) were HIV seropositive. Including HIV serology, 84% (16 of 19) had at least one of the known risk factors for TB (i.e., HIV, history of incarceration, homelessness, alcohol abuse, or intravenous drug use). A review of all drug-treatment regimen records of the W14 cases showed that only one patient had streptomycin as part of TB chemotherapy.

Investigation of 18 cases from NYC identified 107 contacts (mean of 6 contacts per case). Among contacts, 24% were PPD (purified protein derivative) positive. Investigations conducted by the NYC TB Control Program did not link any patients in this cohort or identify any new contacts with active disease.

Discussion

In this study the combination of multiple and independent molecular typing techniques permitted the identification and sub-grouping of a distinct branch of the W phylogenetic lineage, the W14 group. Twenty isolates with an identical IS*6110* pattern (W14) and an additional six (three W23 and three W26) isolates with closely related IS*6110* fingerprint patterns were identified in a fingerprint archive of >13,000 isolates. The W14 group was first typed to the "W" family fingerprint database of 1,498 isolates and then later subgrouped as a distinct branch of the W family. Five independent molecular techniques were used to confirm the distinctiveness of W14 isolates. The IS*6110* fingerprint pattern had two distinct motifs, unique within the PHRI database (Figure 1A). In addition, VNTR, PGRS, and spoligotyping patterns distinguished the W14 group from all other isolates analyzed by these methods in our collection, which includes >1,000 VNTR results, 600 PGRS images, and >2,500 spoligotype samples (Figure 1-3).

All isolates in the W14 group were resistant to high levels of streptomycin (>500 µg/mL), 20 were mono-streptomycin resistant, and the remaining 6 isolates were poly-resistant. Sequencing analysis confirmed that all isolates shared the same mutation in the *rps*L gene in codon 43, a mutation previously associated with high levels of streptomycin resistance (MIC>500; (39). The genetic alteration that directs the change of lysine to threonine in codon 43 of the *rps*L gene is one of the two most frequently reported mutations associated with streptomycin resistance: Together, they account for approximately 53% of streptomycin-resistant

Table. Demographic characteristics of patients in W14 group[a]

Characteristics N=22 (%)	
Gender	
Male	13 (59)
Female	9 (49)
Race	
Non-Hispanic black	17 (77)
Hispanic white	4 (18)
Asian	1 (5)
Age at diagnosis	
Median age (range)	38 (1-83)
25-50 years	16 (73)
HIV serology	
Seropositive	14 (64)
Seronegative	5 (23)
Unknown	3 (13)
Country of birth	
US born	22 (100)
Foreign born	0 (0)
Risk factors	
IVDU[b]	7 (32)
Alcoholic	9 (41)
Incarceration	2 (9)
Homelessness	2 (9)
Year of case report	
1992	2 (9)
1993	7 (32)
1994	4 (18)
1995	1 (4)
1996	3 (14)
1997	3 (14)
1998	0 (0)
1999	2 (9)

[a]All data are presented as number (percentage) except median age.
[b]IVDU = intravenous drug use.

cases (40). Hence, this observation alone would not be sufficient to determine clonal relatedness. However, in combination with the molecular grouping, the data strongly suggest that these strains are clonal and are the progeny of a single streptomycin-resistant predecessor.

Treatment records showed that only one patient received two months of streptomycin in combination with INH and rifampin. This regimen was changed when susceptibility data were available. The absence of streptomycin exposure in the patients with the W14 group of isolates further supports the thesis that each of these patients was infected with a streptomycin-resistant isolate rather than acquiring resistance while on therapy.

There were 295 streptomycin mono-resistant isolates from 295 patients reported in NYC during 1993 and 1995 to 1999; 49% of these were from foreign-born patients. Of the 150 U.S.-born patients with mono-streptomycin resistant TB reported in NYC during the same period, 83 isolates were genotyped (55%); 31% (n=26) belonged to the W14 group. Genotyping data of 187 mono-streptomycin-resistant clinical isolates, including the 83 from NYC, identified the W14 group as the only epidemiologically important cluster of patients. Six additional, unrelated clusters of two cases each were identified in the PHRI fingerprint database. From the PHRI archive, no streptomycin-susceptible isolates with the W14 molecular characteristics have been identified to date from NYC or other locations (samples fingerprinted: n= >12,000; NYC: n= >6,000). Taken together, the molecular data point to the acquisition of streptomycin resistance before dissemination of this strain into the community.

Several strains have continued to acquire additional secondary drug resistances (Figure 4), including INH, ETH, PZA, and EMB (W14: 1 STR^R INH^R, 1 STR^R INH^R EMB^R, 2 STR^R ETH^R, 1 STR^R PZA^R ETH^R; W23: 1 STR^R INH^R). None of the isolates examined acquired resistance to rifampin. In a previous study, we elucidated a plausible pathway for the evolution of the W-MDR strain in NYC (W-index strain) in the early 1990s (17). In that study, the emergence and spread of the W-MDR strain in NYC was believed to have started by acquisition of streptomycin resistance, followed by INH and rifampin resistance, but the outbreak did not occur until the MDR-genotype was developed. In contrast, the W14 group of isolates spread after streptomycin resistance was acquired; subsequently, additional resistance developed, creating a group of poly-resistant variants. Sequencing data in combination with IS*6110* Southern blot hybridization were used to demonstrate that INH resistance had developed on two occasions independently, once in W14 and once in a W23 (Figure 4) isolate. The W23 *katG* substitution on codon 315 is found on the same codon as in the W-MDR index strain but involves a different nucleotide. DNA sequence analysis of different resistance target genes provides molecular markers to speculate the stepwise building of polyresistant strains.

Since the late 1980s, molecular methods have been gradually integrated into the study of TB epidemiology and control. In addition to augmenting conventional epidemiologic investigations, molecular typing has been used to identify previously unrecognized point-source cases and in two reports was used to confirm transmission in a social setting (41,42). More recently, molecular typing with surveillance data uncovered an epidemiologically significant strain cluster (n=43), the W4 group, from New Jersey, that has no apparent common point source or patient links (19). The concordance of demographic and geographic data with molecular methods and the lack of concrete links in the W4 cases suggest a combination of reactivation and recent transmission. Likewise in this study, despite the demographic, geographic and molecular grouping, we could not establish any patient linkage in the W14 group. Nonetheless, the extent and validity of the molecular data demonstrated that these 22 patients were infected with either the same strain (16 W14 cases) or one of two closely related isolates (3 W23 or 3 W26).

This study has a number of limitations. PHRI's collection from NYC reflects a convenience sample that is approximately 44% of the total number of culture-positive TB cases reported to the NYC TB Control Program in 1992 to 1999. Of the W14 group patients, all were U.S.-born, >70% were HIV positive, and most (73%) were 25 to 50 years of age at diagnosis. Despite the demographic homogeneity in these

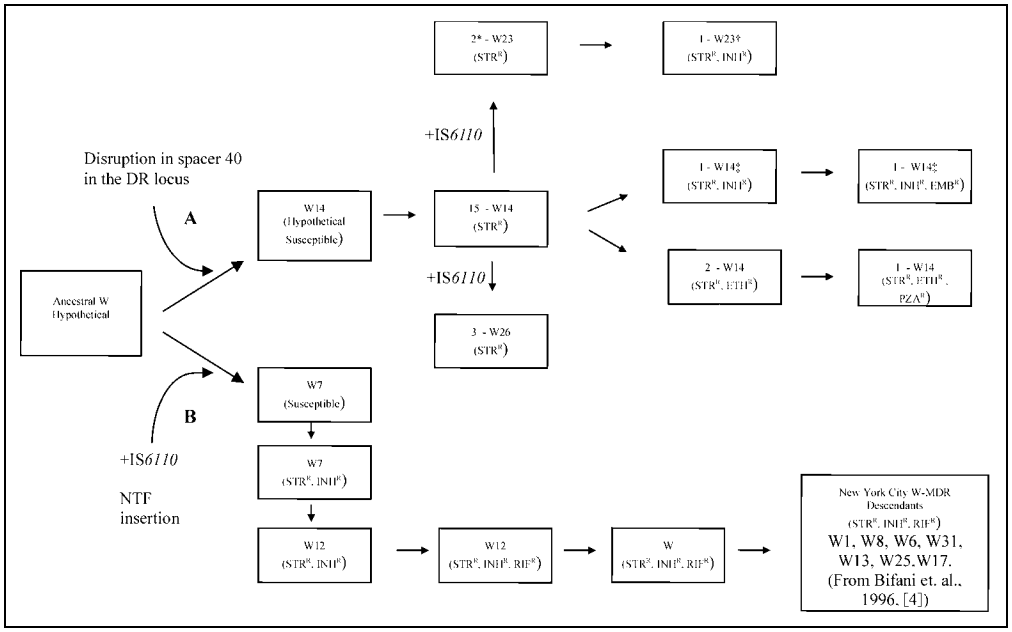

Figure 4. Schematic diagram on a plausible evolutionary pathway of the W14 *Mycobacterium tuberculosis* group, compared with the New York City W-MDR (4). All members of the W14 group lineage have single mutation on codon 43 of the *rps*L target gene associated with high levels of streptomycin resistance (STR^R). Note that isoniazid-resistant (INH^R) W23 and W14 branches have a distinct *katG* mutation. The W23 *katG* substitution on codon 315 is found on the same codon as in the W-MDR index strain but involves a different nucleotide; the W14 *katG* insertion on codon 283 has not been previously reported. ETH^R = ethionamide resistant; PZA^R = pyrazinamide resistant; EMB = ethambutol resistant; R= resistant.

patients, no epidemiologic links were established. Furthermore, the limited sampling suggests an actual denominator of W14 group cases larger than reported in this study, which aids in explaining the inability to establish epidemiologic links in the patients. Patient interviews were initially conducted as part of routine contact investigation; patients were not reinterviewed once molecular cluster information was available. Therefore, in-depth investigation of cases that appeared from molecular and surveillance data to be related was not available, which limits the inferences we can make on the observed lack of epidemiologic links.

Given the lack of overt epidemiologic links indicative of recent transmission between cases, the possibility that the W14 strain group is endemic to the region should be considered. However, the streptomycin monoresistance suggests that this strain must have spread after the introduction and application of this drug for TB treatment in 1944. It is surprising that not a single susceptible, related strain has been identified in the region even following extensive molecular analysis of other W variants. If the W14 or its predecessor were endemic, only the streptomycin-resistant variant has managed to spread, again suggesting that this dissemination depended on a single event, and yet, contact investigation has failed to establish a link. We speculate that the W14 cluster with the large young HIV- seropositive population is likely the result of recent transmission; however, the lack of patient-to-patient links suggests a subgroup of cases caused by an endemic strain that developed in NYC sometime after streptomycin was introduced in 1944.

By using multiple independent molecular markers, we made inferences on the strains' recent evolution. This study highlights the importance of tracking all types of drug-resistant strains to prevent the sequential development of multidrug-resistant strains. The utility that these methods lend to TB control will rely on the efficiency of integrating both surveillance and contact-tracing information with the molecular data in a proactive manner for more well-informed TB investigations.

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Dr. Bifani was a graduate student in the Public Health Research Institute Tuberculosis Center in New York City at the time this article was written. The center was established in January 1992 in response to the reemergence of TB in New York. He is currently at the Institut Pasteur de Lille France working as a postdoctoral fellow.

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Clonal Expansion of Sequence Type (ST-)5 and Emergence of ST-7 in Serogroup A Meningococci, Africa

Pierre Nicolas, Laurent Décousset, Vincent Riglet, Philippe Castelli, Richard Stor, and Guy Blanchet

Institut de Médecine Tropicale du Service de Santé des Armées, World Health Organization Collaborating Center, Marseille Armées, France

One hundred four serogroup A meningococci in our collection, isolated in Africa from 1988 to 1999, were characterized by multilocus sequence typing (MLST). Our results and data from the Internet indicate that sequence type 5 (ST-5) strains were responsible for most of African outbreaks and sporadic cases during this period. In 1995, a new clone, characterized by ST-7 sequence, emerged and was responsible for severe outbreaks in Chad (1998) and Sudan (1999). MLST and epidemiologic data indicate that ST-5 and ST-7 represent two virulent clones. These two STs, which belong to subgroup III, differ only in the *pgm* locus: allele *pgm3* is characteristic for ST-5 and allele *pgm19* for ST-7. Subgroup III strains were responsible for two pandemics in the 1960s and 1980s. Our data show that the third subgroup III pandemic has now reached Africa.

Multilocus enzyme electrophoresis (MLEE) has been the reference method for global epidemiology of *Neisseria meningitidis*. This method identified clusters of closely related strains (for example, subgroup III for serogroup A *N. meningitidis* and ET-5 complex for serogroup B *N. meningitidis*) and permitted monitoring of their clonal spread throughout the world (1-3). This method, however, relies on the indirect assignment of alleles based on the electrophoretic mobility of enzymes. However, indistinguishable variants may be encoded by very different sequences, and results obtained in different laboratories may be difficult to compare. Rather than comparing the electrophoretic mobilities of the enzymes they encode, Maiden et al. adapted this method by identifying alleles directly from the nucleotide sequences of internal fragments of housekeeping genes (4). This new method, called multilocus sequence typing (MLST), is based on the sequencing of DNA fragments belonging to seven housekeeping genes. MLST results are unambiguous and distinguish more alleles per locus, allowing high-level discriminations between isolates. The first data published by Maiden showed good congruence between MLST and MLEE (4).

The aim of our study was to check MLST for the characterization of 104 serogroup A *N. meningitidis* in our collection, isolated in 14 African countries from 1988 to 1999, to determine the feasibility of the technique and which sequence types were circulating in some African countries during this period.

Address for correspondence: Pierre Nicolas, Unité du Méningocoque, IMTSSA, WHO Collaborating Center, BP 46, le Pharo, 13998 Marseille Armées, France; fax: 33 4 91 59 44 77; e-mail: imtssa.meningo@free.fr

Materials and Methods

Bacterial Strains

A total of 104 serogroup A *N. meningitidis* strains isolated in 14 African countries from 1988 to 1999 and received at the World Health Organization Collaborating Centre in Marseilles were included in this study; 101 were isolated from cerebrospinal fluid (CSF), 1 from blood culture of a patient with meningococcal meningitis, and 2 from pharynx (Table). For some outbreaks, we randomly chose three strains for sequencing, if the pulsed-field gel electrophoresis (PFGE) fingerprint patterns were identical (Chad 1988, Central African Republic 1992, and Senegal 1998 outbreaks). Since 1999, all meningococcal strains have been routinely assayed by MLST.

All these strains were stored at -80°C in brain heart broth with 15% glycerol. The identification number for each strain was preceded by Mrs for Marseilles.

Bacterial Identification, Serogrouping, Typing, Subtyping

Bacterial identification was carried out by Gram staining, oxidase test, and tests for biochemical characteristics by using a ready-for-use kit (Neisseria 4H Sanofi Pasteur, Paris, France). *N. meningitidis* strains were serogrouped by agglutination with sera manufactured in Institut de Médecine Tropicale du Service de Santé des Armées (Marseilles). Serotypes and subtypes were determined by using the monoclonal kit from the National Institute of Public Health and the Environment (Bilthoven, the Netherlands) and the whole-cell enzyme immunoassay technique described elsewhere (5,6; Abdillahi, unpub. data).

Whole chromosomal DNA was compared by PFGE of macrorestriction fragments generated by endonuclease *Bgl* II (7). Agar plugs containing bacteria were treated by lysozyme, Proteinase K, and then Pefabloc (Roche, Meylan,

Table. Characteristics of 104 serogroup A meningococci isolated in Africa, from 1988 to 1999[a]

Yr.	Country	No. meningitis cases	No. of isolates	Serogroup	Type	Subtype	Allele no.							Sequence type (ST)
							abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	
1988	Chad	7,867	3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1991	Djibouti	31	1	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1992	Algeria	529	4	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Algeria		1	A	4	P1.10	1	3	1	1	1	1	3	ST-1
	Burundi	2,739	4	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	CAR	1,226	3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1993	Cameroon	5,372	3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Guinea-Bissau		3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1994	Burundi	42	1	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Cameroon	578	2	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Chad	948	1	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Zaire		3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1995	Algeria		1	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Cameroon		2	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Burk. Faso	2,595	1	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Burk. Faso		1	A	4	P1.9	2	1	2	1	3	2	3	ST-580
	Niger	43,203	4	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1996	Burk. Faso	42,129	2	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Cameroon	178	3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Chad	1,079	1	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Niger	16,145	3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1997	Burk.Faso	22,305	1	A	4	P1.9	2	1	2	1	3	2	3	ST-580
	Cameroon	572	2	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Chad	1,123	1	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Mali	11,228	2	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Niger	4,910	6	A	4	P1.9	1	1	2	1	3	2	3	ST-5
1998	Cameroon	2,887	1	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Chad	7,964	2	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Côte d'Ivoire	3	2	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Niger	2,328	4	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Senegal	2,709	3	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Zaire	1,991	2	A	4	P1.9	1	1	2	1	3	2	19	ST-7

Table. Characteristics of 104 serogroup A meningococci isolated in Africa, from 1988 to 1999[a] *(continued)*

Yr.	Country	No. meningitis cases	No. of isolates	Serogroup	Type	Subtype	Allele no. *abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	Sequence type (ST)
1999	Cameroon	2,272	2	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Chad	2,540	2	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Guinea-Bissau	2,836	2	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Niger	5,576	7	A	4	P1.9	1	1	2	1	3	2	19	ST-7
	Senegal	4,939	6	A	4	P1.9	1	1	2	1	3	2	3	ST-5
	Senegal		1	A	4	P1.9	1	64	2	1	3	2	3	ST-581
	Sudan	33,313	11	A	4	P1.9	1	1	2	1	3	2	19	ST-7

[a] Multilocus sequence typing showed the clonal expansion of ST-5 and emergence of ST-7, which was identified for the first time in Algeria in 1995; in Cameroon and Chad in 1997, 1998, and 1999; and in Zaire, Niger, and Sudan in 1999. Allele numbers and reference strains are registered at the MLST website (http://www.mlst.net). CAR=Central African Republic.

France). Plugs were incubated with 25 U of the endonuclease *Bgl* II (Eurogentec, Seraing, Belgium) overnight at 37°C. Electrophoresis was performed with a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA) in 0.5x Tris-borate-EDTA at 14°C, and a voltage of 4.5 V/cm was applied with a pulse-time ramping from 30 seconds to 1 second over 22 hr. Then a pulse of 0.1 to 1 second was applied for 2 hours and 30 minutes with a voltage of 6 V/cm. Fingerprint patterns were analyzed by using Tenover criteria (8).

For MLST, the primers of the housekeeping genes *abc*Z (putative ABC transporter), *adk* (adenylate kinase), *aro*E (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *pdh*C (pyruvate dehydrogenase subunit), and *pgm* (phosphoglucomutase) were synthesized by our institute according to the sequences published by Maiden et al. (4). A seventh locus, *fum*C (fumarase), was added. Primers for amplification and sequencing of *fum*C fragment were synthesized from the sequences given on the MLST web site, http://www.mlst.net. After DNA preparation and amplification by polymerase chain reaction (PCR), each locus sequence was analyzed on an ABI prism 310 Genetic Analyzer (Perkin-Elmer [PE] Applied Biosystems, Foster City, CA) or ABI prism 373 DNA Sequencer (PE Applied Biosystems). The sequence alignment was performed on the Sequence Navigator software (PE Applied Biosystems). The sequences were then compared with the different existing alleles registered on the MLST web site.

Results

Bacterial Identification, Grouping, Typing, Subtyping

All 104 strains were gram-negative diplococci, oxidase positive, and catalase positive. They were classified as *N. meningitidis* on the basis of growth characteristics on selective medium, acidification of glucose and maltose, and gamma-glutamyl transferase activity (9). One hundred three strains were serogroup A, type 4, and subtype P1.9 (A:4:P1.9), the same formula as strains belonging to subgroup III. One strain isolated in Algeria in 1992 was A:4:P1.10.

DNA fingerprint patterns generated with endonuclease *Bgl* II and analyzed by PFGE showed 103 closely related fingerprint patterns. Two profiles could be identified. ST-5 was the first pattern found in Africa and the most frequently isolated from 1988 to 1996 (7). The second profile was the ST-7 pattern, attributable to strains isolated more recently in Algeria, Cameroon, Sudan, Chad, and Niger. The two patterns are closely related but have four band differences (Figure). Most strains analyzed by PFGE were indistinguishable from ST-5 or ST-7. However, one strain (Mrs 99032) isolated in Dakar (1999) from CSF, showed two band differences with the outbreak pattern; and two strains (Mrs 95042, Mrs 97060), isolated in Burkina Faso in 1995 and 1997, showed one band difference with the ST-5 pattern. Strains with the ST-7 profile had almost the same patterns, but one strain (Mrs 98118), isolated in Zaire in 1998, showed one band difference. One serogroup A:4:P1.10 meningococcus (Mrs 92060), isolated in 1992 in Algeria, had a totally different profile; this strain was not related to the other 103 strains. All strains isolated from outbreaks in Chad (1988), Central African Republic (1992), and Senegal (1998) had identical PFGE fingerprint patterns; we sequenced three strains randomly chosen from these isolates (data not shown).

The MLST comparison between sequences and existing alleles allowed us to assign our sequences allele numbers (Table). On the MLST web site, the allele combination assigned 100 of 104 strains to ST-5 or ST-7 because they were identical to the consensus at seven loci. One strain (Mrs 92060), isolated in Algeria in 1992, was assigned to ST-1. Three strains had new STs: two strains (Mrs 95042, Mrs 97060), isolated in Burkina Faso in 1995 and 1997, were *abcZ2* and assigned to ST-580, and one strain (Mrs 99066), isolated during the 1999 Senegal outbreak, was *adk64* and assigned to ST-581.

Discussion

MLST technique was established in our laboratory at the beginning of 1999. Since 2000, all meningococcal strains we have received have been routinely characterized by this method. The seven loci of the 104 serogroup A *N. meningitidis* included in this study were characterized by their sequences, and alleles were assigned directly at the MLST web site (http://www.mlst.net), resulting in identification of sequence types ST-1, ST-5, or ST-7 (Table). An e-mail with sequence trace files was sent to the MLST web site to obtain alleles and sequence types of three strains, subsequently

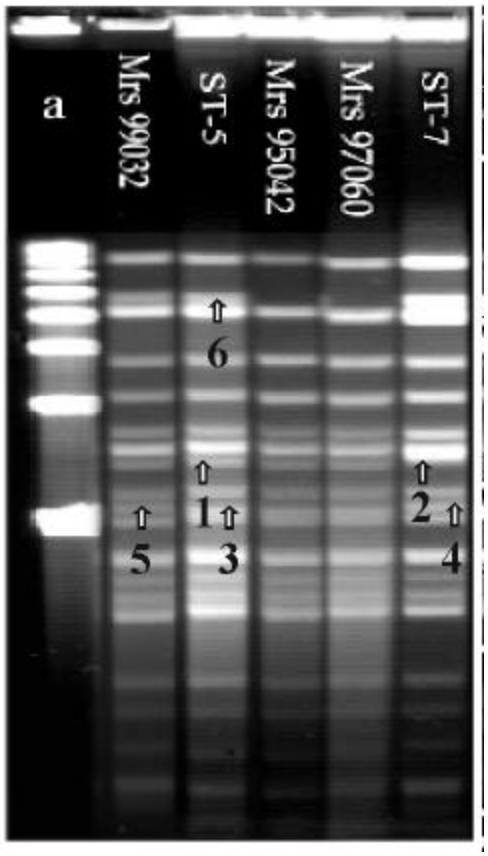

Figure. Pulsed-field gel electrophoresis analysis of chromosomal DNA (ethidium bromide staining) of 104 strains isolated in Africa. DNA macrorestriction fragments were generated with *Bgl*II; 103 out of 104 strains showed closely related profiles. ST-5 pattern was the first pattern found in Africa and the most frequently isolated from 1988 to 1996. The second pattern was the ST-7 pattern, attributable to strains isolated more recently in Algeria, Cameroon, Sudan, Chad, and Niger. ST-7 pattern is closely related to ST-5 pattern but shows four band differences (arrows 1,2,3,4). Mrs 99032, isolated in Dakar in 1999 and belonging to ST-5, showed two band difference with ST-5 pattern (arrows 3,5). Mrs 99066, isolated in Dakar (1999) and belonging to ST 581, showed indistinguishable fingerprint with ST5 pattern (not shown). Strains Mrs 95042 and Mrs 97060, isolated in Burkina Faso in 1995 and in 1997, respectively, and belonging to ST-580, showed one band difference with ST-5 pattern (arrow 6). Lane a, pulsed-field gel electrophoresis marker I (Boehringer Mannheim Biochemicals, Indianapolis, IN).

classified as ST-580 or ST-581 (Table). Except for ST-1, identified in strain Mrs 92061, ST-7, ST-580 and ST-581 differ from ST-5 in only one locus, and these four related STs belong to subgroup III. One hundred of 104 strains were either ST-5 or ST-7. These two STs are closely related, differing only in *pgm* locus: *pgm3* is characteristic for ST-5 and *pgm19* for ST-7. These two alleles differ in sequence of 47 of their base pairs, most likely because of recombination, frequently seen in *N. meningitidis* (10,11). The surface epitopes of strains analyzed in this study (e.g, serogroup, serotype, and serosubtype) are identical in the strains of these two STs. However, a four-band difference was observed in the PFGE patterns, even though there is no *Bgl*II restriction site in either locus *pgm*3 or locus *pgm*19.

MLST and epidemiologic data indicate that strains of ST-5 and ST-7 represent two virulent clones (Table). To date, ST-5 strains have been isolated from several outbreaks: Chad (1988), Central African Republic and Burundi (1992), Cameroon (1993), Niger (1995, 1996), Burkina Faso (1996), Mali (1997), Senegal (1998 and 1999), and Guinea-Bissau (1999) (Table). ST-5 strains were also isolated from a carrier returning from Saudi Arabia in 1987 (Centers for Disease Control and Prevention, unpub. data), Gambia in 1997 (Greenwood, unpub. data), and Ghana in 1997 and 1998 (Popovic, Pluschke, unpub. data). In 1995, when ST-5 strains were widespread, a new clone, characterized by ST-7, emerged. The oldest ST-7 in our collection is from Algeria (1995). ST-7 strains were isolated throughout the "meningitis belt" (12): Chad (1997, 1998, and 1999), Cameroon (1997, 1998, and 1999), Zaire (1998), Niger (1999), and Sudan (1999) (Table).

During the 11-year period 1988-1999, the ST-5 epidemic wave reached all African countries in the meningitis belt. In 1995, the ST-7 clone appeared in Algeria. The origin and emergence of the ST-7 clone might be explained by recombination events' conferring selective advantages to ST-7. Another possibility is that the homogenizing effect of a sequential bottleneck might have selected at random a ST-7 clone among a limited number of different genotypes, resulting in a population uniform for the new variant (13,14). Since their appearance in 1997, only strains of ST-7 have been identified in Chad and Cameroon. Similarly, the 1999 Sudan outbreak was due to an ST-7 clone. Therefore, it appears that ST-7 is a clonal replacement for ST-5 in African countries. Prospective monitoring and analysis of the isolates by MLST will be crucial for assessing the full significance of our observations.

Serogroup A strains of subgroup III were associated with the first pandemic that started in China in the mid-1960s and subsequently spread to Russia, Scandinavia, and Brazil. In the early 1980s, a second wave of meningococcal disease caused by subgroup III clones began in China, spread through Nepal and probably India, and then reached Saudi Arabia in 1987 (2,3,15, 16). Given that this particular clonal group had not been isolated in Africa before 1987, we speculate that subgroup III, and more precisely the ST-5 clone, was introduced into Africa in 1987 by pilgrims returning from Mecca (17-19). By 1988, epidemics of meningococcal disease were recorded in Chad and Sudan, eventually reaching most African countries (20; MLST web site). In 1995 and 1997, the ST-7 clone emerged in Africa and appears to be responsible for a new wave of epidemics.

Achtman et al. showed that the third pandemic caused by subgroup III began in China in 1993, causing large epidemics in Mongolia in 1994 and Moscow in 1996 (21). The strain associated with this pandemic can be readily recognized by the presence of the *pgm19* allele. Results of our study suggest that this third pandemic has now reached Africa.

Implications of the ST-7 clone's replacing ST-5 can be substantial. Chad and probably Sudan experienced epidemics caused by ST-5 in 1988. Although ST-5 and ST-7 are closely related clones, herd immunity due to the presence of the ST-5 strains is now apparently surpassed since these two countries had severe outbreaks caused by the ST-7 clone in 1998 and 1999.

Justified concern is raised now that the new pandemic of subgroup III will spread to other countries of the meningitis belt. It is important to alert those countries, particularly Cameroon and Niger, where sporadic ST-7 strains are already present.

Among our 104 strains, 4 did not belong to ST-5 or ST-7 and were characterized by sequences ST-1, ST-580, and ST-581. The ST-1 strain, belonging to subgroup I, was isolated in Algeria in 1992. Subgroup I has been responsible for epidemics and sporadic cases in Africa since 1961. Although it has not been isolated for many years in the meningitis belt, it was still the predominant clone in South Africa in 1996 (2,3,22). In addition to three strains of ST-5 isolated in Burkina Faso in 1995 and 1996, two strains isolated in 1995 and 1997 were ST-580. This particular type is closely related to ST-5, differing only at the *abcZ* locus. Isolation of only a couple of strains of ST-580 substantially hampers speculation that ST-580 is a genetic variant that could potentially emerge. Finally, the third ST that differed from ST-5 was ST-581, which had a new *adk* allele (Table). A strain of this type was isolated from the CSF of a patient in Senegal in 1999. However, this new clone will probably not emerge as a virulent clone (i.e., to replace ST-5); indeed, it will probably be lost in the future.

Although MLST makes standardization and interlaboratory comparison easier, the technique is time-consuming and expensive. Some simplifications may be possible. For example, 11 strains isolated during the 1999 Sudan outbreak showed the same PFGE fingerprint patterns as well as identical ST. In this case, sequencing only one strain would have been sufficient. However, strain Mrs 99066 of ST-581, isolated in Dakar in 1999, had a PFGE pattern identical to that typically seen in the strains of the ST-5 pattern. With few such exceptions, *Bgl* II PFGE analysis resulted in easy differentiation of ST-5 and ST-7 strains. Their PFGE patterns remained unchanged over several years and in strains isolated in different countries. Although it is accepted that PFGE is not appropriate for long-term comparison purposes, it may discriminate ST-5 from ST-7 strains of *N. meningitidis* serogroup A. Also, in epidemiologic investigations, differentiation of *pgm3* from *pgm19* in serogroup A strains recently isolated in Africa may be useful for differentiating ST-5 and ST-7. That could be accomplished by sequencing or by RFLP-PCR of *pgm* locus. However, the disadvantage of this approach is that strains of ST-580 would be identified as belonging to ST-5.

Since we established MLST in our laboratory in 1999, it has allowed us to obtain reliable and portable data that could be easily compared between laboratories without having to exchange strains. MLST was initially developed for studies of meningococcal population genetics. In this study, MLST was used along with epidemiologic data to identify two virulent clones characterized by ST-5 and ST-7. Strains of the ST-5 were responsible for the second pandemic wave that started in Africa in 1988, and the appearance of ST-7 strains may likely be responsible for the third one. In our study, MLST has proven to be a reliable and useful tool for molecular typing of *N. meningitidis* serogroup A and could replace MLEE as the standard for molecular typing. MLST may aid in identifying and monitoring the global spread of virulent STs to allow rapid implementation of preventive measures.

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Dr. Nicolas is a physician, head of Unité du Méningocoque, World Health Organization (WHO) Collaborating Center in Marseille, France. The laboratory is a reference laboratory on meningococci for French Armed Forces, WHO, and African laboratories. Dr. Nicolas' research interests focus on population genetics and molecular epidemiology of *Neisseria meningitidis*.

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Multidrug-Resistant Tuberculosis in Prison Inmates, Azerbaijan

Gaby E. Pfyffer,* Anni Strässle,* Tamara van Gorkum,† Françoise Portaels,‡ Leen Rigouts,‡ Christine Mathieu,§ Fuad Mirzoyev,§ Hamidou Traore,‡ and Jan D.A. van Embden†

*University of Zurich, Zurich, Switzerland; †National Institute of Public Health and Environmental Protection, Research Laboratory for Infectious Diseases, Bilthoven, the Netherlands; ‡Institute of Tropical Medicine, Antwerp, Belgium; and §International Committee of the Red Cross, Geneva, Switzerland

In a tuberculosis (TB) program in the Central Penitentiary Hospital of Azerbaijan, we analyzed 65 isolates of *Mycobacterium tuberculosis* by IS*6110*-based restriction fragment-length polymorphism (RFLP) and spoligotyping. From 11 clusters associated with 33 patients, 31 isolates had an IS*6110*-based banding pattern characteristic of the Beijing genotype of *M. tuberculosis*. In addition, 15 *M. tuberculosis* isolates with similar RFLP patterns constituted a single group by spoligotyping, matching the Beijing genotype. Multidrug resistance, always involving isoniazid and rifampin, was seen in 34 (52.3%) of 65 isolates, with 28 belonging to the Beijing genotype.

Tuberculosis (TB) is an important health problem in prison settings (1), where factors such as poor general health of inmates, overcrowding, increased risk factors, delayed case finding, and incomplete or inadequate therapeutic intervention contribute to rapid spread (2) and high prevalence (e.g., 500 cases per 100,000 inmates in New York City jails [3] and 2,283 per 100,000 inmates in the General Penitentiary Hospital in Madrid, Spain [4]). In prisons of the former Soviet Union, TB incidence is even higher (e.g., in Siberia [5] or Russia as a whole [6,7]). TB incidence is also associated with length of incarceration (3). Transmission patterns are generally difficult to establish (8), and the rate of unrecognized transmission may be quite high (9). Above all, strains isolated in these settings are often multidrug resistant (MDR) (10,11).

According to the International Committee of the Red Cross (ICRC), the total number of inmates in the Azerbaijan prison system is approximately 25,000. With 4,667 TB cases per 100,000, the incidence in Azeri prisons is nearly 50 times higher than the country average, and the mortality rate may reach 24% (6). In June 1995, the ICRC implemented a TB control program in the Central Penitentiary Hospital in Baku, Azerbaijan, which was the only treatment center for Azeri prisoners with TB. The program was based on directly observed therapy, short course (DOTS), as recommended by the World Health Organization (WHO [12]) and the International Union Against Tuberculosis and Lung Disease (IUATLD [13]). After treatment failures were observed despite completion of strictly supervised therapy, susceptibility testing of inmates' *M. tuberculosis* isolates demonstrated a high rate of multidrug resistance (11).

Address for correspondence: Gaby E. Pfyffer, Swiss National Center for Mycobacteria, Department of Medical Microbiology, University of Zurich, Gloriastrasse 30, 8028 Zurich, Switzerland; fax: 411-634-4918; e-mail: pfyffer@immv.unizh.ch

Several outbreaks of MDR-TB have been reported worldwide from hospitals (affecting patients and health-care workers [14]), the community (15), and prisons (4). In these outbreaks, MDR-TB has predominantly affected immunocompromised patients, and disease was often caused by certain strains of *M. tuberculosis*, in particular the W strain (16-18). This strain and its many variants (e.g., subtypes W_1 [17] and U [19]), form a family lineage that shares a common genotype with the Beijing clone (20). At the molecular level, they exhibit a closely related, unique multiband IS*6110*-based restriction fragment-length polymorphism (RFLP) pattern and contain 9 of 43 polymorphic spacer sequences in the chromosomal DR locus by spoligotyping (21).

We report the genotypic characterization and spread of predominantly MDR *M. tuberculosis* strains in a prison in Azerbaijan. Along with IS*6110*-based RFLP, spoligotyping, and drug-susceptibility profiles, we have determined the type of mutation in the *rpoB* gene responsible for resistance to rifampin (RMP).

Methods

Patients

At the beginning of the ICRC program, 65 male patients 19 to 55 years of age were included in the study. All were from Azerbaijan, and their terms of imprisonment ranged from 6 months to 20 years. Once diagnosed as having smear-positive pulmonary TB, they were transferred to the Central Penitentiary Hospital in Baku, the referral hospital for the Azerbaijan prison system. No clinical histories were available for the time before the ICRC intervention. Personal characteristics have largely been provided by the prisoners themselves (Table). Patients were classified at admission on the basis of their self-recorded anti-TB drug intake as nonresponders (patients who were treated with single or multiple drugs for >1 month and continued to be smear-positive

Table. Characteristics of the cohort of prison inmates with tuberculosis, Azerbaijan

Strain no.	Age of patient (years)	Body mass index	Patient group[a]	Onset of symptoms	Previous treatment (before enrollment in ICRC program)[b]
7933	25	18.2	A	Aug 1996	R,E,S
7887	40	--	A	Jan 1995	H,R,E,S
7909	40	16.1	A	Jun 1995	S (4 mo. in 1995) S (1 mo. in 1996)
7920	22	16.3	A	Mar 1995	H(?),R(?),S
5695	22	17.2	B	Feb 1996	S
5698	43	--	A	1976	R,E
7101	20	--	B	Jan 1996	S
5691	26	--	A	Feb 1995	S
7915	21	16.2	B	Mar 1996	None
7922	33	16.1	A	Spring 1996	H,R,E,S
5689	24	19.7	A	1993	H,R,E,S (1 to 2 mo.)
7924	40	17.4	B	Apr 1996	H,R,E,S
7934	36	--[c]	A	Jul 1996	S
7908	32	16.1	B	Mar 1996	R,E,S
7930	21	16	B	Aug 1996	R,E,S
7935	23	17.3	B	Mar 1996	R
7925	22	19.3	A	Feb 1996	S (monotherapy initially); R,E,S (2 to 3 mo.)
7931	21	16	B	Jul 1996	R,S
7914	34	15.7	A	Nov 1995	R,E,S (not completed)
5713	25	15.6	B	Dec 1995	H,R,E,S
7910	32	17.5	A	Feb 1996	S,E (not completed)
7906	22	19.5	B	Aug 1996	R,S
5719	32	--	C	1991	H,R,E,S (9 mo. in 1991)
7923	34	13.8	B	Jan 1996	R,E,S
7899	33	15.8	A	Jan 1996	H (4 mo.), R (10 d), E (4 mo.), S,Z (4 mo.) (not completed)
5684	29	17.2	A	1993	H,R,E,S (in 1994) S (in 1995)
7900	?	20.2	A	Jul 1995	H,R,S (in 1995) S (in 1996)
5697	19	17.4	B	Mar 1996	none
5692	22	--	A	Feb 1995	H,R,E,S
7916	32	18.2	A	1994	H,R,E,S,K (several $\leq$3-mo.intervals of treatment)
7932	22	18.1	C	Nov 1993	H,R,E,S,K (several intervals of treatment: 1 mo., 4 mo., 12 mo.)
5700	34	18	B	Feb 1996	none
5702	26	18.1	A	Sep 1995	S
5706	23	--	B	Feb 1996	S
5716	24	19	B	Feb 1996	R,S
7888	30	--	A	Oct 1994	H,R,E,K (<2 mo. in 1994) H,R (in 1995)
5693	26	17.2	B	Jan 1996	S
5722	26	17.1	B	Dec 1995	H,R,E,S,K
7895	19	18.5	B	Jun 1996	H,R,S
5724	37	17.3	B	Dec 1995	H,R,E,S
7886	46[c]	--	A	Mar 1995	R,E,S (not completed)
7917	55	--	C	1987	R,E,S (11 mo.)

Table. (continued) Characteristics of the cohort of prison inmates with tuberculosis, Azerbaijan

Strain no.	Age of patient (years)	Body mass index	Patient group[a]	Onset of symptoms	Previous treatment (before enrollment in ICRC program)[b]
7933	25	18.2	A	Aug 1996	R,E,S
5718	20	17.6	B	Oct 1995	H,K
7889	?	--	A	?	?
7921	22	19.4	B	Jul 1996	S
7926	20	--[c]	B	Aug 1996	none
7918	33	16.4	B	Jul 1996	H,R,E,S
7927	32	18.4	A	Mar 1996	H,E,S (not completed)
7904	21	16.8	B	May 1996	H,S
7929	47	26.3	A	May 1996	R,E,S (not completed)
7894	28	17.3	B	Apr 1996	None
7897	20	13.8	A	Jan 1996	R,E,S (not completed)
7905	26	18.6	B	Apr 1996	S
7907	19	14	B	Dec 1995	H,S
7902	25	19.2	A	1995	H,S (6 mo.) R,E, (7 d)
5686	29	--	A	May 1995	H,R,E,S (not completed)
5694	29	17.3	A	Jan 1995	H,S (1 mo. in 1995) E,S (1 mo. in 1996)
5726	44	16.9	A	?	H,R,E,S (3 to 4 mo. in 1991) H,R,E,S (in 1995)
7911	34	17.9	B	Mar 1995	R,E,S,K
5688	37	--	A	Sep 1994	R,E,S
5687	41	20.3	B	Feb 1996	E,S
5729	40	16.3	C	1994	R,S(3 mo. in 1994) S (in 1996)
7890	19	--	A	1994	H,E,S (3 mo. in 1994) R,E,S (in 1996)
5699	22	--	B	Mar 1996	H,S

[a]Group A = nonresponders; Group B = new cases (never treated or treated for <1 month); Group C = relapsed cases
[b]H, isoniazid; R, rifampin; E, ethambutol; S, streptomycin; Z, pyrazinamide; K, kanamycin
[c]Patient too weak to be weighed

[group A; n = 30]), new cases (smear-positive patients who had never been treated or were treated for <1 month [group B; n = 31]), and relapsed cases (patients who had active TB and were treated and considered clinically cured but became smear-positive again [group C; n = 4]).

Microbiologic Diagnosis and Drug Susceptibility Testing

Sputum specimens were analyzed for the presence of acid-fast bacilli (Ziehl-Neelsen staining) by the local microbiology laboratory, which was supervised by the ICRC. Processing of specimens, culture, and identification of *M. tuberculosis* were done in Zurich or Antwerp according to standard procedures (22). Susceptibility testing of *M. tuberculosis* to primary drugs (isoniazid, RMP, ethambutol, and pyrazinamide) plus streptomycin was performed in liquid medium by the radiometric BACTEC 460 TB system (Becton Dickinson, Sparks, MD) (23) and confirmed by the agar proportion method (22).

Molecular Characteristics of RMP Resistance

All *M. tuberculosis* isolates were analyzed by the INNO-LiPA-Rif TB Assay (Innogenetics, Ghent, Belgium) (24). This reverse hybridization-based line probe assay carries one oligonucleotide probe for the detection of *M. tuberculosis* complex strains and nine probes to detect nucleotide changes in the relevant part of the *rpoB* gene.

Molecular Epidemiology

P*vu*II-digested DNA of *M. tuberculosis* was probed with the insertion element IS*6110* according to the standardized protocol of van Embden et al. (25), with some modifications (26). "Cluster strains" had 100% identical RFLP patterns.

Cluster Analysis

IS*6110*-based RFLP patterns were analyzed by GelCompar software (Windows 95, version 4.0; Applied Math, Kortrijk, Belgium) with an HP ScanJet 4c (Hewlett Packard, Greeley, CO; scanned at 190 dpi). The mobility of the IS*6110* fragments was compared with the molecular weight marker lambda-DNA *Eco*RI/*Hin*dIII (Roche Diagnostics, Rotkreuz, Switzerland), which was used as external size marker. In addition, the accuracy of the procedure was tested by comparing the IS*6110* banding pattern of *M. tuberculosis* isolate Mt14323, which was present on each autoradiograph. Patterns were compared by the UPGMA clustering method with the Dice coefficient (1.2%). Spoligotype patterns (27) generated from all 65 isolates were compared visually.

Results

Except for two patients in whom the first symptoms of TB had appeared 9 and 20 years previously, the patients had recent onset of TB disease (Table). Most prisoners were substantially undernourished (as indicated by low body mass indices) and in poor clinical condition, many with unilateral or bilateral pulmonary infiltrates and cavities. Most of the nonresponding patients (group A) had been treated inadequately before the ICRC intervention (Table).

Based on morphologic and biochemical characteristics, all 65 isolates were identified as classical *M. tuberculosis*. Hybridization of *Pvu*II-digested chromosomal DNA with the 245-bp fragment of IS*6110* resulted in variable patterns, although to a limited degree (Figure 1). Within a cluster (each cluster comprising a minimum of 2 and a maximum of 10 patients), isolates had identical IS*6110* patterns. Overall, we found 11 clusters containing a total of 33 isolates. Apart from cluster 1, which consisted of 2 isolates with 10 IS*6110* copies, the isolates belonging to clusters 2 to 11 were characterized by 15 to 18 IS*6110* fragments and similar banding patterns (similarities of >90% for clusters 2 to 9 and ≥80% for clusters 10 and 11 with the former group). In addition to the 31 isolates of clusters 2 to 11, 15 other isolates of *M. tuberculosis* displayed similar IS*6110* patterns, differing in the presence or absence of one or two bands or in the IS*6110* fragment length (Figure 1). These 15 isolates were highly related (≥68%) to those of clusters 2 to 11. DNA fingerprints of the 31 isolates in clusters 2 to 11 and the 15 closely associated isolates strongly resembled the Beijing genotype of *M. tuberculosis* (Figure 1, asterisk).

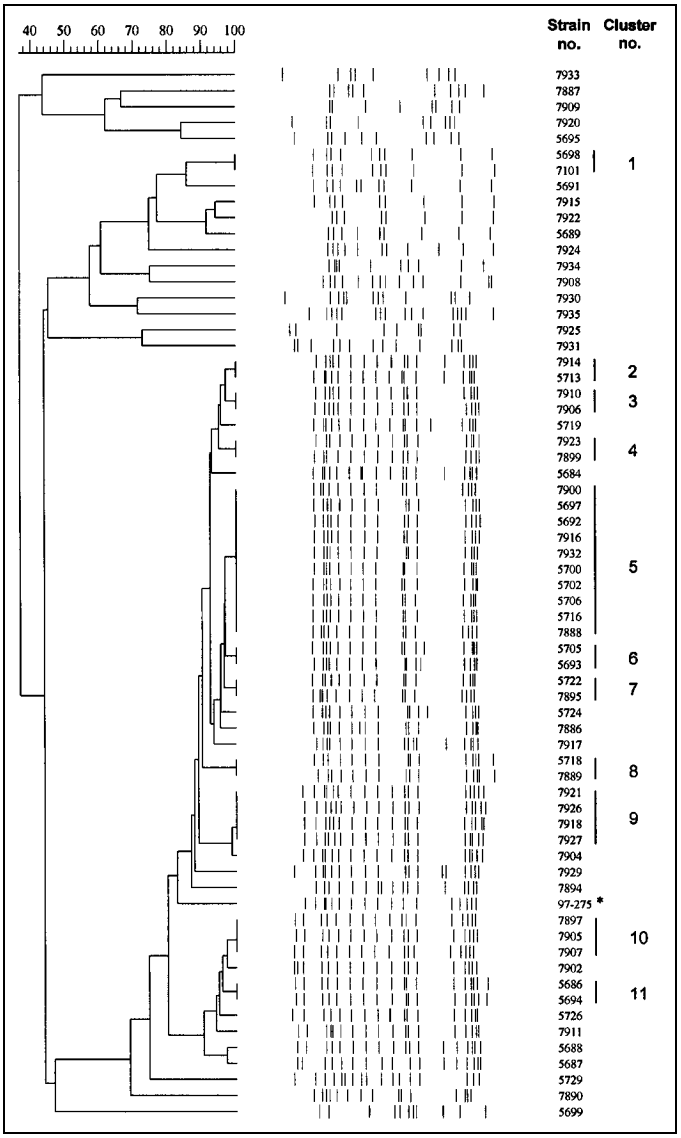

Figure 1. Computer-generated lane maps of genomic fingerprints of *Mycobacterium tuberculosis* strains isolated from the patient cohort (n = 65). *Pvu*II-digested chromosomal DNA was probed with IS*6110*. Clusters 1 to 11 are indicated by vertical lines at the right margin. Asterisk indicates strain 97-275 (Beijing clone, obtained from K. Kremer, RIVM/NL) for comparison. Strains 7914 through 5687 show the Beijing genotype restriction fragment-length polymorphism pattern. The molecular weight marker and internal reference strain of *M. tuberculosis* (Mt 14323) are not shown.

Spoligotyping grouped those 46 isolates into one genotype with identical spoligotypes that lacked all but 9 of the 43 DR spacers and thus resembled the Beijing family of *M. tuberculosis*. The remaining 19 isolates of the patient cohort resulted in different, mostly individual spoligotypes (Figure 2). While cluster 1 was confirmed by spoligotyping, other isolates, mostly with a high degree of similarity in their RFLP patterns, were also grouped together (isolates 7887/5695/7920; isolates 5691/5689; isolates 7922/7915; and isolates 7908/7935; Figure 2).

Regardless of Beijing or other types, the susceptibility patterns differed, ranging from fully susceptible to resistant to one or more drug(s). Overall, 52.3% of the strains isolated from the cohort (Figure 2) and 60.9% (28/46 isolates) in the group of Beijing type strains were MDR. While none of the RMP-susceptible strains carried a mutation in the relevant part of the *rpoB* gene, all strains resistant to RMP (by growth-based susceptibility testing) were correctly identified by the INNO-LiPA-Rif assay. For 22 of them, the type mutation was detected: D516V (R2; n = 1), H526Y (R4a; n = 2), H526D (R4b; n = 1), and S531L (R5; n = 18). S531L was predominant in the Beijing family (17/28 RMP^R isolates = 60.7%; Figure 2. MDR strains of *M. tuberculosis* were found in 19 of 30 patients in group A, 14 of 31 patients in group B, and 1 of 4 patients in group C. Full drug susceptibility was also seen in all groups of patients (3 of 30 in group A, 7 of 31 in group B, and 1 of 4 in group C).

Conclusion

Analyzing the TB patients in the Central Penitentiary Hospital in Baku was complicated by constraints and biases inherent in the prison environment. Clinical information on the prisoners was limited and mainly based on self-reported data. Conclusions based on analysis of 65 of the approximately 300 TB patients in that hospital are largely fragmentary and may not be truly representative. However, enrolling more patients into our pilot study was not considered, mainly because of frequent transfer of prisoners and high mortality rates. When the DOTS program was implemented by the ICRC, many of the TB patients were either untreated or had received inadequate drug regimens for years.

Although we analyzed a limited number of patients, our study demonstrates that most strains (70.8%) belonged to the Beijing family of *M. tuberculosis* and that multidrug resistance was a serious problem (52.3%) in this setting. The Beijing genotype, characterized by 15 to 18 IS*6110* copies and a specific spoligotype pattern, was identified in 10 of the 11 clusters (2 to 11) and included isolates of 31 patients, plus

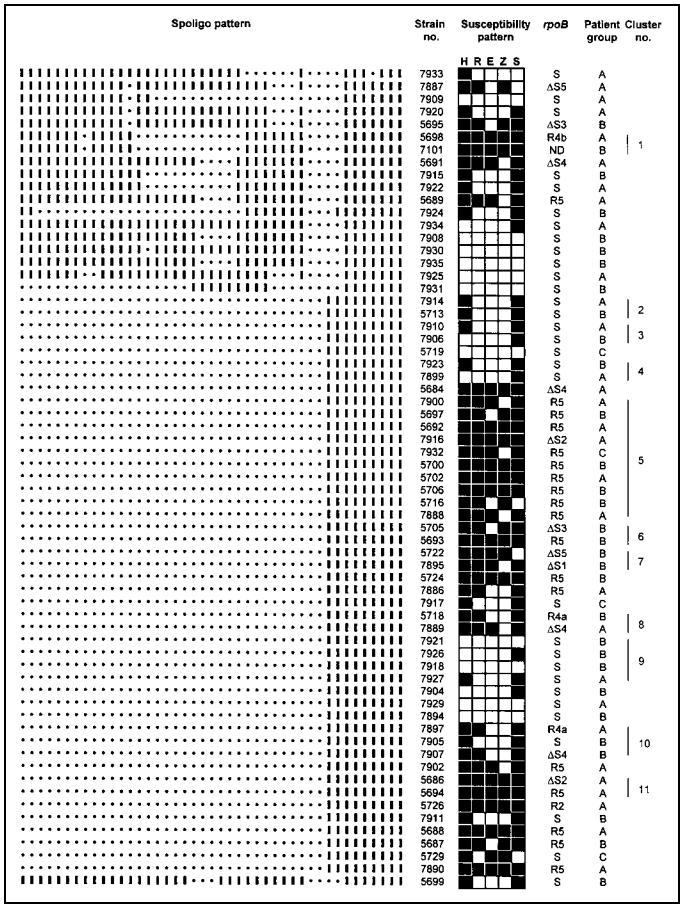

Figure 2. Spoligotypes of the 65 strains of *M. tuberculosis* analyzed, drug susceptibility patterns, and type of mutation on the *rpoB* gene conferring rifampin resistance (*rpoB*). H = isoniazid; R = rifampin; E = ethambutol; Z = pyrazinamide; S = streptomycin. Black fields indicate resistance; blank ones susceptibility. S = wild type (RMP^S); mutations (RMP^R): ΔS1; DS2; ΔS3; ΔS4; ΔS5; R2; R4a; R4b; R5; ND = not done. Group A = nonresponders; Group B = new cases; and Group C = relapsed cases.

isolates from an additional 15 patients that had a high similarity with the cluster strains on IS*6110*-based DNA fingerprinting. Sharing most of the IS*6110*-containing restriction fragments and the polymorphism associated with other repetitive DNA elements (28), the Beijing clone *of M. tuberculosis* has been isolated predominantly from patients in the People's Republic of China and neighboring Asian countries such as Mongolia, Korea, and Thailand (20). The emergence of the Beijing clone is now also well documented in Vietnam: Anh et al. have shown that 304 (54%) of 563 isolates studied were of that genotype and associated with younger patients and, hence, with active transmission (29).

In the three patient groups, only 11 isolates of *M. tuberculosis* were fully drug susceptible (2 patients in group A; 7 patients in B; and 1 patient in C). Dual resistance to isoniazid and streptomycin was seen in 10 cases (4 in A and 6 in B). Multidrug resistance was particularly associated with the Beijing genotype (28/46 = 61% versus 6/19 = 31% with the non-Beijing type) and, as expected, most frequent in patients of group A (19 in A, 14 in B, and 1 in C). For strains of the Beijing group whose mutation types could be determined, substitution at ser^{531} (mutation type R5) predominated. Published reports indicate that this mutation is the most common point mutation conferring RMP resistance (30,31). Recently, Naroditskaya et al. (unpub. data) studied 40 MDR-TB isolates from inmates of a prison in Tomsk (Russia) and reported that as many as 77.5% of the isolates had a substitution of $ser^{531} \geq$leu. Of these isolates, 15 had an RFLP pattern (W148) specific for the so-called W-strain family, which is highly drug-resistant and belongs to the phylogenetic lineage of the Beijing family. W and W-like strains have been associated with large MDR-TB outbreaks in the United States (16,17) and elsewhere (e.g., South Africa [19]). With high disease and mortality rates, MDR outbreaks caused by the W strain family and its variants have mainly affected HIV-infected persons (17,18) but affect HIV-negative persons as well (19,32). The HIV status of our cohort remained unknown, since the prisoners had not been tested. We assume that HIV infection has had little influence, since its incidence in the general Azeri population is low (33).

The difficulty in establishing transmission links among prisoners is well known. Although ample clinical information about their patients was available, Jones et al. (8) were unable to identify specific person-to-person lines of transmission among most inmates in a North American jail. Having been unable to establish accurate links among inmates or to define index patient(s) in the Baku Penitentiary Hospital, we can only hypothesize possible modes of transmission; for example, some patients may have contracted TB in the community long before they were imprisoned. Political constraints prevented us from studying TB strains in the Azeri population, which would have allowed us to analyze the molecular genetic composition of the strains circulating in the community, as well as to estimate the prevalence of the Beijing genotype strains. Acquisition of TB disease within the prison walls is, however, more likely. Identical RFLP patterns and spoligotypes, as well as identical susceptibility patterns, including the same point mutation in the *rpoB* gene (e.g., isolates 5692, 5700, 5702, 5706, 7932, and 7888 of cluster 5) suggest recent person-to-person transmission of TB. Nevertheless, we could not establish that the isolates of the large cluster carrying an S531L mutation are all derived from the same index strain. Since this mutation is by far the most frequent one, it could also have arisen independently in some of these strains.

Recent transmission within the prison is questionable for those cluster strains that are identical by RFLP, spoligotype and susceptibility profiles but have distinct mutations in the *rpoB* gene (e.g., isolate 7916 [cluster 5: ΔS2] versus all other isolates of this cluster [R5]; isolates 7897 and 7907 [cluster 10: R4a and ΔS4]; isolates 5686 and 5694 [cluster 11: ΔS2 and R5]). Thus, in all these cases TB is most likely not the result of recent transmission from a single source, which would require an RMP-susceptible progenitor strain. The fact that different susceptibility patterns were detected among the Beijing strains may indicate that transmission in the prison primarily involved RMP-susceptible strains, with resistance to RMP (and other drugs) evolving gradually in individual patients.

From this study, several conclusions can be drawn: MDR-TB is a substantial problem in Azeri prisons; the Beijing clone of *M. tuberculosis* was found in all three patient groups (nonresponders, new cases, and relapsed cases) and had a high rate of multidrug resistance; modes of transmission remain unclear, i.e., TB may have been acquired in different ways (outside or inside the prison); and MDR-TB in

this cohort may result from recent transmission in the prison, but may also have evolved as a result of longstanding, inadequate treatment regimens.

Unless WHO/IUATLD-recommended treatment schemes are followed, the problem of TB, in particular of MDR-TB, may become more widespread (8). In response, both WHO and ICRC have developed guidelines for managing TB in prisons (2). Special emphasis has to be put on settings where TB strains have a high degree of resistance, since regimens with first-line antibiotics may not be sufficient, as demonstrated by Coninx et al. (34). To achieve TB control in prisons, multiple goals need to be attained: efficient diagnosis to permit early case finding, prompt initiation of standardized treatment, monitoring of patient response to treatment, and provisions for ample supply of anti-TB drugs that meet quality standards.

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Dr. Pfyffer is professor of medical microbiology, Department of Medical Microbiology, and head of the Swiss National Center for Mycobacteria, both at the University of Zurich. Her research interests include the chemical and molecular taxonomy of mycobacteria, DNA fingerprinting and other typing methods, and new techniques for drug susceptibility testing and growth and detection of mycobacteria.

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Rapid Emergence of Ciprofloxacin-Resistant Enterobacteriaceae Containing Multiple Gentamicin Resistance-Associated Integrons in a Dutch Hospital

Alex van Belkum, Wil Goessens, Cindy van der Schee, Nicole Lemmens-den Toom, Margreet C. Vos, Jan Cornelissen, Elly Lugtenburg, Siem de Marie, Henri Verbrugh, Bob Löwenberg, and Hubert Endtz

Erasmus University Medical Center Rotterdam, Rotterdam, the Netherlands

In a hematology unit in the Netherlands, the incidence of ciprofloxacin-resistant *Enterobacter cloacae* and *Escherichia coli* increased from <0.5% to 20.7% and <0.5% to 64%, respectively, from 1996 to 1999. Clonal spread of single genotypes of both ciprofloxacin-resistant *E. coli* and *Enterobacter cloacae* from patient to patient was documented by pulsed-field gel electrophoresis and random amplification of polymorphic DNA. In addition, genetically heterogeneous strains were isolated regularly. Integrons associated with gentamicin resistance were detected in *Enterobacter cloacae* and *E. coli* strains. Integron-containing *E. coli* were detected in all hematology wards. In contrast, in *Enterobacter cloacae* strains two integron types were encountered only in the isolates from one ward. Although in all patients identical antibiotic regimens were used for selective decontamination, we documented clear differences with respect to the nosocomial emergence of ciprofloxacin-resistant bacterial strains and gentamicin resistance-associated integrons.

Patients with severe neutropenia (neutrophil count <500/µL) are at risk for bacterial and fungal infections. In addition to exogenous routes of infection, the endogenous intestinal bacterial flora is a potential source of life-threatening bacteremia caused by gram-negative microorganisms. Patients are especially vulnerable who have prolonged periods of neutropenia (>10 days), a very low neutrophil count (<100/µL), and mucositis due to anticancer chemotherapy. To reduce the incidence of bacteremia, such patients often receive antibiotic prophylaxis, called selective decontamination of the gut. This prophylaxis is intended to eliminate potentially pathogenic bacterial species while maintaining native anaerobic flora. The fluoroquinolones (e.g., ciprofloxacin) appear to combine excellent activity, good bioavailability, and high concentrations in the gut, and thus provide an important component of the standard selective decontamination in many centers (1). Successful implementation of this method has been reported for liver transplant patients (2), patients with cirrhosis and acute upper gastrointestinal hemorrhage (3), and hematooncology patients (4,5). However, a drawback is that the use of broad-spectrum antibiotics may eventually lead to selection of antimicrobial resistance (6-8), especially when patients have received prior courses of antibiotic treatment (9). Concern about emergence of multidrug-resistant microorganisms is one of the main reasons that decontamination procedures have not been generally accepted.

Bacteria such as *Escherichia coli* and *Enterobacter cloacae* exist in the human gut, as well as the environment. These bacteria represent an important class of opportunistic pathogenic microorganisms. The number of strains that are resistant, especially to antibiotics that are frequently used for selective decontamination, is increasing. In the United States, Canada, Latin America, Sicily, Spain, and Kuwait, for example, ciprofloxacin is no longer the most effective drug for treating bloodstream infections caused by gram-negative bacilli (9-12). Recent French and Belgian studies presented detailed, nationwide analyses of the emergence of antibiotic-resistant strains of *Enterobacter aerogenes* and associated risk factors (13-15). The emergence of nosocomially disseminating clones can be explained by the fact that ciprofloxacin resistance is due to point mutations in DNA gyrase and topoisomerase genes. The latter genes are not known to be interchangeable or prone to recombination among resistant and susceptible enterobacterial isolates. Most bacterial strains derived from bloodstream infections acquired in northern European countries were still highly susceptible to ciprofloxacin (16,17). An additional, complicating factor is that antibiotic-resistance genes are frequently trapped in cassettes, the so-called integrons (18-20), which provide an efficient means for capturing and exchanging resistance genes. This implies that both bacterial strain characteristics and the exchange and dissemination of resis-

Address for correspondence: Alex van Belkum, Erasmus University Medical Center Rotterdam EMCR, Department of Medical Microbiology and Infectious Diseases, Dr. Molewaterplein 40, 3015 GD Rotterdam, the Netherlands; fax: 00-31-10-4633875; e-mail: vanbelkum@bacl.azr.nl

tance genes need to be considered during ongoing outbreaks of infection (21).

Our molecular epidemiologic study was undertaken as part of an effort to understand the rapid spread of ciprofloxacin-resistant enterobacteriaceae in a hematooncology department. Subsequent microbiologic analysis of the *E. coli* and *E. cloacae* strains involved revealed a high prevalence of integron-associated gentamicin resistance as well. In addition to whole genome typing of the bacterial isolates, integron polymorphism was assessed with the help of a polymerase chain reaction restriction fragment-length polymorphism (PCR-RFLP) assay. The study environment comprised three clinical wards of one department of hematology, housed in two buildings: one at the south bank of Rotterdam harbor, containing two hematology wards where patients are treated for hematologic malignancies, and the other at the north bank, containing one similar ward. The prevalence of several antimicrobial resistance traits was assessed, isolates were characterized by pulsed-field gel electrophoresis (PFGE) or random amplification of polymorphic DNA (RAPD) analysis, and the presence and variability of integrons were determined.

Materials and Methods

Patients

All isolates were cultured from patients admitted to one of the three hematology wards (Table 1). Units A and B were located in the same building. Units B and C consisted of single-person rooms with anterooms and private bathrooms, negative air flow, and HEPA-filtered air supply. Gloves and gowns were worn by staff on all occasions. To prevent possible food-related infections, all foods were specifically selected: items likely to be contaminated with large numbers of bacteria (uncooked vegetable such as lettuce, fermented cheese, and several fruit species) were avoided. Unit A was made up of shared rooms only (up to four patients per room). All patients received the same selective decontamination procedure, which was started on the day of admission. The standard regimen involved ciprofloxacin (2 doses a day, either 500 mg orally or 400 mg intravenously). If ciprofloxacin-resistant gram-negative bacilli were present in surveillance cultures, patients received 4 doses a day of 200 mg colistin plus 5 mL of a 1 mg/mL colistin-containing solution, combined with 3 doses a day of 1 mL of 80 mg/mL tobramycin. Surveillance cultures were taken weekly from nose, throat, urine, vagina, and rectum.

Bacterial Strains and Ciprofloxacin Resistance

All strains belonging to the species *E. cloacae* or *E. coli* cultured in the microbiology laboratory of the Erasmus University Medical Center Rotterdam (EMCR), having an MIC for ciprofloxacin >2 mg/mL and isolated from November 1998 to October 1999, were selected from stock cultures. A general antimicrobial-resistance profile was generated by the commercial MicroScan Walk-A-Way system (Dade Behring, Paris, France) according to the manufacturer's instructions. On the basis of this analysis, all ciprofloxacin-resistant strains were identified and included in the study. The MICs of ciprofloxacin, ceftazidime, gentamicin, tobramycin, amikacin, cotrimoxazole, imipenem, and colistin were determined by agar dilution testing according to guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (22). Strains were classified as susceptible (S), intermediate (I), or resistant (R) according to NCCLS breakpoints. Finally, to define the nature of the gentamicin resistance, strains were phenotypically analyzed with the Vitek2 Advanced Expert System (bioMerieux, Lyon, France). Gram-negative susceptibility cards (ASTN-010) were inoculated with a 0.6 McFarland suspension of the strains involved. A description of the aminoglycoside resistance genes is provided. From November 1998 to October 1999, 164 isolates of ciprofloxacin-resistant enterobacteriaceae were retrieved from 45 patients. A total of 159 isolates were identified as either *E. coli* or *E. cloacae*; the five other isolates could not be identified unambiguously. Overall, 108 of 159 isolates from 33 patients (from 1 to 7 per patient) were available for further study. Fifty-one appeared to be *E. coli* and 57 belonged to the species *E. cloacae*. Of 33 patients, 6 showed clear evidence of clinically significant infection with *E. coli* (n = 5) or *E. cloacae* (n = 1). The other 27 patients were colonized with these resistant organisms, but had no clear symptoms or signs of clinical infection.

DNA Isolation

DNA isolation from individual colonies of cells was performed with the guanidinium-Celite protocol described by Boom et al. (23). DNA concentration was assessed by agarose gel electrophoresis of aliquots and staining by ethidium bromide in comparison with known amounts of lambda DNA. DNA was stored at a concentration of 5 ng/mL in 10 mM tris-HCl pH 8.0, 0.1 mM EDTA at -20°C.

Amplification and Characterization of Integrons

PCR mapping of integrons can be done on the basis of their 5' and 3' conserved regions. PCR was carried out in 100-mL volumes, essentially as described by Levesque et al. (24). The sequences for the 3' and 5' CS primers were GGCATCCAAGCAGCAAG and AAGCAGACTT-GACCTGA, respectively. Amplifications included 50 ng of bacterial DNA and cycling consisted of 35 repetitions of 1 min at 94°C, 1 min at 60°C, and 5 min at 74°C. Amplification products were analyzed by agarose gel electrophoresis. Besides length assessment, RFLP was determined for all amplicons. For these experiments, the endonuclease *Alu*I was used according to the manufacturer's instructions (Boehringer-Mannheim, Mannheim, Germany).

Random Amplification of Polymorphic DNA (RAPD)

RAPD was performed for the *E. coli* strains essentially as described (25). Primers for amplification were ERIC-1 and ERIC-2 (26). The PCR protocol consisted of 40 cycles of denaturation (1 min at 94°C), annealing (1 min at 25°C) and chain extension (2 min at 74°C). Reaction products were analyzed by agarose gel electrophoresis; single differences in banding patterns among strains led to the definition of novel genotypes.

Pulsed-Field Gel Electrophoresis (PFGE)

Before PFGE, strains were grown overnight on *Brucella* blood agar plates (bioMerieux, Lyon, France). Cells were embedded in 0.5% low melting point InCert agarose (FMC Bioproducts, Landgraaf, the Netherlands) buffered in 5 mM tris-HCl pH 8.0, 50 mM Na_2EDTA, 5 mM EGTA. The blocks

Table 1. Survey of epidemiologic and genetic data for the ciprofloxacin-resistant enterobacteriaceae isolated in the three hematology wards

Patient strain no.	Material	Date of isolation	Dept.	Integron tests		PFGE	Res. type	Aminoglycoside enzyme
				Size	RFLP			
Enterobacter cloacae								
1-1	fc	12-30-98	A			A	SL	
2-1	bc	10-23-99	C	2,000/2,200	A	B	CGTASL	E
2-2	bc	10-23-99	C		A	B	CGTASL	E
3-1	re	07-13-99	A			C	SL	
3-2	re	08-05-99	A			C	SL	
3-3	ur	08-11-99	A			C	SL	
3-4	re	10-04-00	A			C	SL	
4-1	re	06-07-99	A			C	SL	
4-2	re	06-21-99	A			C	SL	
5-1	re	02-01-99	B	2,000	B	D	CGTSE	
5-2	re	02-04-99	B	2,000	B	D	GTS	E
5-3	ur	02-04-99	B	2,000	B	D	GS	BCDE
5-4	re	02-08-99	B	2,000	--	E	GTS	E
6-1	re	07-05-99	A	--	--	C	SL	
6-2	re	07-26-99	A	--	--	C	SL	
6-3	ur	07-27-99	A	--	--	C	SL	
6-4	--	--	A	--	--	C	SL	
7-1	fc	12-18-98	A	2,000/2,200	A	F	GSL	B
7-2	re	12-21-98	A	2,000/2,200	A	G	GCTAS	B
8-1	re	06-28-99	A			C	SL	E
8-2	ur	06-28-99	A			C	SL	
9-1	fc	01-15-99	A			H	--	
9-2	fc	01-18-99	A			H	S	
9-3	fc	01-21-99	A			H	S	
9-4	fc	01-25-99	A			H	S	
9-5	fc	01-28-99	A			H	CS	
10-1	re	04-19-99	B	2,000	B	E	GTS	E
11-1	re	06-07-99	C	2,000/2,200	A	B	CGTASL	E
11-2	re	06-18-99	C	2,000	B	I	CGTAL	E
11-3	re	06-21-99	C	2,000/2,200	A	J	CGTASL	D
11-4	re	07-05-99	C	2,000/2,200	A	B	CGTASL	
11-5	we	07-11-99	C	2,000/2,200	A	B	CGTASL	
11-6	re	07-12-99	C	2,000/2,200	A	B	CGTASL	
11-7	ur	08-04-99	C	2,000/2,200	A	B		
12-1	fc	12-26-98	A			C	SL	
12-2	sk	01-15-99	A			C	SL	
12-3	wd	01-15-99	A			C	CSL	

Table 1. *(continued)* Survey of epidemiologic and genetic data for the ciprofloxacin-resistant enterobacteriaceae isolated in the three hematology wards

Patient strain no.	Material	Date of isolation	Dept.	Integron tests		PFGE	Res. type	Aminoglycoside enzyme
				Size	RFLP			
Enterobacter cloacae								
12-4	fc	01-18-99	A			C	SL	
13-1	fc	03-08-99	C	2,000/2,200	A	B	CGTAE	
13-2	ur	03-09-99	C	2,000/2,200	A	K	CGTSE	
13-3	fc	03-15-99	C	2,000/2,200	A	B	CGTSL	E
13-4	ur	03-16-99	C	2,000/2,200	A	Q	CGTASL	F
13-5	re	03-22-99	C	2,000/2,200	A	J	CGTSL	E
14-1	ur	08-05-99	A			--	--	
15-1	re	07-26-99	C	2,000/2,200	A	L	CGTASL	E
15-2	re	08-02-99	C	2,000/2,200	A	M	CGTASL	E
15-3	fc	08–06-99	C	2,000/2,200	A	M	CGTASL	
15-4	re	08-26-99	C	2,000/2,200	A	M	CGTASL	
15-5	re	08-30-99	C	2,000/2,200	A	?	CGTASL	E
15-6	--	--	C	2,000/2,200	A	N	CGTASL	E
15-7	re	09-03-99	C	2,000/2,200	A	M	CGTASL	E
15-8	re	09-06-99	C	2,000/2,200	A	L	CGTASL	E
15-9	re	09-30-99	C	2,000/2,200	A	L	CGTASL	
16-1	re	06-21-99	A			C	SL	
17-1	re	09-17-99	B	2,000/2,200	A	P	CGTS	E
18-1	bc	--	--	2,000/2,200	A	M		
Escherichia coli								
1-1	bc	12-31-98	A	2,000	-	L	S	
20-1	re	10-07-99	B			M		
20-2	re	10-11-99	B			M		
20-3	re	10-18-99	B			M		
21-1	fc	11-19-98	B	1,000/1,800	C	L	GTS	AB
21-2	fc	11-12-98	B	1,000/1,800	C	L	GTS	
21-3	fc	11-15-98	B	1,000/1,800	C	L	GTS	
21-4	fc	11-17-98	B	1,000/1,800	C	L	GTS	
21-5	fc	11-17-98	B	1,000/1,800	C	L	GTS	AB
22-1	fc	01-04-98	A	2,700	D	N	TS	
22-2	re	01-11-99	A	2,700	D	N	TS	
22-3	re	02-25-99	B	2,700	D	N	S	
22-4	ur	02-25-99	B	2,700	D	N	TS	
22-5	re	04-06-99	B	2,700	D	N	TS	
22-6	re	04-06-99	B	2,700	D	N	TS	
23-1	re	09-04-99	A	1,800	E	O	GTS	AB
23-2	re	09-06-99	A	1,800	E	O	GTS	
23-3	re	09-13-99	A	1,800	E	O	GTS	

Table 1. *(continued)* Survey of epidemiologic and genetic data for the ciprofloxacin-resistant enterobacteriaceae isolated in the three hematology wards

Patient strain no.	Material	Date of isolation	Dept.	Integron tests		PFGE	Res. type	Aminoglycoside enzyme
				Size	RFLP			
Enterobacter cloacae								
23-5	ur	10-21-99	A	1,800	E	O	GTS	AB
24-1	re	09-26-99	A	1,500	F	A	GS	ABCD
24-2	re	10-11-99	A	1,500	F	A	GS	
25-1	fc	01-11-99	A			J	S	
25-2	fc	01-13-99	B			K	S	
26-1	re	10-11-99	B	1,500	F	I	GTS	ABCDE
27-1	ur	03-09-00	C			G	S	
27-2	--	--	C			G	S	
27-3	ur	03-10-99	C			H	S	
27-4	--	--	C			H	S	
27-5	ur	03-22-99	C			H	S	
27-6	ur	03-25-99	C			C	S	
28-1	re	02-01-99	C			--		
28-2	ur	02-01-99	C			A		
28-3	ur	02-15-99	C	1,500	F	A	GS	BCD
28-4	va	02-27-99	C	1,500	F	A	GS	ABCD
28-5	ur	03-15-99	C	1,500	F	A	GS	
29-1	tr	02-01-99	A	1,500	G	A	GS	ABCD
29-2	tr	02-08-99	A			B	CG	CD
29-3	tr	02-23-99	B	1,500	G	A	GS	
30-1	ur	12-07-99	A	1,000/ 1,300/1500	I	A	GS	
30-2	fc	12-10-98	A	2,700	D	C	S	F
30-3	fc	12-10-98	A	1,000/ 1,300/1500	I	D	-	
31-1	fc	12-07-98	B	1,500/2,000	G	A	S	F
32-1	re	02-11-99	A	1,800	F	A	CS	
32-2	re	02-22-99	A	1,800	F	A	CS	
32-3	re	03-01-99	A	1,800	F	A	CS	F
32-4	cx	03-23-99	A	1,800	F	A	S	
33-1	bc	11-05-99	C	2,000	J	A	S	
34-1	bc	06-21-99	A			E	GS	AB
35-1	ur	09-23-99	A	1,800	?	F	GTS	AB

Clinical materials from which the samples were derived were feces (fc), blood (bc), rectal swabs (re), urine (ur), wounds (wd), skin (sk), vaginal swabs (va) or cervical swabs (cx). All strains were resistant to ciprofloxacin, additional antibiotics tested to determine the antibiogram were ceftazidime (C), gentamicin (G), tobramycin (T), amikacin (A), cotrimoxazole (S), imipenem (I) and colistin (L). The nature of the aminoglycoside modifying enzymes involved was only tested for those strains for which a result is mentioned. Enzymes are encoded as follows: A: resistant aminoglycoside acetyl transferase AAC(3)-II; B: resistant aminoglycoside nucleotidyl transferase ANT(2'); C: heterogeneous aminoglycoside phosphotransferase APH(3') + AAC(3)-I; D: resistant AAC(3)-I; E: AAC(6'); F: resistant APH(3').

were deproteinated by overnight incubation in the same buffer containing 1% sodium dodecyl sulfate and 1 mg/mL of proteinase K (Sigma Chemicals, St. Louis, MO) at 37°C. After extensive washing, blocks were stored at 4°C. Approximately 3x5-mm portions of the blocks were incubated in the presence of 40 units of the restriction endonuclease *Xba*I (Boehringer-Mannheim, Mannheim, Germany) in the appropriate buffer. Incubation at 37°C continued for 18 hours, after which the blocks were incorporated in 1% Seakem GTG agarose slabgels (FMC BioProducts, Rockland, ME). The restriction fragments were separated at a field strength of 6 V/cm for 20 hours at 14°C. The pulse time linearly increased from 5 sec to 35 sec during electrophoresis (27). Concatemers of lambda DNA were used as molecular size markers (Bio-Rad, Veenendaal, the Netherlands). Gels were stained with ethidium bromide postelectrophoresis and photographed (Mitsubishi Copy Processor, Progress Control, Waalwijk, the Netherlands). DNA fingerprints were inspected visually. Single band differences were scored for the definition of separate genotypes, which is stricter than the guidelines of Tenover et al., which were not intended for outbreaks of infection or colonization monitoring (28).

Statistical Analysis

We used Fisher Exact or chi-square tests for analysis of the differences in incidence of fluoroquinolone resistance. A two-tailed test p-value of <0.05 was considered statistically significant.

Results

Prevalence of Ciprofloxacin-Resistant Enterobacteriaceae

Our study was prompted by the apparent rise in the incidence of ciprofloxacin-resistant enterobacteriaceae in the hematology wards of the EMCR, as well as a fatal case of ciprofloxacin-resistant *E. cloacae* septicemia in a neutropenic patient with cancer. In the hospital as a whole, the incidence of ciprofloxacin resistance among isolates of *E. coli* and *E. cloacae* has been relatively stable, increasing slightly from 1996 to 1999 from 3.5% (65 of 1,879 isolates, one per patient) and 3.4% (12 [4.7%] of 358) to (111 of 2,367; p = 0.06) and 4.2% (27 of 549; p = 0.3) per species. Among patients in the hematology wards, however, the incidence of ciprofloxacin-resistant *E. coli* increased from <0.5% in 1996 to 20.7% (21 of 82; p = 0.0004) in 1999. Even more notable, the incidence of ciprofloxacin-resistant *E. cloacae* rose from <0.5% to 64% (16 of 25; p = 0.01) during the same period. Therefore, the small increase in the overall incidence of ciprofloxacin resistance among enterobacteriaceae in the entire hospital was in part attributable to the rise observed in the hematology department.

Overall Antimicrobial Resistance Patterns in Ciprofloxacin-Resistant Enterobacteriaceae

Resistance patterns were derived from MIC determinations in ciprofloxacin-resistant isolates from individual patients (Table 1). For patient 1, both a ciprofloxacin-resistant *E. coli* and an *E. cloacae* isolate were detected. Clear differences were observed in the proportions of *E. coli* or *E. cloacae* strains resistant to the various antibiotics tested (Table 2). Apparently, the *E. cloacae* strains are more often resistant toward ceftazidime, tobramycin, amikacin, cotrimoxazole, and, most notably, colistin. No strain was found to be resistant to imipenem.

Many of the enterobacters appeared multiresistant. The most prevalent types of resistance were CGTASL (17 [30%] of 56) and SL (16 [29%] of 56). The *E. coli* strains were more diverse with respect to their antibiograms, with the GTS- (12 [24%] of 50) and GS-types (9 [18%] of 50) the most prevalent multiresistant isolates. These figures are biased, since multiple isolates were included per patient, but also on an individual basis the SL type is the most prevalent of the enterobacter strains, with 7 of 18 patients colonized. For the *E. coli* strains, the S type is the most frequently encountered on a patient-to-patient basis (8 of 27). In most cases, serial isolates from individual patients shared the same antibiogram, but exceptions to this rule were observed.

In addition to the aminoglycoside MIC determinations, the aminoglycoside-modifying enzymes involved were defined by the Vitek2 Advanced Expert System (Table 1). The aminoglycoside resistance trait of the *Enterobacter* spp. is primarily determined by the putative presence of an AAC(6') gene (aminoglycoside resistance type E; Table 1). Incidentally, other combinations are documented. This type

Table 2. Proportions of ciprofloxacin-resistant *Enterobacter* spp and *Escherichia coli* strains resistant to other antibiotics[a]

	Enterobacter			*E. coli*		
Antibiotic	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ceftazidime	24 (47)	0	27 (53)	46 (92)	0	4 (8)
Gentamicin	21(41)	6(12)	24(47)	28(56)	0	22(44)
Tobramycin	23 (45)	0	28 (55)	32 (64)	11 (22)	7 (14)
Amikacin	31 (61)	18 (35)	2 (4)	50 (100)	0	0
Cotrimoxazole	0	0	51 (100)	7 (14)	0	43 (86)
Imipenem	51 (100)	0	0	50 (100)	0	0
Colistin	12 (24)	0	39(76)	48(96)	0	0

[a]The figures express the absolute number of susceptible (S), intermediate (I) and resistant (R) strains defined according to the 1999 NCCLS Guidelines [22].

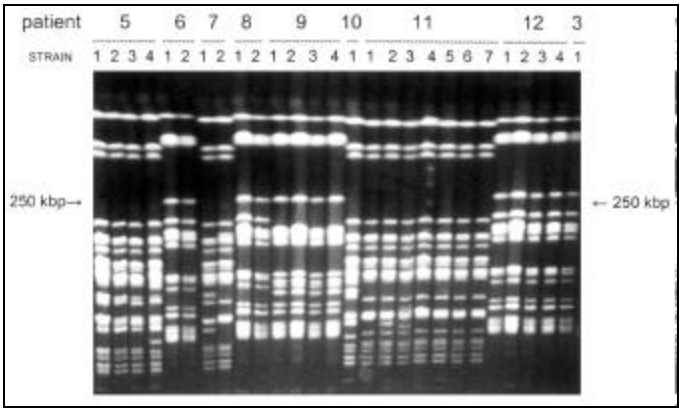

Figure 1. Pulsed-field gel electrophoresis of *Xba*I macrorestriction fragments for the ciprofloxacin-resistant *Enterobacter cloacae* strains. Examples of different sets of patient isolates are shown. The interpretation of these experimental data is given in Table 1. Note the minor differences, for example, among strains 11-1, 11-2, and 11-3. Not all fingerprints for the strains analyzed for each of the patients are presented. The arrows on the left and right indicate the position of the 250-kbp marker molecule.

of resistance always correlated with the presence of an integron. For the *E. coli* strains, more heterogeneous aminoglycoside resistance gene contents were documented. Aminoglycoside resistance was never recorded in the absence of an integron.

Genetic Typing of the Enterobacteriaceae

Genetic heterogeneity among the *E. cloacae* strains was assessed by PFGE (Figure 1). A relatively large proportion (approximately 33%) of the *E. coli* strains appeared to be reproducibly nontypeable by PFGE. During two independent experiments, no distinct banding patterns were generated, and only smears of degraded DNA were seen. Therefore, we decided to type all strains by using independent RAPD assays. With this approach, all strains appeared to be typeable. Moreover, the *Not*I PFGE data that were available for the PFGE-typeable subset of the strains were in full agreement with the RAPD data (results not shown). A survey of all PFGE- or RAPD-based genetic codes is presented in Table 1. *E. cloacae* strains were isolated from diverse clinical specimens derived from 18 patients. Sixteen PFGE types were determined. Among five patients who were colonized or infected by multiple types, two patients had three types and one patient was colonized by as many as four different PFGE genotypes. Several genotypes were encountered in more than one patient. Types B were isolated from three patients, and types E-J-M were detected in two patients each. These patients had not been clustered in time or space, so a common infectious source may exist that is unrelated to any of these patients. Type B occurred in 3 of 5 patients housed in Unit C, indicating that transmission is also a possibility in the highly restricted wards. Shared strains were also documented for patients in the high-level isolation, single-person units with anterooms and separate bathrooms, but could not be explained on the basis of patient-to-patient contacts. Most specific genotypes were confined to a single patient, where they were persistently found. The six patients who carried type C were admitted to ward A during overlapping time intervals (Figure 2), indicating that this type is an important nosocomial pathogen. The strains were derived from a variety of clinical specimens.

The isolates of *E. coli* were retrieved from 17 patients, and 15 different RAPD types were documented. Two patients were colonized with two strains, and two other patients with three different strains. One of these patients was transferred to another unit during his stay in the hematology department. Several RAPD type-strains were found in more than one patient. Types L and C were encountered twice, and type A was found in seven patients. For the type A strain, limited overlap in time and space was documented for the patients involved (late 1998 to early 1999). Four of them were in unit A (40% of all patients in that ward during the screening period were colonized with ciprofloxacin-resistant strains) (Figure 2). The possibility of a non-patient-associated environmental source remains, especially because bacterial dissemination was not restricted to the A unit, where physical isolation was less strict and several patients shared rooms. Putative explanations for spread are carriage by staff members or contamination of showers or toilets, but neither potential source was examined systematically during the study.

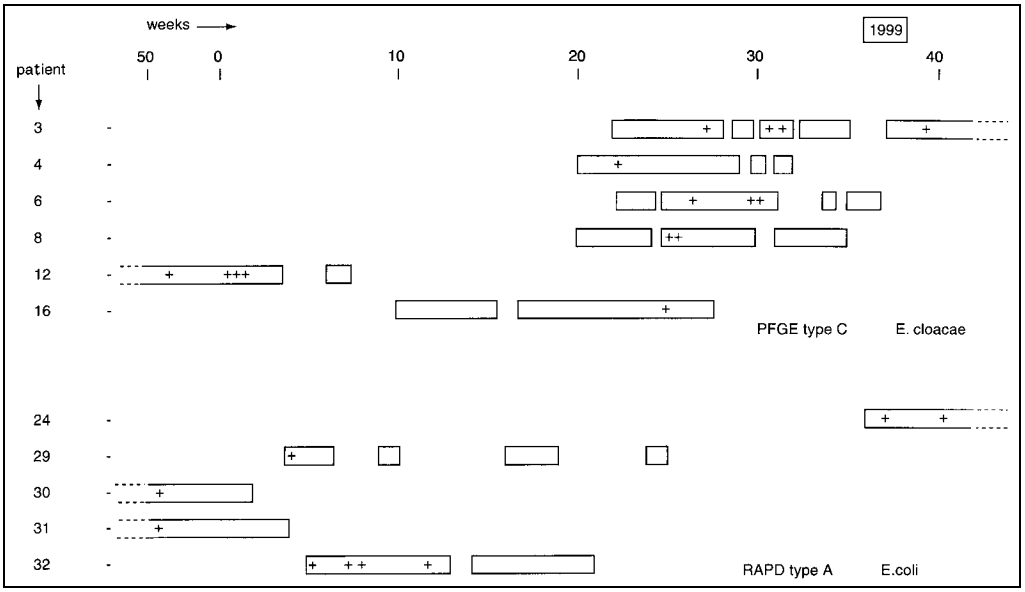

Figure 2. Clustering in time of the pulsed-field gel electrophoresis type C strains of *Enterobacter cloacae* and the RAPD type A strains of *Escherichia coli* for isolates from the low-care hematology Unit A. Note that RAPD type A *E. coli* strains were also retrieved from two patients in another unit (patients 28 and 33). The dates on which positive cultures were obtained are indicated by +; the horizontal bars indicate the periods during which the patients were in Unit A.

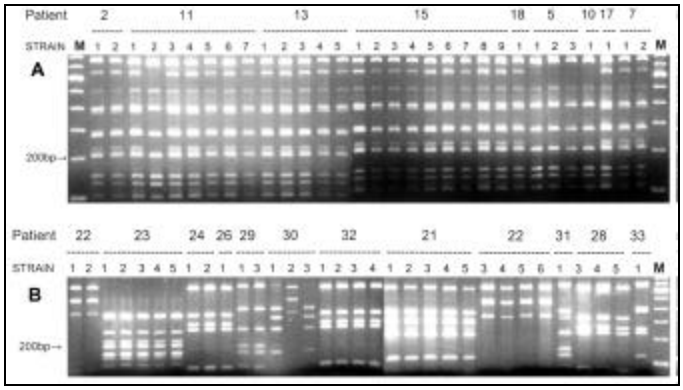

Figure 3. Amplification fragment length polymorphism analysis analysis of the *Enterobacter cloacae* and *Escherichia coli* integron amplicons. A. *Alu*I restriction fragment length polymorphism (RFLP) patterns for the integrons amplified from the *E. cloacae* strains. Patients and strains are identified above the lanes. Lanes marked M contain molecular length markers; the 200-bp fragment is identified on the left. Two different patterns are identified: only strains 11-2, 5-1, 5-2, 5-3, and 10-1 show a slightly different mixture of restriction fragments (2 are missing). B. *Alu*I RFLP patterns for the integrons amplified from the *E. coli* strains. Patients and strains are identified above the lanes; lanes marked M contain molecular length markers, with the 200-bp fragment is identified on the left. Note the clear difference in complexity in comparison with the homogeneous patterns shown in figure 3A.

Integron Analysis

Detection of integron sequences was performed with a straightforward PCR assay. The results of these amplifications and the molecular sizes of the amplicons are summarized in Table 1. The integrons among the *E. cloacae* strains are clearly similar in structure and composition. In 26 (46%) of 56 strains, two different integrons were detected. In six additional strains, only the smaller one (2,000 bp) was detected. When the amplicons were digested with *Alu*I, homogeneous RFLP patterns were observed, suggesting similarity of the two integrons (Figure 3A). The presence of the integrons correlates with resistance of the strains to gentamicin, tobramycin, and amikacin. Among the *E. coli* strains, greater diversity of PCR products was detected (Table 1) (Figure 3B). At least seven different-sized PCR products were visualized. Moreover, similar-sized integron amplicons can give rise to different RFLP patterns, indicating even more extensive heterogeneity. Thirty-five (70%) of 50 strains had one or more integrons. The association between integron presence and gentamicin resistance is still present, but is not as strong as for the *E. cloacae* strains.

Discussion

We describe a study in a hematology department with patients housed in three clinical units in two buildings. As the antibiotic policy, especially the use of ciprofloxacin for selective decontamination, is identical throughout the department, the occurrence and means of spread of ciprofloxacin-resistant gram-negative bacilli can be measured against patient-related or environmental features. This study was initiated after we observed that the overall incidence of bacterial ciprofloxacin resistance in the hematology department had substantially exceeded that of the hospital as a whole. Moreover, these resistant organisms resulted in clinical infections in a significant proportion of the patients. Of the patients that we could track, 6 (18%) of 33 at any given time had bacteremia with a ciprofloxacin-resistant microorganism. Our study does not allow definite conclusions to be drawn on the relationship between the increased incidence of resistance on the one hand and bacterial genetic variability on the other. This would require analysis of susceptible strains as well, both from the hematology wards and other wards of the same hospital. However, our observations do provide insight into the molecular epidemiology of ciprofloxacin-resistant organisms and, separately, gentamicin resistance encoding integrons in the hematology department.

Outbreaks Caused by Ciprofloxacin-Resistant Microorganisms

PFGE has been used to study the dissemination of ciprofloxacin-susceptible *Enterobacter* strains in detail in various clinical settings (14,27,29-31). As such, the method is well accepted and has proven epidemiologic efficacy. We show dissemination of a PFGE type C *E. cloacae* strain in Unit A. This finding emphasized the need for adequate implementation of robust barriers in nursing hematology patients. Hand disinfection, both for patients and personnel, and the use of gloves and gowns by staff members are indispensable. After patients were specifically instructed on how to wash their hands after using the bathroom, the incidence of ciprofloxacin-resistant strains decreased (data not shown). However, in light of the overlapping genotypes, all clinical personnel involved should be aware of the potential for resistant bacteria to spread from patient to patient, within the environment, or among persons acting as potential vectors.

On two separate occasions, PFGE failed to type 33% of all ciprofloxacin-resistant *E. coli* strains. Therefore, we turned to random amplification of polymorphic DNA (RAPD) analysis, which has been used successfully to elucidate epidemic spread of *E. coli* (25,26) and appeared to be useful here as well. *E. coli* strains were 100% typeable with this latter technique. A possible nosocomial outbreak was assessed for RAPD type A. This type also occurred in the multiperson rooms in Unit A. The level of barriers (e.g., negative airflow, use of gloves and gowns) seems to be associated with the likelihood of an outbreak. A recent study performed in another Dutch university hospital identified 21 hematologic cancer patients colonized (79%) or infected (21%) with a ciprofloxacin-resistant *E. coli* strain over a 5-year period (32). An increase in incidence was noted over the years, whereas limited RAPD analyses suggested that nosocomial transmission had occurred. Overall, the observations from the two Dutch university hospitals suggest that the use of ciprofloxacin may predispose to outbreaks due to ciprofloxacin-resistant *E. coli* strains.

Integron Epidemiology

An integron is a genetic element that possesses a site at which cassettes of DNA can be integrated by site-specific recombination. The integron encodes an integrase enzyme that mediates the recombination events (18,19). Integrons are not independently mobile but may be found as part of transposons or plasmids, and the genes that they contain may not always be expressed with equal effectiveness (33). Such features may favor or limit the successful spread of integrons and may also provide a likely explanation for their ubiquity among gram-negative bacilli (34,35). A pan-Euro-

pean study recently revealed that >40% of all gram-negative clinical isolates harbor integrons and that the presence of integrons is associated with increased frequency of multiresistance and distinct resistance against aminoglycosides, quinolones, and beta-lactam compounds (36). In addition, the same authors suggested in a follow-up study that the conserved nature of the integrons that were identified could be an indication of the spread of entire integrons rather than the cassettes only (37). Integrons are assumed to play an important role in the dissemination of antimicrobial resistance (38). The contribution of integron cassettes to the prevalence of transferable aminoglycoside resistance has been demonstrated in a French hospital (39).

We have decribed the frequent occurrence of integron-encoded gentamicin resistance among nosocomial isolates of ciprofloxacin-resistant *E. cloacae* strains. Among the *E. cloacae* strains, two different integron types were encountered against a diverse background of chromosomes. This could be indicative of intraspecies dissemination of these particular elements, either as a whole or as the cassette content only, suggesting a strong species barrier. In case of the *E. coli* isolates, the integron types had greater diversity. Furthermore, the *E. cloacae* integron types were primarily confined to certain hematology units. Surprisingly, this suggests that a species barrier exists, prohibiting integron transfer between *Enterobacter* sp. and *E. coli*. In contrast to this observation, and substantiated by the fact that indiscriminate integrons occur in various bacterial strain types, exchange of integrons between strains of a single species seems to be effective. Furthermore, some integron types seem to be confined to a certain patient unit. In addition, our data indicate that in clinics where relatively large amounts of antibiotics are prescribed, resistance determinants may accumulate, especially in strains that show patient-to-patient transmission.

In conclusion, we describe differences in the nosocomial epidemiology of ciprofloxacin-resistant enterobacteriaceae. Besides variation in integron content, different species-specific integrons appear to circulate within the shared clinical environment. The observed differences between the spread of strains compared with antimicrobial resistance traits encoded by integrons merit additional investigations. We hypothesize that the use of various antibiotics, whether or not in combination, may have acted as a selective force in the emergence and dissemination of resistant microorganisms or their resistance-associated traits.

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Dr. van Belkum is a molecular microbiologist working in the Department of Medical Microbiology and Infectious Diseases of the Erasmus University Medical Center in Rotterdam, the Netherlands. His research interests are in the field of molecular epidemiology of infectious agents and characterization of molecular determinants of host-microbe interactions during colonization and disease.

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Topical Insecticide Treatments to Protect Dogs from Sand Fly Vectors of Leishmaniasis

Richard Reithinger,*† Ueslei Teodoro,† and Clive R. Davies*
*London School of Hygiene & Tropical Medicine, London, United Kingdom; and †Universidade Estatal de Maringá, Maringá, Paraná, Brazil

We compared the susceptibility of sand fly vectors to four topical insecticide treatments applied to domestic dogs, a reservoir of human leishmaniasis. Dogs were exposed to sand flies pretreatment and at 1 week, 1 month, and 2 months posttreatment. Sand fly bloodfeeding and survival rate of both fed and unfed flies were significantly reduced by the permethrin, deltamethrin, and fenthion treatments, but diazinon had no effect. The survival rate of bloodfed sand flies was reduced by up to 86% with deltamethrin collars. The antifeeding effect suggests that deltamethrin collars may be recommended to dog owners to protect their pets from sandfly-borne diseases. The combined effects on sand fly feeding and survival indicate that epidemiologic, community-based trials are warranted to test whether deltamethrin collars could reduce the incidence of canine and, hence, human leishmaniasis.

Leishmaniases are a group of zoonotic diseases transmitted to humans and animals by the bite of phlebotomine sand flies (1). Worldwide, the leishmaniases are the third most important vector-borne disease (after malaria and sleeping sickness), accounting for an estimated 1.98 million disability-adjusted life-years and 57,000 deaths annually (2). In the past 20 years, the number of human leishmaniasis cases has dramatically increased, a trend that shows no signs of abating (3). Much of this increase may result from adaptation of *Leishmania* transmission cycles to the peridomestic environment as a response to deforestation and urbanization (4). Another explanation is that the leishmaniases are a common opportunistic infection in HIV-infected persons (5).

In Latin America, as in North Africa and Asia, *Leishmania* infections in dogs have great public health importance, as dogs are the reservoir hosts of zoonotic visceral leishmaniasis (ZVL) caused by *Leishmania infantum (=Le. chagasi*). Dogs are also the suggested reservoir hosts of American cutaneous leishmaniasis (ACL) caused by *Le. (Viannia)* spp. (6) and may have a role in the transmission cycle of cutaneous leishmaniasis in the Old World caused by *Le. tropica* (7). Canine leishmaniasis is mainly a veterinary problem in Europe (where estimates suggest that up to 7 million dogs are at risk for infection [8]) and the United States (where there has been a recent widely publicized outbreak in foxhounds in New York [9]).

In addition to treating patients and spraying houses with insecticide, ZVL control programs (notably in Brazil and China) often target the reservoir hosts by culling infected dogs. A similar culling policy has been suggested for controlling ACL where domestic transmission has been demonstrated. However, the impact of dog-culling programs on (human and canine) ZVL incidence has been questioned on theoretical and practical grounds (10,11), and results of controlled intervention trials are contradictory (6,12-14). Treating infected dogs with antileishmanial drugs (e.g., pentavalent antimonials, amphotericin B, aminosidine, or allopurinol) is not a practical control policy, not only because of the prohibitive cost involved but also because of high relapse rates (up to 74% [15]) among treated and clinically cured dogs. Moreover, a high proportion of clinically cured dogs remain parasitologically positive and therefore infectious to the sand fly vector (16).

While awaiting a leishmanial vaccine (17), alternative canine leishmaniasis control strategies are urgently needed. One proposal has been to cut transmission by treating domestic dogs with insecticides (18)–lotions or insecticide-impregnated dog collars (1) originally developed for flea and tick control (19). Our work directly compares the anti-feeding and lethal effects of deltamethrin-impregnated (DM) dog collars with those of alternative topical insecticide applications. The study also increases the number of sand fly species against which insecticide-impregnated dog collars have now been tested and is the first such study to test their impact on a vector of ACL (*Lu. intermedia*), which is known to feed on both humans and dogs (20).

Materials and Methods

Study Site and Protocol

All 17 dogs used in the experimental trial came from the Fazenda Palmital, a large farm 40 km from Maringá, Estado do Paraná, Brazil (23°40'S, 52°25'W). Mongrel dogs were stationed individually inside cages (50-cm width x 60-cm height x 60-cm depth) and were exposed overnight (±22.30 to 5.30 hours, i.e., for 7 hours) to an average 96 (69 to 121) wild-caught sand flies introduced through sleeves of closed net curtain tents (100 cm x 180 cm x 130 cm). Sand flies had been caught the same night by mouth aspirator inside open chicken pens at the Fazenda Marista (21.00 to 22.00 hours),

Address for correspondence: Richard Reithinger, Disease Control & Vector Biology, Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom; fax: 44-20-7467-9536; e-mail: rreithinger@yahoo.co.uk

a neighboring farm. Previous studies have shown that >85% of all collections at the Fazenda Marista are *Lu. intermedia* (Teodoro et al., unpub. data), which was confirmed by microscopically identifying to species a subset of caught sand flies: Of 200 unfed and 40 bloodfed sand flies, 172 (86%) and 35 (88%) were identified as *Lu. intermedia*, respectively; the remainder were *Lu. whitmani*. Sand flies were collected from the tents the following morning by using a mouth aspirator, placed into suspended gauze cubic cages, and maintained on sucrose solution for a further 17 hours at 24°C to 26°C and 90% to 95% relative humidity. After 24 hours, flies were identified by sex and scored as either dead or alive, and bloodfed or unfed.

All dogs were exposed to sand flies before treatment (day 0) and again at 5 to 12 days posttreatment (dpt), 32 to 36 dpt, and 58 to 65 dpt. Three time points were compared: 1 week, 1 month, and 2 months. Four treatments were compared: 1) 40 mg/g DM-impregnated collars (Scalibor, Intervet International GmbH, Wiesbaden, Germany) (n = 5); 2) 15% diazinon-impregnated collars (Canovel, Pfizer, United Kingdom) (n = 3); 3) application of 1 mL of 0.65 g/mL permethrin topical lotion (Pulvex, Coopers Brasil Ltda, São Paulo, Brazil) (n = 3); and 4) application of 1 mL of 15% fenthion topical lotion (Pulfim, Bayer S.A. Brasil, Barueri, Brazil) (n = 3). Collars were attached around the necks of the dogs. Topical lotions were applied directly onto the skin after the dog's hair was separated at the nape of the neck. Three untreated dogs (negative controls) were exposed to sand flies at the same time points to adjust for any background changes in sand fly feeding and survival rates over time. Changes in climatic conditions (temperature and relative humidity) were measured at the start and end of each bioassay.

Data Analysis

The effects of the different treatments (in relation to the negative control) at each time point were tested by using General Linear Models (21) in the computer package STATA, i.e., by analyses of deviance, specifying binomial errors, of the log odds that the sand fly bloodfeeding rate was diminished as a result of treatment or that the sand fly death rate was increased as a result of treatment. Any significant overdispersion was corrected by rescaling the model using the ratio of the residual deviance to residual degrees of freedom. Maximal models incorporated the effects of dog age and size and climatic conditions along with treatment. The significance of each variable was tested by back-step analysis of deviance, i.e., by observing whether these variables explained a significant ($p < 0.05$) proportion of the deviance remaining after their removal from the model. Variables were excluded from the models in order of least significance until only significant variables were retained in the minimum adequate model.

Results

An average of 49 (19 to 86) female and 22 (2 to 47) male sand flies were recovered from the tents the following morning (mean recovery rate 74%). The sex ratio was remarkably constant throughout the experiment, with no significant differences detected with time or treatment (median proportion of females recovered: 0.68; 25% to 75% quartiles: 0.61 to 0.75). Sand fly bloodfeeding and death rates were unaffected by dog age and size or climatic conditions throughout the trial. No dogs had visible side effects from the different treatments.

Sand Fly Bloodfeeding Rate

In the absence of treatment, the average sand fly bloodfeeding rate was 42% (Table 1) (Figure). There was no significant difference between the bloodfeeding rate on negative control dogs and the rates on any of the four treatment groups before treatment ($p > 0.2$ for all four comparisons). Neither were there any significant differences in bloodfeeding rates on treated and untreated dogs at the first time point (1 week), although there was some suggestion of a reduction on the dogs treated with permethrin ($p = 0.088$) and DM ($p = 0.083$). Bloodfeeding rates were significantly lower on dogs treated with DM, permethrin, and fenthion (compared with untreated dogs) at both 1 month ($p < 0.001$; $p = 0.010$; and $p = 0.005$, respectively) and 2 months ($p < 0.001$; $p = 0.004$; and $p = 0.018$, respectively). At both time points, the reduction in bloodfeeding rate was greatest on DM-treated dogs (Table 2), although the difference with permethrin- and fenthion-treated dogs could not be demonstrated statistically. The greatest antifeeding effect in all trials was detected on DM-treated dogs after 2 months, when bloodfeeding rates were reduced by 69% (95% confidence intervals [CI] 43,78). A similar pattern was demonstrated when bloodfeeding rates posttreatment were compared with bloodfeeding rates pretreatment on the same dogs (rather than with bloodfeeding rates on control dogs at the same time points). In these analyses, a significant reduction in bloodfeeding was detected on DM-treated dogs at all three time points: at 1 week ($p = 0.012$), 1 month ($p < 0.001$), and 2 months ($p < 0.001$). In contrast, bloodfeeding rates only dropped significantly by 1 month on fenthion-treated dogs and by 2 months on permethrin-treated dogs. No significant reduction in bloodfeeding was detected on diazinon-treated dogs at any time point, when compared with either negative control dogs or pretreatment controls.

Sand Fly Death

In the absence of treatment, the average sand fly death rate of unfed and bloodfed sand flies was 12% and 2%, respectively (Table 1) (Figure). There was no significant difference between the death rate of bloodfed sand flies on negative control dogs and the equivalent rates on any of the four treatment groups before treatment ($p > 0.4$ for all four comparisons). In comparison with untreated dogs, deaths of bloodfed flies was significantly increased at 1 week by 11%, i.e., 5.7-fold (95% CI 1.1, 20.5) as a result of permethrin treatment ($p = 0.037$) and at 1 month by 33%, i.e., 27-fold (4.4, 66) by fenthion treatment ($p = 0.001$) and by 30%, i.e., 25-fold (4.2, 63) by DM treatment ($p = 0.001$). No significant treatment effects on deaths of bloodfed sand flies were detected at 2 months.

Before treatment, there was no significant difference between the death rate of unfed sand flies on negative control dogs and the equivalent rates on three of the treatment groups ($p > 0.4$ for all three comparisons), but deaths of unfed sand flies in the group allocated to have DM treatment were significantly less ($p = 0.020$) than for the control group. The effect of this minor pretreatment bias was to make it harder to detect any significant increase in deaths as a result of DM treatment. With this caveat, we were unable to detect any

Table 1. Experimental dog trial comparing effects of topical insecticide applications on feeding and survival rates of female sand flies[a]

Dog	TM	0 dpt		5-12 dpt		32-36 dpt		58-54 dpt	
		B	U	B	U	B	U	B	U
1	C	40 (1)	37 (3)	37 (0)	38 (6)	25 (0)	34 (4)	23 (1)	30 (4)
2	C	43 (1)	34 (8)	28 (0)	27 (2)	26 (1)	39 (6)	25 (1)	33 (3)
3	C	31 (1)	44 (5)	24 (2)	40 (7)	30 (0)	29 (2)	14 (3)	37 (8)
4	DM	20 (1)	28 (2)	21 (2)	31(10)	14 (3)	34(14)	8 (1)	36(11)
5	DM	24 (0)	54 (4)	16 (1)	27 (5)	7 (3)	37 (8)	ND[b]	ND[b]
6	DM	47 (1)	39 (2)	9 (1)	53 (7)	8 (2)	37(14)	5 (1)	43 (9)
7	DM	26 (0)	28 (3)	3 (0)	20(10)	4 (2)	31(20)	4 (2)	34(15)
8	DM	25 (1)	34 (1)	14 (0)	26 (5)	6 (2)	26(23)	5 (1)	23(19)
9	DZ	38 (0)	40 (5)	32 (0)	20 (3)	23 (1)	18 (6)	ND[c]	ND[c]
10	DZ	26 (0)	38 (4)	36 (2)	8 (4)	29 (1)	27 (7)	ND[c]	ND[c]
11	DZ	16 (0)	34 (5)	15 (1)	21 (2)	13 (0)	39 (6)	17 (0)	29 (4)
12	PM	17 (0)	27 (6)	6 (2)	35 (7)	9 (1)	21 (4)	8 (1)	23 (8)
13	PM	16 (1)	39 (3)	10 (1)	24(11)	21 (1)	38(19)	11 (0)	38(11)
14	PM	22 (0)	14 (3)	15 (1)	31 (9)	13 (2)	36(10)	10 (1)	39 (9)
15	F	22 (1)	47 (7)	15 (2)	15 (6)	6 (0)	17 (9)	9 (1)	28(13)
16	F	29 (1)	22 (8)	27 (1)	15(13)	11 (4)	28(19)	16 (0)	37 (7)
17	F	10 (1)	21 (2)	16 (0)	44 (5)	4 (3)	15(10)	8 (3)	33 (8)

[a] Dead sand flies (after 24 hrs) are in parentheses.
[b] Dog 5 was killed by his owner
[c] Dogs 9 and 10 moved.
Abbreviations: B, bloodfed sand flies; C, control; DM, deltamethrin-impregnated dog collar; dpt, days posttreatment; DZ, diazinon-impregnated dog collar; F, fenthion topical lotion; PM, permethrin topical lotion; ND, not done; TM, treatment; U, unfed sand flies.

significant differences in the death rate of unfed sand flies at 1 week. However, in comparison with untreated dogs, death of unfed sand flies was significantly increased by 41%, i.e., 4.1-fold (1.7 to 6.6) at 1 month by DM treatment ($p = 0.004$) and by 58%, i.e., 5.4-fold (2.3 to 7.6) by fenthion treatment ($p = 0.001$). The death rate of unfed sand flies at 2 months was significantly increased by 29%, i.e., 2.6-fold (1.02 to 4.7) by DM treatment ($p = 0.046$).

Finally, we analyzed the combined effects of treatment on bloodfeeding and the deaths of bloodfed flies, i.e., how treatment affected the proportion of females that both took a bloodmeal and survived 24 hours. As expected, the diazinon collar had no effect at any time point. None of the treatments had a significant effect at 1 week (although the effects of DM and permethrin were of borderline significance: $p = 0.064$ and $p = 0.053$, respectively). At 1 month, DM ($p < 0.001$), permethrin ($p = 0.003$), and fenthion ($p < 0.001$) all caused a significant reduction, but the effect of DM was significantly greater than that of permethrin ($p = 0.001$). A significant reduction was again detected at 2 months for DM ($p < 0.001$), permethrin ($p = 0.008$), and fenthion ($p = 0.015$); and the effect of DM was significantly greater than that of both permethrin ($p = 0.019$) and fenthion ($p = 0.01$).

Discussion

The observed reduction in *Lu. intermedia* bloodfeeding and increase in sand fly deaths support the hypothesis that topical insecticides, including collars, could protect dogs against leishmaniasis. Bloodfeeding rates of sand flies were reduced from 1 month posttreatment not only on dogs with collars impregnated with deltamethrin but also on dogs treated with topical lotions of permethrin and fenthion. No effect was detected for dogs with diazinon collars. Although no significant difference between the antifeeding effects of DM, permethrin, and fenthion was detected, the reduction due to DM was greater than that due to either permethrin or fenthion at both 1 month and 2 months after treatment (Table 2). The failure to detect any significant effect on bloodfeeding 1 week after treatment is probably due to the time required for the lipophilic insecticides to spread in the dermal secretions over the dog's body (22). The manufacturers currently recommend that the DM collar be put on approximately 2 weeks before an anticipated sand fly challenge. However, a significant fivefold increase in death rates was detected for bloodfed sand flies on permethrin-treated dogs after 1 week, suggesting that topical application of permethrin lotion can have a relatively immediate effect. This effect is relatively short-lived, however, as no lethal effect on bloodfed sand flies was detected at 1 month or 2 months posttreatment, and no effect on unfed flies was detected at any time point. In contrast, topical fenthion application showed greater persistence, significantly increasing death rates of both unfed and bloodfed flies at 1 month after treatment. The lethal effect of the DM collar was the most persistent treatment, with significant effects on bloodfed flies after 1 month and on unfed flies at both 1 month and 2 months posttreatment. The failure to detect a significant impact on bloodfed flies after 2 months may be due to the relatively few bloodfed flies at that time point (due to the high antifeeding effect). The diazinon-treated collar did not affect the death rate of either bloodfed or unfed flies at any time.

The first evidence that topical insecticides could be used to

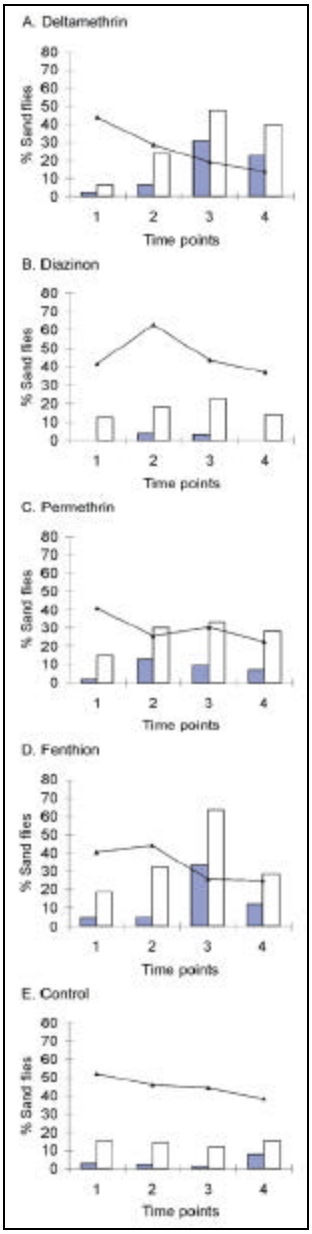

Figure. Comparison of various topical insecticide applications to protect dogs from sand fly bites. Represented are percentage bloodfed (line), dead bloodfed (dark columns), and dead unfed (white columns) sand flies. Time point (TP) 1: 0 days posttreatment (dpt); TP2: 5-12 dpt; TP3: 32-35 dpt and TP4: 58-65 dpt.

control ZVL came from experimental studies in China. After it was demonstrated that DM bath treatment reduced the bloodfeeding rate and survival rate of *Phlebotomus chinensis* sand flies exposed to an unnatural host (hamsters) (23), researchers found that the bloodfeeding rates of *P. chinensis* exposed to dogs for 8 hours were significantly reduced (from 62% to 4%) by dipping dogs in 25 ppm DM and that none of the sand flies (unfed or fed) exposed to treated dogs survived (18); this effect persisted for up to 104 days. Field evidence for the impact of topical insecticides comes from a community-based trial in China, where ZVL transmission was apparently interrupted after 2 years of treatment (two rounds per year) of all village dogs in 50 ppm DM baths (24). However, the trial results must be interpreted with caution, as no control villages were included in the study.

In contrast to insecticide lotions, the effects of DM collars on sand fly bloodfeeding and survival persisted for up to 8 months under experimental conditions in France using colonized *P. perniciosus* (25). Once collars had been applied for 2 weeks, blood-feeding consistently dropped by 90% and death rates increased by 51% during the experiment. When similar trials were carried out in Spain (26), the antifeeding effect dropped significantly from >90% during the first 4 months to 84% after 6 months, and the lethal effect dropped steadily from 76% after 2 weeks to 42% after 6 months. Both trials demonstrate that, when collars are used, the effects persist much longer than when dogs are dipped in DM. The potentially wide applicability of DM collars for protecting dogs against sand flies has since been demonstrated by experimental trials with *P. papatasi* in Iran (27).

Comparisons of the results of our trial with those of similar studies with DM collars (25-27) are difficult because of differences in experimental protocols. For example, we used wild-caught *Lu. intermedia,* whereas Killick-Kendrick et al. (25) and Lucientes (26) used 7- to 15-day-old, colonized *P. perniciosus.* This may account for the relatively low lethal effect detected in our trial, as old and colonized flies tend to be more sensitive to low doses of insecticide. Second, whereas dogs were exposed to high sand fly densities (mean 155 female flies per dog recovered after 2 hours) in the study by Killick-Kendrick et al. (25), our dogs were exposed to low fly densities (mean 49 female flies per dog recovered after 7 hours). The longer exposure time in our experiments, which was chosen to increase sand fly bloodfeeding rates and contact time with the various treatments, may have contributed to the decreased sand fly recovery rates we observed. Third, in contrast to all previously reported studies, we chose not to sedate the dogs to make the trial conditions as natural as possible. This provides a further reason for the slightly lower recovery rates of sand flies after exposure, compared with previous studies.

The potential protection against sand fly-transmitted diseases afforded by collars to individual dogs depends solely on their antifeeding effect. Although trials measuring impact on dog infection rates are still required, the entomologic results we report strongly indicate that DM collars may be recommended to dog owners to protect their dogs from leishmaniasis. This could include dog owners traveling to leishmaniasis-endemic countries, such as in southern Europe, as imported leishmaniasis cases of dogs with a travel history to Mediterranean leishmaniasis-endemic areas are increasingly common (15,28). With the recent change in the United Kingdom quarantine laws, this is likely to be of increasing concern to British dog owners.

The putative epidemiologic impact of DM collars on leishmaniasis transmission will depend on reducing not only the number of sand flies feeding on dogs but also the survival of those flies that do feed so that they are less likely to transmit *Leishmania* when taking a subsequent bloodmeal on a susceptible dog or human. Combining the effects on the bloodfeeding and death rates, we calculate that DM collars reduced the number of bloodfed flies that survive 24 hours by an average of 91% at 1 month posttreatment and by 81% at 2 months posttreatment. This was greater than the reductions of 61% (not significant) and 37% (p = 0.010) for

Table 2. Percentage reduction in sand fly bloodfeeding after application of topical insecticide

	Percent reduction in sand fly bloodfeeding (95% CI)					
TM	5-12 dpt		32-36 dpt		58-65 dpt	
DM	37.6	(5.3, 68.0)	56.7	(38.7, 70.5)	68.5	(42.8, 77.6)
DZ	-37	(-77.1, 13.3)	1.4	(-23.1, 24.7)	3.4	(-40.1, 40.0)
PM	44.1	(-7.2, 76.2)	31.6	(7.8, 51.4)	49.2	(14.3, 61.6)
F	4.2	(44.8, 48.3)	41.4	(13.3, 62.9)	43.1	(53.0, 56.0)

Abbreviations: CI, confidence intervals; DM, deltamethrin-impregnated dog collar; dpt, days post treatment; DZ, diazinon-impregnated dog collar; F, fenthion topical lotion; PM, permethrin topical lotion; TM, treatment.

fenthion treatment, and 37% (p = 0.001) and 41% (p = 0.019) for permethrin treatment, at 1 month and 2 months, respectively. Thus, there appears to be a clear advantage in terms of effectiveness in using DM collars versus the two topical lotions. Another advantage of collars is that their presence on dogs, when following up treated dogs during a control campaign, is proof that the insecticide is applied. Balancing these advantages, pour-on lotions are probably easier to use, and wear and tear is not a concern. Further studies are clearly needed to decide the optimal mechanisms of insecticide application and delivery (topical lotions, sprays, powders, dips, collars, or ear tags) before widespread implementation can be recommended.

In conclusion, the entomologic results reported here are sufficiently encouraging to warrant the undertaking of trials measuring the epidemiologic impact of communitywide DM collar implementation. Trials are required in a variety of endemic ZVL zones, as the impact of targeting domestic dogs in a given zone will be determined by the relative importance of sylvatic canids or other mammals as alternative reservoir hosts. Where topical insecticide applications are shown to be effective in intervention trials, the choice of whether to use topical lotions or insecticide-impregnated dog collars as a public health tool will ultimately depend on 1) the relative strength and persistence of their effects on sand fly bloodfeeding and survival; 2) the cost of the intervention; and 3) the practical applicability of these tools in the field (e.g., the willingness of the community to apply the topical formulation to their dogs or the efficiency with which they replace collars that have detached). The implementation of any topical insecticide treatment (including collars) is more likely to have the consent of a population at risk than the highly unpopular dog culling policy that is currently practiced.

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Mr. Reithinger is a doctoral student at the London School of of Hygiene & Tropical Medicine. He has been working on leishmaniasis epidemiology research projects in South America. His research interests include molecular diagnostics, epidemiology, immunology, control, and economics of infectious diseases.

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Ehrlichia-Infected Ticks on Migrating Birds

Anneli Bjöersdorff,*† Sven Bergström,‡ Robert F. Massung,§ Paul D. Haemig*‡, Björn Olsen*‡*

Kalmar County Hospital, Kalmar, Sweden; †Lund University, Lund, Sweden; ‡Umeå University, Umeå, Sweden; and §Centers for Disease Control and Prevention, Atlanta, Georgia, USA

During the spring of 1996, an estimated 581,395 *Ehrlichia*-infected ticks were imported into Sweden by migrating birds. *Ehrlichia* gene sequences found in ticks collected from these migrating birds were identical to those of granulocytic ehrlichiosis found in domestic animals and humans in Sweden. These findings support the idea that birds may play a role in dispersing *Ehrlichia*.

The genus *Ehrlichia* contains several species of intracellular bacteria capable of causing clinical disease in humans and animals. Ehrlichiosis caused by *Ehrlichia* of the *Ehrlichia phagocytophila* genogroup has been diagnosed in horses, dogs, and cats in Sweden, as well as in cattle and sheep (1). Cases of ehrlichiosis have been reported among humans in Scandinavia (2), and in Sweden, *Ehrlichia* have been detected in the tick *Ixodes ricinus* (3).

A common behavior of migrating birds is to feed and rest at stopover sites along their routes (4,5). At these sites, ticks and other ectoparasites may attach, and later detach along the migration route or in breeding areas. New foci of tick-borne diseases can be established in this way (6,7). Several investigations in Europe and the Middle East have examined the role of birds as carriers of ticks infected with tick-associated arboviruses and the Lyme borreliosis agent, *Borrelia burgdorferi* sensu lato (7). However, the involvement of birds in the ecology and epidemiology of ehrlichiosis has not yet been studied.

The Study

Our investigation was designed to determine the frequency of *Ehrlichia*-infected ticks on migrating birds in Sweden and estimate the number of *Ehrlichia*-infected ticks being imported and exported by these birds. Ticks were collected from migratory passerine birds at a stopover site in southern Sweden (east coast of Öland), identified, and checked for the presence of *Ehrlichia* by polymerase chain reaction (PCR). To determine if different genomic species of *Ehrlichia* could be found in bird-borne ticks and indirectly in the birds, all *Ehrlichia*-positive PCR products were further subjected to DNA sequencing analysis.

A total of 3,054 birds (1,204 in the spring and 1,850 in the fall) of 56 species were caught and examined for ticks. One hundred sixty-five ticks, all *I. ricinus*, were collected from 73 birds of 18 species (Table), for a tick infestation frequency of 2.4% (73/3,054) and a relative density of 0.054 ticks per bird (165/3,054). Fifty-one larvae, 112 nymphs, and 2 adult females of *I. ricinus* were found (Table), suggesting that nymphs are more common than larvae on birds and that adult *I. ricinus* rarely feed on birds.

Ehrlichia DNA was detected in none of the 51 larvae, in 8.0% (9/112) of the nymphs, and in none of the adult ticks. *Ehrlichia* DNA was detected in 8.9% (7/79) of the nymphs in the spring and 6.1% (2/33) in autumn.

Ehrlichia 16SrRNA gene sequences in the nine positive nymphs were analyzed by DNA sequencing. The gene sequences (base positions 20-570 corresponding to base numbering for the U.S. human granulocytic ehrlichiosis (HGE) agent 16S rDNA; GenBank Accession Number U02521) were identical to *E. phagocytophila* genogroup sequences found in clinical cases of granulocytic ehrlichiosis in horses, dogs, cats, and humans in Sweden (1).

Conclusions

Every spring, approximately 100 million birds migrate through Sweden, importing large numbers of potentially infectious ectoparasites. If the frequency of infected ticks is assumed to be similar to that in this study (7 *Ehrlichia*-infected ticks on 1,204 birds), 581,395 *Ehrlichia*-infected ticks were imported into Sweden by birds during the spring migration of 1996. The bird species we found carrying *Ehrlichia*-infected ticks (Table) breed in FennoScandia and arrived from wintering grounds in continental Europe, North Africa, and sub-Saharan Africa. During the fall migration, the frequency was lower (2 *Ehrlichia*-infected ticks found on 1,850 birds). However, even if the same number of birds migrated in the fall as in the spring, an estimated 108,108 *Ehrlicha*-infected ticks were exported from Sweden that fall by the birds. These figures support previous studies indicating that migratory birds are an important component in the dispersal of ticks infected with medically important pathogens (8).

Only one species of tick (*I. ricinus*) was recorded in this study. This species is the main vector of *B. burgdorferi* sensu lato, *E. phagocytophila*, and tick-borne encephalitis virus, both in Sweden and the rest of western and central Europe. The facts that a large number of birds are infested with this tick and that the *Ehrlichia* 16S rRNA gene sequences found in these ticks are identical to the gene sequences found in HGE patients in Sweden, Slovenia, and the USA suggest that birds may play an important role in the dispersal of I. ricinus infected with *Ehrlichia* and may contribute to the distribution of granulocytic ehrlichiosis (9). We found no *I.*

Address for correspondence: Björn Olsen, Research Center for Zoonotic Ecology and Epidemiology, Department of Infectious Diseases, Kalmar County Hospital, SE-391 85 Kalmar, Sweden; fax: 46 90-13 30-06; e-mail: Bjornol@ltkalmar.se

Table. Migrating birds carrying *Ixodes ricinus* ticks and the frequency of infection with *Ehrlichia* sp. in bird-borne ticks, Sweden, 1996

Bird species[a]	No. of birds infested/ no. examined (%)	No. of ticks	*Ehrlichia* DNA detected by PCR of ticks examined		
			Larvae	Nymphs	Adults
Starling *(Sturnus vulgaris)*	1/10 (10)	1			0/1
Dunnock *(Prunella modularis)*	1/33 (3)	1		0/1	
Reed Warbler *(Acrocephalus scirpaceus)*	1/5 (20)	1		0/1	
Icterine Warbler *(Hippolais icterina)*	1/12 (8)	1	0/1		
Garden Warbler *(Sylvia borin)*	2/79 (2.5)	2	0/1	0/1	
Blackcap *(S. atricapilla)*	3/26 (11.5)	3	0/1	1/2	
Whitethroat *(S. communis)*	1/29 (3.4)	1	0/1		
Greenish Warbler *(Phylloscopus trochiloides)*	1/1 (100)	1	0/1		
Willow Warbler *(P. trochilus)*	3/1,025 (2.9)	4	0/4		
Goldcrest *(Regulus regulus)*	1/892 (0.1)	1		0/1	
Pied Flycatcher *(Ficedula hypoleuca)*	1/22 (4.5)	1		0/1	
Collared Flycatcher *(F. albicollis)*	1/2 (50)	1		0/1	
Redstart *(Phoenicurus phoenicurus)*	7/76 (9.2)	28	0/11	1/17	
Robin *(Erithacus rubecula)*	33/655 (5)	54	0/14	3/40[b]	
Thrush Nightingale *(Luscinia luscinia)*	5/27 (18.5)	22	0/8	2/14	
Blackbird *(Turdus merula)*	5/41 (12.2)	27	0/7	1/19[c]	0/1
Song Thrush *(T. philomelus)*	4/24 (16.7)	14	0/2	1/12	
Bullfinch *(Pyrrhula pyrrhula)*	2/27 (7.4)	2		0/2	
Total	73/2,986 (2.4)	165	0/51	9/112	0/2

[a]Bird species sampled that did not carry ticks are not listed.
[b]One of these infected ticks was collected in the autumn.
[c]This infected tick was collected in the autumn.

ricinus larvae infected with *Ehrlichia*, which may suggest that birds are incompetent reservoirs of *Ehrlichia* but act as carriers of infected ticks. In this way, avian transport of *Ehrlichia* might differ from that of *B. burgdorferi* sensu lato. In the latter disease, birds not only carry infected ticks of all stages but can also carry latent infections of *Borrelia* that can be reactivated by the stress of migration (10). However, the potential of a vertebrate to function as a tick host and a reservoir for *Ehrlichia* depends on many factors, including the host's density in the tick habitat, the degree of contact between the potential tick vector and the host, and its infectivity. Therefore, the question of whether birds act as reservoirs of *Ehrlichia* has not yet been conclusively answered, and additional studies of various bird host species should be conducted over longer time spans than our study. Moreover, the reservoir competence of birds should be studied by isolation or detection of *Ehrlichia* after natural or experimental infection.

The apparent increase of granulocytic ehrlichiosis in animals and humans during the last few decades may be the result of several factors, such as increased awareness of this disease and the distribution of reservoirs, vectors, and host animals. Whether passerine birds have affected this increase has not yet been conclusively demonstrated. However, our data suggest that birds are important in the dispersal of *Ehrlichia*.

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Dr. Björsdorff is a consultant in medical microbiology in the Department of Clinical Microbiology and at the Research Center for Zoonotic Ecology and Epidemiology, Kalmar, Sweden.

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Mycobacterium tuberculosis Beijing Genotype Strains Associated with Febrile Response to Treatment

Reinout van Crevel,* Ron H.H. Nelwan,† Wilma de Lenne,*
Yelilsan Veeraragu,‡ Adri G. van der Zanden,§ Zulkifli Amin,†
Jos W.M. van der Meer,* and Dick van Soolingen¶

*University Medical Center Nijmegen, Nijmegen, the Netherlands; †University of Indonesia, Jakarta, Indonesia; ‡Trisakti University, Jakarta, Indonesia; §Gelre Hospitals, Apeldoorn, the Netherlands; and ¶National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands

DNA fingerprinting has demonstrated predominance of the Beijing genotype among *Mycobacterium tuberculosis* strains isolated in Southeast Asia. We prospectively examined the occurrence of Beijing genotype strains in tuberculosis patients in Indonesia. Early in treatment, patients infected with Beijing genotype strains more often had fever unrelated to disease severity, toxicity, or drug resistance, indicating that Beijing genotype strains may have specific pathogenic properties.

In 1995, DNA fingerprinting showed that >80% of a collection of *Mycobacterium tuberculosis* isolates from China belonged to a genetically closely related group of bacteria, the Beijing genotype family (1). Strains of this genotype family also dominated in neighboring countries in Southeast Asia, whereas prevalence was lower on other continents (1). In a recent study, 50% of 563 isolates from Vietnam belonged to the Beijing genotype; moreover, the occurrence of these strains correlated strongly with young age and hence with active transmission of tuberculosis (TB) (2). Strains of the Beijing family were thought to have expanded recently from an evolutionary lineage with an unknown selective advantage over other *M. tuberculosis* genotypes (1). Strain W, a highly drug-resistant strain that caused large nosocomial outbreaks in New York City in the early 1990s (3,4), is an evolutionary branch of the Beijing genotype family (5).

Worldwide, Indonesia has the third highest number of TB patients, with an estimated 591,000 cases in 1998 (6). No data have been published from Indonesia on the distribution of *M. tuberculosis* genotypes.

The Study

We prospectively collected demographic and clinical data and performed DNA fingerprinting of *M. tuberculosis* isolates from a cohort of patients in Jakarta. We assessed prevalence of the Beijing genotype strains and compared drug resistance and clinical course of patients infected with Beijing and other genotype strains of *M. tuberculosis.*

From December 1998 through March 1999, 121 consecutive patients were enrolled at Perkumpulan Pembertasan Tuberkulosa Indonesia, an outpatient TB clinic in a densely populated area in Jakarta. Informed consent was obtained from all patients. Demographic data, symptoms and signs, risk factors for TB, and details about previous antituberculosis therapy were recorded. From three large-volume sputum samples, microscopy for acid-fast bacilli (AFB) and culture for *M. tuberculosis* were performed. Chest X-rays taken before start of treatment were evaluated by two experienced pulmonologists. HIV status was determined. When TB was bacteriologically confirmed, a standard four-drug regimen (isoniazid [INH], rifampicin, pyrazinamide, and ethambutol) was prescribed (7). During follow-up examinations, symptoms, body temperature, and weight were recorded.

After standard processing of sputum samples, culture was performed in 3% Ogawa's medium. Twice a week, slants were examined for colonies. At the National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands, susceptibility testing of patient isolates was done by using serial dilutions of five antituberculosis drugs on Middlebrook's medium (8). For pyrazinamide, the same method was used after pH of the medium was adjusted to 5.70–5.75. MICs used to define drug resistance were INH 0.2 mg/L, rifampicin 1 mg/L, ethambutol 5 mg/L, streptomycin 5 mg/L, and pyrazinamide 50 mg/L.

Genotyping of mycobacterial isolates was done by restriction fragment length polymorphism (RFLP) typing. Extraction of DNA from *M. tuberculosis* strains and Southern blotting with labeled IS*6110* DNA as a probe were done by a standard DNA fingerprinting method (9). Spacer oligonucleotide typing (spoligotyping) of *M. tuberculosis* DNA from patient isolates was done as described (10). This method reliably identifies Beijing genotype strains on the basis of specific reaction with only 9 (35 to 43) of the 43 spacers used in spoligotyping (1,2). All the identified Beijing genotype strains also had highly similar but in most cases not identical IS*6110* RFLP patterns, with a high number of bands. From culture-negative patients, *M. tuberculosis* DNA for spoligotyping was directly isolated from sputum smears (11). Computer-assisted analysis of IS*6110* fingerprints and

Address for correspondence: Reinout van Crevel, Department of Internal Medicine, University Medical Center Nijmegen, P.O Box 9101, 6500 HB Nijmegen, the Netherlands; fax: 0031-243541734; e-mail: r.vancrevel@aig.azn.nl

spoligotyping patterns was done with Bionumerics 4.0 (Applied Maths, Kortrijk, Belgium).

Descriptive result sare reported as median (range) for continuous variables and as percentages for categorical data. The Mann-Whitney U test was used for comparison of continuous variables and the Pearson chi-square test for proportions. The level of significance was $p<0.05$.

In 121 consecutive patients with a clinical diagnosis of TB, direct examination of at least one sputum smear was positive for AFB in 89 (73%), and *M. tuberculosis* was cultured from 83 (73%) of 113 cultures performed. IS*6110* restriction fragment patterns were analyzed in combination with the respective spoligopatterns of these samples (Figure). Most (90%) strains had a fingerprint pattern that differed from the RFLP patterns of other strains in this study.

Four miniclusters of two identical RFLP patterns were found. Besides visiting the same clinic, no epidemiologic connection could be made between patients infected with these strains. Laboratory contamination seems unlikely, as patient specimens were collected on separate days. DNA of two isolates did not show hybridization with the IS*6110* probe, but yielded positive reactions in spoligotyping. Such *M. tuberculosis* strains without IS*6110* DNA have been described (12). Twenty-seven strains had a high number of IS*6110*-containing restriction fragments and a high degree of similarity (≥66%) among patterns. This homogeneous group of isolates represented the Beijing family of genotypes, as confirmed by spoligotyping (Figure). Direct spoligotyping on sputum smears of culture-negative patients added another four patients with Beijing strain infections. Of 92 *M. tuberculosis* strains analyzed (83 cultured isolates by IS*6110* RFLP and spoligotyping and 9 stained sputum smears by spoligotyping only), 31 strains (34%) were Beijing genotypes.

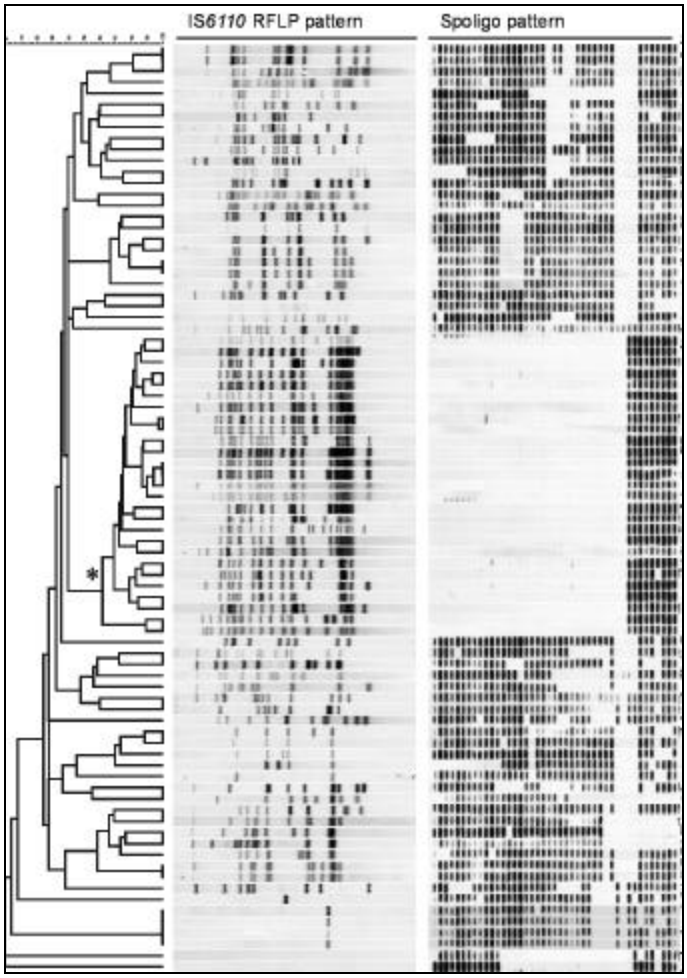

Figure. Dendrogram showing similarity of the 83 IS*6110* restriction fragment length polymorphism patterns of *Mycobacterium tuberculosis* isolates from Jakarta, in combination with the respective spoligotype patterns. The branch in the dendrogram representing Beijing genotype isolates is indicated with an asterisk (*).

Cultures became positive for *M. tuberculosis* after 4.7 weeks for Beijing strains, compared with 5.2 weeks for other strains. As in a recent study in Vietnam, drug susceptibility testing showed a trend toward higher prevalence of resistance to INH (37% vs. 20%; p=0.09) and streptomycin (15% vs. 5%; p=0.16) in Beijing strains compared with other strains; however, these differences did not attain statistical significance. (Table). The two groups did not differ significantly in prevalence of multidrug resistance (7% vs. 4%). For both Beijing and other strains, drug resistance was found equally among age groups (data not shown).

The characteristics of patients infected with Beijing genotypes (n=31) were compared with those of patients infected with other strains (n=61) (Table). The age distribution in the two groups was similar, although no patients <16 years of age were evaluated. No relation was found between genotype and BCG vaccination status (Table). Of the patients infected with Beijing strains, 7 (23%) had received one or more antituberculosis drugs before, compared with 18 (29%) of patients infected with other strains. Only two patients (one in each group) had been prescribed a standard four-drug regimen for 6 months. On entering the study, patients did not differ significantly in the presence of fever, dyspnea, or hemoptysis, or in duration of symptoms (data not shown). The nutritional status in both groups of patients was similar, as judged by body mass index (17 vs. 16.9 kg/m^2). The number of smear-positive patients and the AFB density in sputum smears were similar in both groups (data not shown). Patients did not differ significantly by X-ray evaluation; the number of lung fields involved was similar, and an equal percentage in both groups had pulmonary cavities (Table). Only one patient, whose initial diagnosis was cavitary TB, was HIV positive; this patient was infected with a Beijing strain.

Patients were evaluated weekly. Most patients had an early, beneficial response to treatment. In both groups, 20% continued to lose weight during the first 2 months of the study. This loss was small (approximately 1 kg) and may have been due to observance of the Ramadan fast. Body weight increased a median of 2 kg (range 0 to 8 kg) in the remaining patients in both groups. No relation was found between drug resistance and changes in body weight. At 6 months, patients in both groups had gained weight equally (median 5 kg). No patient had active disease at this point, but treatment was extended in patients infected with strains resistant to multiple drugs.

Thirty-two percent of patients developed fever (>38°C, maximum 39.3°C) during the first weeks of treatment. No patient reported shaking chills during this period. This transient febrile response, which lasted 2 to 3 weeks, was found in 15 (48%) of 31 patients infected with Beijing strains compared with 13 (21%) of 61 patients with other strains (relative risk 2.3; 95% confidence interval 1.1–4.7). Drug resistance could not account for this finding: 46% of

Table. Drug resistance and patient characteristics by *Mycobacterium tuberculosis* genotype, Indonesia

Patient characteristics	Genotype	
	Beijing, n=31 (%)	Other, n=61(%)
Male	19 (61)	35 (57)
Age in years, median (range)	31 (19-68)	29 (17-70)
BCG scar	8 (26)	14 (23)
Chest X-ray		
cavities	16 (52)	29/55[a] (53)
bilateral disease	24 (77)	40/55 (73)
Febrile response to treatment[b]	15 (48)	13 (21)
Patient strain resistant to[c]	n = 27 (%)	n = 56 (%)
Isoniazid	10 (37)	11 (20)
Rifampicin	2 (7)	2 (4)
Pyrazinamide	0	3 (5)
Ethambutol	1 (4)	3 (5)
Streptomycin	4 (15)	3 (5)
Any prescribed drug	11 (39)	14 (25)

[a]Six chest X-rays in the non-Beijing group were evaluated by only one pulmonologist.

[b]Significant difference between groups (Mann-Whitney U-test; p=0.02).

[c] Eighty-three isolates were available for drug susceptibility testing.

patients infected with a fully susceptible Beijing strain compared with 19% of patients infected with susceptible non-Beijing strains had a febrile response (p=0.06). Neither did severity of disease account for the difference, as the febrile response was not associated with nutritional status or presence of pulmonary cavities (data not shown). Drug toxicity, which may also induce fever, was not established.

Conclusions

Worldwide, DNA fingerprinting has revealed extensive heterogeneity of *M. tuberculosis* genotypes (13). However, a distinct and predominant *M. tuberculosis* genotype, termed Beijing, has been found in the People's Republic of China and neighboring countries (1,14). Our study demonstrates that Beijing strains are also present in the Indonesian archipelago.

The prevalence of Beijing strains in China (85%), Mongolia (50%), South Korea (43%), Thailand (37%), Vietnam (50%), and Indonesia (34%) suggests that this clone spreads in Southeast Asia, where TB is endemic. Beijing genotype strains also account for a substantial proportion of multidrug-resistant TB cases in Azerbaijan and Estonia (15,16) and Cuba (17). Strain W, which also belongs to the Beijing genotype family (5), caused major outbreaks of multidrug-resistant TB during the past decade in the United States (3,4) and South Africa (18). In summary, all reports on the occurrence of Beijing genotypes show a clear correlation with drug resistance. In our study, 37% of the Beijing strains were resistant to INH. However, multidrug resistance was limited, making drug resistance unlikely as a single explanation for the predominance of Beijing strains in this population.

Different transmission rates may account for an unequal distribution of genotypes. In a TB outbreak in the United States, a particular *M. tuberculosis* genotype caused extensive transmission, as evaluated by skin test conversion (19). In Indonesia, outbreak investigations like these seem impossible in light of the high prevalence of TB and standard BCG vaccination, which hampers interpretation of tuberculin skin tests. However, indirect evidence supporting increased transmission of Beijing strains comes from a recent study in Vietnam, which demonstrated that Beijing strains were more prevalent among young patients (2). We could not confirm this in the Indonesian patients, but we did not investigate patients <16 years of age. In agreement with the study in Vietnam, we found no correlation between vaccination status and genotypes.

Disease severity in patients infected with Beijing or other genotypes seemed similar. However, our prospective evaluation revealed a different response to treatment. Forty-eight percent of the patients infected with Beijing strains and 21% of the patients infected with other strains had a transient febrile response shortly after start of treatment. Disease severity, drug toxicity, and drug resistance could not account for this difference. The increased risk of febrile response in patients infected with Beijing strains is unusual and suggests that these strains induce a different host response. In support of this hypothesis, Beijing genotype strains were recently found to elicit an altered cytokine response in two animal models (D. van Soolingen, unpub. data). This differential response may be related to the rapid spread of Beijing genotype strains. There may be an interesting parallel with the outbreak in the United States (19), since the causative strain in that outbreak, designated CDC1551, induced a more rapid and robust in-vitro production of pyrogenic cytokines such as interleukin-6 and tumor necrosis factor-α (20). Whether Beijing strains also elicit a different cytokine response in TB patients is a subject for future study.

Acknowledgment

We thank staff members of the outpatient clinic of Perkumpulan Pembertasan Tuberkulosa Indonesia in Central Jakarta for their help, Dr. Judanarso Dawud for providing laboratory facilities, and especially Dr. Julianti Gunawan of the Microbiology Department of Persahabatan Hospital. Dr. Iskandar Zulkarnain, head of the Division of Tropical Medicine and Infectious Diseases, University of Indonesia, provided staff to conduct this study. In the Netherlands, Jan Henraat and Mirjam Dessens performed drug susceptibility testing of patient isolates, and Petra de Haas conducted DNA fingerprinting and helped with data analysis.

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Dr. van Crevel is a resident in internal medicine at the University Medical Center Nijmegen, the Netherlands. His main research interest is the role of host defense in TB. In addition he is involved in clinical, bacteriologic, and operational studies on tuberculosis in Indonesia.

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Lactococcus lactis subsp. *lactis* Infection in Waterfowl: First Confirmation in Animals

Joaquín Goyache, Ana I. Vela, Alicia Gibello, María M. Blanco, Víctor Briones, Sergio González, Sonia Téllez, Cristina Ballesteros, Lucas Domínguez, and José F. Fernández-Garayzábal

Facultad de Veterinaria, Universidad Complutense, Madrid, Spain

We report the first description, confirmed by bacteriologic and molecular (polymerase chain reaction and pulsed-field gel electrophoresis) analysis, of an infection in animals caused by *Lactococcus lactis* subsp. *lactis*, affecting waterfowl.

Until recently, members of the genus *Lactococcus* were considered opportunistic pathogens (1,2). They are often misidentified as enterococci or streptococci (3,4), and the difficulties in correctly identifying them have probably hindered elucidation of their clinical significance. However, the number of clinical cases associated with infections by these microorganisms has increased in the last decade in both humans and animals (5-7). *Lactococcus lactis* subsp. *lactis*, *L. piscium*, and *L. garvieae* are recognized as the species with clinical significance for human and veterinary medicine (2,8). In humans, *L. garvieae* and *L. lactis* subsp. *lactis* have been associated with endocarditis (9,10) and have also been isolated from clinical samples of blood, skin lesions, and urine (5,7). In veterinary medicine, *L. garvieae* and *L. piscium* are pathogenic for various fish species (8,11,12), and *L. garvieae* causes mastitis in ruminants (13,14). However, infection by *L. lactis* subsp. *lactis* in animals has not previously been reported. We present the first microbiologic and molecular evidence for infection produced by *L. lactis* subsp. *lactis* in waterfowl.

The Study

From September to November 1998, a mass die-off was detected among waterfowl in southwestern Spain, affecting >3,000 birds (0.6% of the total waterfowl population in the area). The species most affected were coots (*Fulica atra*) (26.9%), shovelers (*Anas clypeata*) (25.1%), and mallards (*Anas platyrrhynchos*) (13.8%). Overall, 20% of the birds died. Affected birds showed general weakness, evidenced by drooping wings and sluggishness; approximately 50% had respiratory distress. At necropsy, most animals had mild lung congestion; no other lesions were found at postmortem examination.

Samples from the lungs, liver, and spleen of five diseased birds (one mallard, S-15; three shovelers, S-16, S-18, and S-19; and one coot, S-17) were submitted to the Animal Health Department at the School of Veterinary Medicine of Madrid for microbiologic analysis. After 48 hours of incubation at 37ºC, pure cultures of weakly α-hemolytic catalase-negative cocci were obtained on blood agar plates from samples of lung (S-15, S-16, and S-17) and liver and spleen (S-15, S-16, S-17, and S-18). All 11 clinical isolates had an identical biochemical profile, which was identified as *L. lactis* subsp. *lactis* by the Rapid ID 32 Strep system (bioMérieux España, S.A., Madrid).

L. lactis subsp. *lactis* and *L. garvieae* are the two species more frequently found in human and animal infections (2). Routine clinical microbiologic diagnosis requires accurate discrimination of the two species, as their similar biochemical reaction patterns may lead to misidentification (2,4). Although physiologic tests, differences in antimicrobial susceptibility, whole-cell protein, and DNA or RNA analysis (4,7,13) have been proposed to distinguish them, some of these techniques are not reliable or may be too time-consuming, limiting their use for routine identification. For these reasons, the clinical isolates were also identified by a polymerase chain reaction (PCR) assay, which has been successfully used to identify many other pathogens (15,16).

Specific primers LLF 5'-GCAATTGCATCACTCAAAGA and LLR 5'-ACAGAGAACTTATAGCTCCC were designed from diagnostic regions of the *L. lactis* subsp. *lactis* 16S rRNA gene sequence (accession number M58837). PCR amplifications were performed in a 100-µL reaction volume containing 150 ng each of the two primers, 1 mM each of deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase (Biotools, Inc., Madrid, Spain), and 25 ng of template DNA in 1x reaction buffer. The amplification was carried out in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA), under the following conditions: initial denaturation at 94ºC for 2 minutes, followed by 30 cycles of denaturation for 1 minute at 92°C, primer annealing for 1.5 minutes at 50°C, primer extension for 2 minutes at 72°C, and a final extension of 5 minutes at 72°C. The following bacterial strains were used to test the specificity of the PCR assay: *L. lactis* subsp. *lactis*, ATCC 19435 and ATTC 11007; *L. garvieae*, NCFB 2155; four clinical isolates of *Lactococcus garvieae* (1336, 1458, 1982, and 4294, isolated from lactococcosis in trout); *L. piscium*, NCFB 2778; *Streptococcus iniae*, ATCC 29187; *Vagococcus fluvialis*, NCFB 2497; and *Enterococcus faecalis*, CECT 481. All the *L. lactis* subsp. *lactis* clinical isolates generated an expected PCR amplification product of 650 bp. No amplification was observed with any other *Lactococcus* species tested, indicating the specificity of the PCR assay (Figure 1). These results confirmed those of the biochemical identification, as well as the utility of this PCR assay for spe-

Address for correspondence: José F. Fernández-Garayzábal, Departamento Patología Animal I (Sanidad Animal), Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain; fax: +34 91 3943908; e-mail: garayzab@eucmax.sim.ucm.es

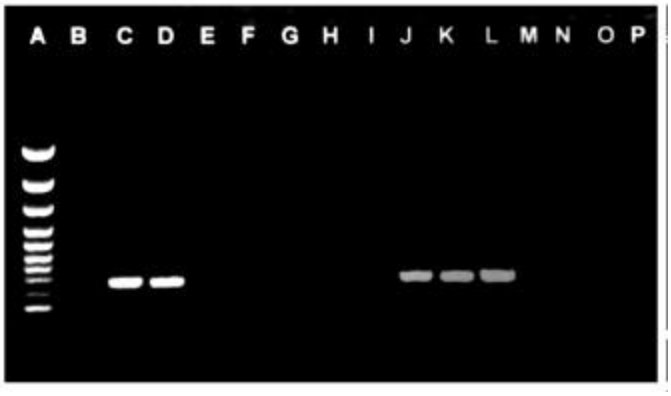

Figure 1. Polymerase chain reaction (PCR) products formed from total DNA of lactococcal strains with primers LLF and LLR. Lane A, molecular size marker; lane B, negative control. *Lactococcus lactis* subsp. *lactis* ATCC 19435 (lane C), *L. lactis* subsp. *lactis* ATCC 11007 (lane D), and the clinical isolates (lanes J, K, and L) generated a PCR amplification product of 650 bp. No amplification was observed from *L. garvieae* NCFB 2155 (lane E), clinical isolates of *L. garvieae* (lanes F, G, H and I), *L. piscium* NCFB 2778 (lane M), *Streptococcus iniae* ATCC 29187 (lane N), *Vagococcus fluvialis* NCFB 2497 (lane O), and *Enterococcus faecalis* CECT 481 (lane P).

cific, rapid, and accurate identification of this microorganism. In addition, the fact that no PCR amplification was observed with clinical isolates when tested with a PCR specific for *L. garvieae* (17) corroborates the identification (data not shown).

Pulsed-field gel electrophoresis (PFGE) with the enzyme *Sma*I was used for molecular characterization of the clinical isolates, as described by Vela et al. (18). This technique has been successfully applied for strain identification and epidemiologic investigations of lactococci (19,20). Indistinguishable restriction patterns were obtained from all clinical isolates (Figure 2), which were clearly distinct from the pulsotypes of *L. lactis* subsp. *lactis* ATCC 19435, *L. garvieae*, and *L. piscium* (data not shown). These PFGE results indicate that infection was produced by a single strain of *L. lactis* subsp. *lactis* in all the animals studied. The bacteriologic and molecular results clearly confirm the isolation of *L. lactis* subsp. *lactis* in waterfowl, the first confirmation of infection in animals caused by this microorganism.

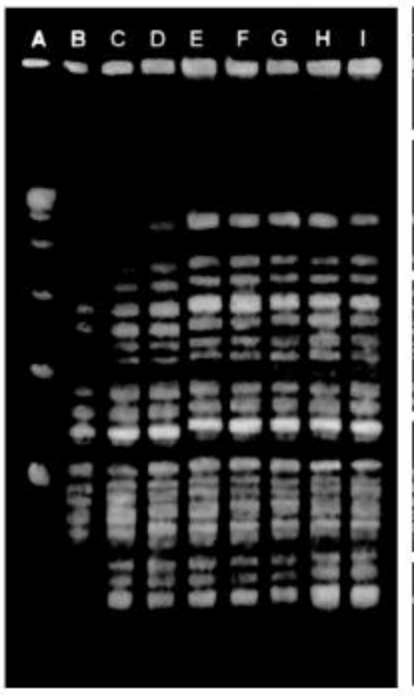

Figure 2. Pulsed-field gel electrophoresis patterns of *Sma*I digests of genomic DNA of *Lactococcus lactis* subsp. *lactis* clinical isolates. Lane A, molecular weight marker; lanes B, D, F, and H, liver isolates from samples S-15, S-16, S-17, and S-18; lanes C, E, and G, lung isolates of samples S-15, S-16, and S-17; and lane I, spleen isolate from sample S-18.

As *L. lactis* subsp. *lactis* is considered nonpathogenic for animals (1,2) and no additional histopathologic or toxicologic studies could be carried out in the diseased animals, we cannot rule out other possible causes for the mass deaths. Therefore, although the PFGE results, together with the recovery of *L. lactis* subsp. *lactis* in pure culture from the clinical samples, may suggest clinical significance, no direct link between the *L. lactis* subsp. *lactis* infection and this episode can be established. Further studies are necessary to elucidate the exact pathogenic potential of this microorganism for waterfowl.

Wild animals, including waterfowl, are known reservoirs for various pathogens (21). We can only speculate about the possibility that waterfowl may be a reservoir for this bacterium. However, wild animal reservoirs for other species of lactococci have been described (22).

Dr. Goyache is associate professor, Facultad de Veterinaria, Universidad Complutense de Madrid. His responsibilities include research and teaching related to clinical microbiology of wildlife and exotic animals.

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First Epidemic of Echovirus 16 Meningitis in Cuba

Luis Sarmiento,* Pedro Mas,* Angel Goyenechea,* Rosa Palomera,* Luis Morier,* Virginia Capó,* Ibrahim Quintana,† and Manuel Santin†

*"Pedro Kouri" Tropical Medicine Institute, Havana, Cuba and †Ministry of Public Health, Havana, Cuba

From April to September 2000, an epidemic of aseptic meningitis spread throughout Cuba, with 16,943 reported cases. Virologic studies identified echovirus 16 as the cause of this epidemic. This is the first reported isolate of echovirus 16 from patients with viral meningitis in Cuba.

The Study

From April 30 to September 2000, Cuban health authorities reported a marked increase in acute aseptic meningitis cases. The peak incidence occurred in July (49.6 cases per 100,000 population) (Figure 1). The index cases appeared in Cienfuegos Province, in the central part of the country. Subsequently, the disease was widely distributed: 16,943 cases were reported from April to September 2000.

Most patients were children ≤15 years old. The age-specific peak incidence occurred in infants ≤1 year of age, but none were neonates (Figure 2). Vomiting (91.5%), headache (88.1%), and fever (72.8%) were the predominant clinical manifestations; few patients had diarrhea (11.8%) or skin rash (6.8%). Cerebrospinal fluid (CSF) cell counts showed >100 leukocytes/mm^3 in 40% of patients. The rest of the CSF counts were 10-100 leukocytes/mm^3. No deaths related to aseptic meningitis were reported, and all patients recovered completely.

To establish the outbreak-associated enterovirus, 54 CSF, 76 fecal samples, and 31 paired sera from acute and convalescent phases were obtained from 98 children with symptoms suggestive of aseptic meningitis. CSF and fecal samples were collected only once per child, at onset of symptoms. The children were admitted into hospitals in 11 of Cuba's 14 provinces. Specimens were collected from May 5 to August 11, 2000, and transported frozen to our laboratory.

We used conventional methods for diagnosis of enterovirus and an in-house-developed reverse transcriptase-nested polymerase chain reaction (RT-N-PCR) assay of CSF specimens. For the enterovirus genome detection, RNA was extracted from 250 µL of CSF using TRIzol (Life Technologies TM, GIBCO BRL; Grand Island, NY), according to the manufacturer's instructions. RNA amplification was performed by the method of Kilpatrick et al. (1), except that two amplification rounds were used. Oligonucleotides were derived from the 5'noncoding region (5'NCR), a highly conserved zone in enterovirus serotypes that allows a near-universal amplification of the enteroviruses (2,3).

Specificity of the RT-N-PCR assay was confirmed by detection of amplification products of RNA extracted from cell culture fluids infected with polioviruses sabin 1-3, coxsackievirus A9 and A16, and the most common epidemic types of echoviruses (echovirus 4,6,9,11,30), as well as by the absence of amplified cDNA with RNA extracted from herpesvirus family-infected and noninfected monkey kidney cells (Vero). The recognized sensitivity (0.01 50% tissue culture infective dose) and specificity of our enterovirus-RNA detection assay allowed us to detect specific enterovirus RNA sequences in 46.3% (25 of 54) of CSF specimens. We do not believe that positive results were due to PCR contamination because universal precautions were adopted (4). An equal number of test samples and reagent controls were processed in each test batch to prevent contamination of RNA extrac-

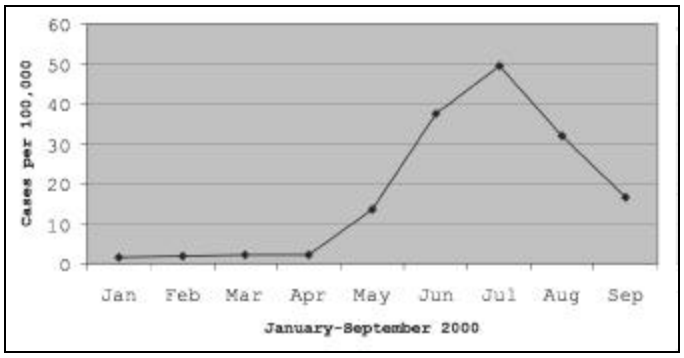

Figure 1. Monthly distribution of aseptic meningitis incidence (cases

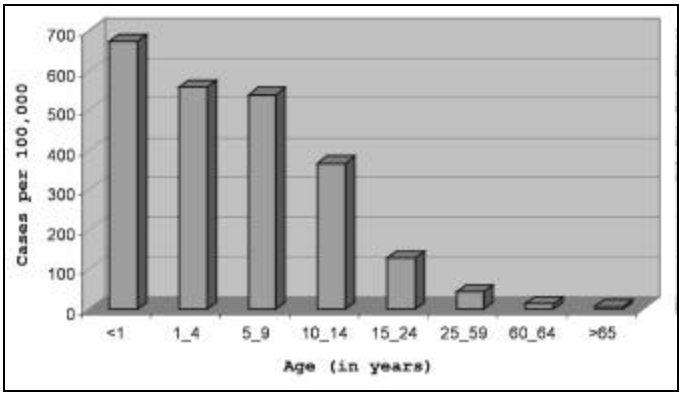

Figure 2. Age-specific incidence (cases per 100,000 population) of reported aseptic meningitis cases in Cuba, January through September 2000.

Address for correspondence: Luis Sarmiento Perez, Department of Virology, "Pedro Kouri" Tropical Medicine Institute, Autopista Novia del Mediodía, km 6, PO Box 601, Marianao 13, Havana, Cuba; fax: 53-7-220633 and 53-7-246051; e-mail: sarmiento@ipk.sld.cu

tion by extraneous nucleic acid sequences; PCR reagents were amplified by PCR. The reagent controls' reaction verified the absence of contamination at all stages of the PCR process.

The application of this in-house RT-N-PCR assay guaranteed the rapid etiologic diagnosis of this epidemic. However, our RT-N-PCR is unable to determine the enterovirus serotype, which is necessary to understand the epidemiology of enterovirus infections.

For enterovirus isolation, 200 µL of CSF and fecal specimens were inoculated in duplicate into tubes covered with monolayers of fibroblastic diploid embryonic human cells (PhuE-1) and Vero cells. From the 76 fecal specimens inoculated, 45 (59.2%) induced cytopathic effect (CPE). This CPE was only evident in the PhuE-1 cell monolayers; Vero cells remained unchanged. Viral isolation was possible from all the tubes showing CPE. However, no enterovirus strains were recovered by cell culture isolation from any CSF specimen. These discrepancies could be explained by the massive excretion of viral particles in feces. Moreover, previous studies have demonstrated that enteroviruses are isolated from CSF in only a few cases with acute aseptic meningitis because viral particles are present in low titers in CSF (5-7). Nevertheless, a higher positivity was obtained from 20 CSF samples when RT-N-PCR was used than for 20 samples of feces from the same patients when evaluated by viral isolation. Only 10 of the 16 CSF specimens positive by PCR could be correlated with a positive fecal culture, whereas the 4 PCR-negative CSF samples correlated well with the absence of viral isolation from the related fecal specimens.

CPEs produced by isolated strains were typical of those characterizing enteroviruses (e.g., cell rounding followed by shrinkage and degeneration of the cell sheet). However, at the beginning of the epidemic, isolated strains produced a CPE that progressed slower (slow-CPE) than typical enterovirus isolates do. The inoculated cultures had to be subpassaged weekly at least 5 times to obtain the typical degenerative CPE of enterovirus. By the late stages of the epidemic, CPE produced by isolated strains became evident at the first passage (fast-CPE). No slow-CPE isolates were detected late in the epidemic, and there was no evidence of "fast CPE-to-slow CPE" reversion of isolates late in the epidemic. Nevertheless, no changes in the clinical outcome of the infection were observed during the epidemic. It is tempting to speculate that the genetic constitution of the selected viral population could be substantially different from that of the original strain. This phenomenon could explain the variation in CPE during the epidemic.

All strains from the epidemic were identified as echovirus 16 by a neutralization test using Lim-Benyesh-Melnick antisera pools. To corroborate the infecting serotype, presence of a fourfold or greater increase of type-specific virus-neutralizing antibody titers between acute- and convalescent-phase serum specimens was determined; 54.8% (17 of 31) of the patients' sera exhibited a significant rise of neutralizing antibody titer against the isolated strains. The geometric mean titers of the first and second sera were 1:3.4; and 1:22.4, respectively.

Previous studies in Cuba have estimated the circulation of enterovirus in patients with meningitis. From 1972 to 1999, seven meningitis outbreaks caused by an enterovirus occurred: echovirus 4 (1972 and 1985-86), coxsackievirus B5 (1976 and 1995), coxsackievirus A9 (1990-1991), echovirus 30 (1994), and echovirus 9 (1999). Other enteroviruses (coxsackievirus and numerous echoviruses) were identified from sporadic cases of viral meningities during nonepidemic periods (6). Before this epidemic, aseptic meningitis caused by echovirus 16 had never been recognized in Cuba.

Nevertheless, the age distribution in this outbreak suggests previous exposure and immunity to echovirus 16 in older persons (Figure 2). The age group distribution was also similar to that in previously described outbreaks of aseptic meningitis in Cuba (6). A possible explanation for this age distribution is that an antigenically related virus without potential to cause acute aseptic meningitis circulated in the population before year 2000 and induced an immune response. Alternatively, maternal antibodies against this virus may protect neonates from infection.

Factors influencing the prevalence of enteroviruses are poorly understood. The ease with which a type of nonpolio enterovirus can be isolated, however, is likely to be a major determinant of the frequency with which it is reported (5,8). Echovirus 16 isolates associated with sporadic cases of aseptic meningitis have probably been infrequently reported in previous years because of the difficulties in tissue culture propagation.

According to data collected through the U.S. National Enterovirus Surveillance System, echovirus 16 is routinely isolated, but the frequency of its isolation is low in most years (i.e., 3 to 5 cases per year) (Centers for Disease Control and Prevention, unpub. data).

To our knowledge, the last Cuban outbreak of aseptic meningitis associated with echovirus 16 infections occurred in Tajimi City, Gifu prefecture, in 1984. Diverse etiologic agents (coxsackievirus B1, B4, and B5) were also reported during the outbreak (9).

The emergence of echovirus 16 associated with a very large-scale meningitis epidemic in Cuba should alert public health officials to the potential for epidemics associated with this serotype in other areas of the world.

The overall genetic diversity and molecular evolution in echovirus 16 strains and the correlation with the epidemiologic features of echovirus 16-associated disease have not yet been studied. The availability of the viral isolates, together with the massive clinical and epidemiologic data from this epidemic, represents an unprecedented opportunity to study the emergence of echovirus 16 strains and their subsequent molecular evolution.

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Lic. Sarmiento is a virologist at the "Pedro Kouri" Tropical Medicine Institute. His research interests include enteroviral disease outbreak investigations and pathogenesis of enteroviral diseases.

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Expanding Global Distribution of Rotavirus Serotype G9: Detection in Libya, Kenya, and Cuba

Nigel A. Cunliffe,* Winifred Dove,* James E.G. Bunn,† M. Ben Ramadam,‡ James W.O. Nyangao,§ Raul L. Riveron,¶ Luis E. Cuevas,† and C. Anthony Hart*

*University of Liverpool, Liverpool, United Kingdom; †Liverpool School of Tropical Medicine, Liverpool, United Kingdom; ‡Misurata Teaching Hospital, Misurata, Libya; §Kenya Medical Research Institute, Nairobi, Kenya; and ¶Centro Havana Children's Hospital, Havana, Cuba

Serotype G9 may be the fifth most common human rotavirus serotype, after serotypes G1 to G4. In three cross-sectional studies of childhood diarrhea, we have detected serotype G9 rotaviruses for the first time in Libya, Kenya, and Cuba. Serotype G9 constituted 27% of all rotaviruses identified, emphasizing the reemergence of serotype G9 and suggesting that future human rotavirus vaccines will need to protect against disease caused by this serotype.

Group A rotaviruses are firmly established as the most important etiologic agents of dehydrating gastroenteritis in infants and young children worldwide (1). Severe rotavirus disease is preventable by vaccination. The expected impact of rotavirus vaccines in reducing disease and death from rotavirus infection will be most evident in developing countries, where rotavirus causes up to 500,000 childhood deaths annually (2).

Rotaviruses are nonenveloped viruses whose genome comprises 11 segments of double-stranded RNA (dsRNA), contained in the core of the mature, triple-layered particle (1). Rotavirus serotypes are determined by neutralizing antibody responses to each of the two outer capsid proteins, VP7 (termed G serotype) and VP4 (termed P serotype). Ten G serotypes and 7 P serotypes have been identified in humans (1). Since serotypes G1, G2, G3, and G4 together account for >80% of global human rotavirus strains, some vaccines aim to provide serotype-specific protection against these four serotypes (1). However, in certain geographic settings, other G types (e.g., G5, G8, and G9) may be epidemiologically important (1).

Since their first report in 1987 in the United States, rotaviruses of serotype G9 had been rarely detected in the human population (3). Since 1995, however, serotype G9 has been documented in India, Brazil, Italy, the United States, Bangladesh, Malawi, the United Kingdom, France, and Australia (4). Recent reports from Ireland (5), the Netherlands (6), Japan (7), and Thailand (8) further emphasize the wide geographic distribution of this serotype. We recently characterized rotavirus strains detected during studies of diarrheal disease in children in Libya, Kenya, and Cuba. We identified serotype G9 rotaviruses in each of these countries, providing further evidence that serotype G9 has reemerged as a globally important human serotype.

Address for correspondence: C. Anthony Hart, Department of Medical Microbiology and Genito-Urinary Medicine, University of Liverpool, Duncan Building, Daulby Street, Liverpool L69 3GA, United Kingdom; fax: 44 151 706 5805; e-mail: cahmm@liverpool.ac.uk

The Study

In all three studies, fecal specimens were collected from children <5 years of age who were hospitalized for treatment of acute gastroenteritis. In Libya, the study was conducted during April and May 2000 and was based at the Misurata Teaching Hospital in Misurata. The samples from Kenya were collected from January to March 2000 from children hospitalized at the Gertrude's Garden Children's Hospital in Nairobi. The third group of samples was obtained during April and May 2000 from children hospitalized at the Centro Havana Children's Hospital, Havana, Cuba.

Fecal specimens were stored at 4°C until they were shipped to the University of Liverpool. Rotavirus infections were diagnosed by negative stain electron microscopy. All rotavirus-positive samples were selected for rotavirus strain characterization.

Rotavirus dsRNA was extracted from fecal samples by a guanidine and silica method (9). The 11 dsRNA segments were separated by polyacrylamide gel electrophoresis and stained with silver to identify the two main RNA profiles, or electropherotypes (long and short). Rotavirus G and P types were determined by using hemi-nested reverse transcription-polymerase chain reaction (RT-PCR). The genotyping methods, which serve as a proxy for serotype determination by virus neutralization, have been described (9,10). A combination of consensus and type-specific primers have been designed to amplify strains of the common rotavirus serotypes, as well as some uncommon serotypes (9-12).

Briefly, for G typing, consensus primers 9con1 and 9con2 were used in a first-round RT-PCR (10 cycles) to generate a 905-bp VP7 gene fragment; 9con1 was then used in a second-round PCR (30 cycles) with type-specific primers 9T-1 (G1), 9T-2 (G2), 9T-3P (G3), 9T-4 (G4), MW8 (G8), and 9T-9B (G9). For P typing, consensus primers con2 and con3 were used in a first-round RT-PCR (10 cycles) to generate a 877-bp fragment of gene 4; con3 was then used in a second-round PCR (30 cycles) with type-specific primers 1T-1 (P[8]), 2T-1 (P[4]), 3T-1 (P[6]), 4T-1 (P[9]), and 5T-1 (P[10]). For strains that failed to give type-specific products, alternative typing prim-

ers were used that included a G1-specific primer (nac9) and a P[8]-specific primer (nac10). These primers were designed at the 9T-1 and 1T-1 primer binding regions, respectively, of divergent serotype G1 and P[8] lineages that we have recently identified in Malawi (13). Strains that could not be typed even by using the alternative primers were recorded as nontypeable (NT). RT-PCR products were resolved by electrophoresis on 2% agarose gel and then visualized by ultraviolet illumination after ethidium bromide staining.

Thirty-five (48%) rotaviruses were detected in 73 fecal samples from Libyan children. These comprised strains P[8], G1 (n = 16, 46%); P[8], G9 (n = 12, 34%); P[6], G1 (n = 4, 11%); P[4], G2 (n = 1, 3%); and P[NT], G1 (n = 2, 6%). Seven serotype G1 strains and 23 genotype P[8] strains required alternative primers for efficient typing. Long electropherotype profiles were visualized for serotype G1 and G9 strains, and the single serotype G2 strain had a short profile.

Twenty (41%) rotaviruses from 49 cases of diarrheal disease were characterized from Kenya. The most commonly identified strain was P[8], G1 (n = 12, 60%), followed by P[8], G9 (n = 2, 10%). One strain (5%) of each of the following genotypes made up the rest: P[6], G9; P[4], G8; P[6], G1; P[8], G3; P[NT], G1; and P[NT], G8. Alternative primers were required to type three serotype G1 strains and eight genotype P[8] strains. Overall, serotype G9 was detected in three (15%) of specimens. Most strains, including serotype G1 and G9 strains, had long electropherotype profiles. Both serotype G8 strains had short electropherotypes.

Five (9%) rotaviruses were detected in 55 fecal samples from Cuban children. Three P[8], G4 strains, a single P[8], G9 strain, and a single P[NT], G4 strain were identified. All strains had long electropherotype profiles.

The G9 specificities of representative serotype G9 strains from Libya and Kenya and the single Cuban G9 strain were confirmed by partial nucleotide sequencing of full-length RT-PCR VP7 gene products obtained by using primer pair beg9/end9 (10) (data not shown).

Age data were available for 54 (90%) of the children, including 16 children infected with serotype G9 rotaviruses and 38 infected with other rotavirus strains. The age distribution of serotype G9-infected children (median 15 months; range 6-30 months) was similar to that of children excreting other rotavirus serotypes (median 11.5 months; range 4-57 months).

Conclusions

These three small cross-sectional studies have documented the presence of serotype G9 in each of the countries surveyed. Serotype G9 was the second most commonly detected serotype in Libya and Kenya, accounting for 34% and 15%, respectively, of G types in those countries. It was also the only G type, apart from serotype G4, to be detected in five samples from Cuba. No previous studies have characterized rotaviruses from either Libya or Cuba. A recent study examined the distribution of rotavirus serotypes in Kenya (14), but to our knowledge this is the first time that serotype G9 has been reported there. Furthermore, this is only the second published report of serotype G9 from Africa, following an earlier report from Malawi (12).

Many serotype G1 and P[8] strains from Libya and Kenya could be typed only by using alternative typing primers, since type-specific products could not be obtained for them with conventional primers. Variation in the sequence of the primer binding region of common serotypes, as well as the occurrence of uncommon serotypes, should be considered when nontypeable rotaviruses are identified in strain characterization studies (13).

Most serotype G9 strains in these studies had long electropherotypes and P[8] VP4 specificity, but a single P[6] VP4 type (also with a long electropherotype) was associated with a serotype G9 strain in Kenya. The reference serotype G9 strains, WI61 (3) and F45 (15), have long electropherotypes and P[8] VP4 specificity. More recently, serotype G9 has been associated with both long and short electropherotypes and a variety of P types including P[6], P[8], and P[11], and molecular characterization of a representative sample of these strains suggests that genomic reassortment played an important role in their evolution (4).

The recent upsurge in published reports of serotype G9 rotaviruses may partly reflect more widespread application of improved methods for their detection. However, increasing evidence indicates that serotype G9 has reemerged in the human population and has recently been imported into certain countries. For example, molecular characterization of serotype G9 strains identified in the United Kingdom demonstrated a high degree of sequence homology among VP7 genes, suggesting that they had been imported relatively recently (16). The observation that children in the United Kingdom infected with serotype G9 strains were older and had more severe disease than children infected with other serotypes supports this hypothesis, since the population would lack neutralizing antibody to this serotype (17). We did not find serotype-specific age differences in this study; however, the numbers may have been too small to allow detection.

More recently, a protracted outbreak of rotavirus diarrhea caused by P[6], G9 strains occurred in a neonatal ward in the Netherlands, which was surprising since rotavirus infection of neonates is typically asymptomatic (6). The unusually high proportion of symptomatic cases may be partly explained by lack of protective, passively acquired maternal neutralizing antibodies to serotype G9 in the affected neonates, since G9 had not previously been identified in the Netherlands (6).

Although our studies are limited by small sample size, we have demonstrated the presence of serotype G9 in each of the three countries, and this serotype represented 16 (27%) of 60 strains overall. These and other recent data suggest that serotype G9 has emerged as the fifth most common global rotavirus serotype. Continued rotavirus surveillance will be necessary to monitor the spread and persistence of this serotype. Future rotavirus vaccines will likely need to provide adequate protection against disease caused by serotype G9 rotaviruses.

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Dr. Cunliffe is a clinical lecturer in the Department of Medical Microbiology, Liverpool University. He is a Wellcome Trust Trop-

ical Research Fellow working in Blantyre, Malawi, and Liverpool, examining both the diversity of rotaviruses and their effect in HIV-infected children.

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Human Herpesvirus-8 and Other Viral Infections, Papua New Guinea

Giovanni Rezza,* Robert T. Danaya,† Theresa M. Wagner,* Loredana Sarmati,‡ Ifor L. Owen,§ Paolo Monini,* Massimo Andreoni,‡ Barbara Suligoi,* Barbara Ensoli,* and Edoardo Pozio*

*Istituto Superiore di Sanità, Rome, Italy; †Port Moresby General Hospital, Port Moresby, Papua New Guinea; ‡Tor Vergata University, Rome, Italy; and §National Veterinary Laboratory, Department of Agriculture and Livestock, Boroko, Papua New Guinea

We studied residents of remote villages and the capital (Port Moresby) of Papua New Guinea to determine the distribution of human herpesvirus-8 (HHV-8) infection. Our data suggest that HHV-8 has been endemic on the island for a long time and that the epidemiologic pattern of HHV-8 is more similar to that of herpes simplex virus-2 than hepatitis C virus.

The distribution of human herpesvirus-8 (HHV-8) infection and its main clinical consequence, Kaposi sarcoma (KS), appears to vary greatly by geographic area; however, its global distribution has not been determined (1). HHV-8-related viruses have been found not only in lower African and Asian simians and South American primates (2) but also in great apes, such as chimpanzees (3,4). Although the precise time of divergence from related viruses of nonprimates is not known, evidence indicates that interspecies transmission to humans occurred on an evolutionary time scale of tens of thousands of years. HHV-8 seroprevalence studies of remote populations from different areas of the world may contribute to understanding the geographic origin and spread of the infection.

Paralleling the distribution of KS, HHV-8 rates are high in central Africa and low in the United States and Europe, except for Mediterranean countries, which have intermediate rates (1). Little information is available about the distribution of HHV-8 infection and KS in indigenous populations of Australasia. HHV-8 seroprevalence appears to be low in countries of Southeast Asia, such as Malaysia (5), where KS is considered very rare. In Papua New Guinea, little information is available about KS, although cases have been reported among children, suggesting that it does occur, although it is probably relatively uncommon (6).

To verify whether HHV-8 infection is endemic in Papua New Guinea and to determine seroprevalence rates, we studied sera from Melanesian residents of remote villages in Western Province, Papua New Guinea. In Port Moresby, the capital, Melanesian residents were studied as a reference population group. Serologic tests for other viruses were also done to characterize the study population and explore modalities of transmission of HHV-8 infection by comparing the patterns of these infections with that of HHV-8.

Address for correspondence: Giovanni Rezza, Laboratory of Epidemiology and Biostatistics, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161, Rome, Italy; fax: 0039–06-49387210; e-mail: g.rezza@iss.it

The Study

The Melanesian study participants live in six remote villages (total population 524) in a flat, rural area called the Bensbach in southwestern Papua New Guinea. Participants from these villages were recruited for a study on trichinellosis, a parasitic infection recently reported in this area; at least 5 mL of venous blood had been collected from each participant in 1999, and these specimens were separated and treated as described elsewhere (7). Participants from Port Moresby were teachers and students at a local high school.

For serologic testing for antibodies to HHV-8 and other viruses, serum samples were stored at –20°C in the laboratories of Tor Vergata University, Rome, Italy. As described (8,9), antibodies to lytic and latent antigens of HHV-8 were detected by using two immunofluorescence assays based on the BCBL-1 cell line. Samples reactive at >1:20 dilution were considered positive.

Antibodies to herpes simplex 2 (HSV-2) were detected by an immunoglobulin (Ig) G-based type-specific enzyme-linked immunosorbent assay (ELISA; Gull Laboratories, Inc., Salt Lake City, UT). An ELISA was also used to detect antibodies to the Epstein-Barr virus (EBV) gp125 viral capsid antigen (Gull Laboratories, Inc.). Antibodies against hepatitis C virus (HCV) were detected by a third-generation enzyme immunoabsorbent assay (Innotest HCV Ab III, Innogenetics, Ghent, Belgium).

Seroprevalence ratios for each of the four viruses were calculated to assess differences stratified by age group, sex, and geographic area. These associations were evaluated by the chi-square test and the Cochran-Armitage test for trend. The viral correlates of HHV-8 infection were evaluated by estimating crude and adjusted prevalence ratios and their associated p-values by univariate and multivariate log-binomial linear regression analysis (GENMOD procedure in SAS, version 6.12). The relationship between antilytic and antilatent antibodies was assessed by calculating the proportion of antilatent positive persons by level of antilytic titer.

Sera from 150 participants, 56 from the villages and 94 from Port Moresby, were studied. Fifty-one (34%) were female; the proportion of females was the same among village (19 [34%] of 56) and urban (32 [34%] of 94) residents.

Median age was 22.5 years; residents of the remote area were older (median 35 years, range 15 to 85) than those recruited in the town (median 18 years, range 16 to 49 years).

Seroprevalence data for HHV-8 and the other viruses were stratified by age, sex, and area of residence (Table 1). HHV-8 and HSV-2 seroprevalence tended to increase with age. These increases, while different for each virus, were similar for urban and rural residents (for HHV-8, from 18.1% for those <25 years of age to 40.0% for those >35 in the city and from 18.2% to 41.4% in the villages; and for HSV-2, from 1.4% to 33.3% in the city and from 18.2% to 39.3% in the villages). Overall, age was significantly associated with infection with both HHV-8 (test for trend p=0.01) and HSV-2 (test for trend p=0.001). No significant difference was observed between women and men, although HHV-8 prevalence was slightly higher in women. HHV-8 infection was more common in rural dwellers and HCV in the capital, but the differences were not statistically significant. Rural participants were four times more likely to be infected with HSV-2 than those in the city (p=0.001), but this disparity was attributable to the differing age distribution of the two groups. No difference was seen in prevalence of EBV.

The analysis of serologic correlates of HHV-8 infection showed a significant association with HSV-2 infection (Table 2). However, after data were adjusted for age and other variables, the prevalence ratio of HHV-8 infection was no longer different from 1 for HSV-2-positive vs. -negative participants.

Of the 37 HHV-8 antilytic-positive participants, 16 had a titer of 1:20, 10 of 1:80, 9 of 1:160, and 2 of 1:320. Ten of these 37 also had antilatent antibodies. The proportion of antilatent positivity changed according to the antilytic titer: none of the 16 participants with an antilytic titer of 1:20 had antilatent antibodies, but 2 (20%), 7 (77.7%), and 1 (50%) of those with antilytic titers of 1:80, 1:160, and 1:320, respectively, were also antilatent-positive. One antilytic-negative participant also had antilatent antibodies, with a titer of 1:160. Because the 16 residents with an antilytic titer of 1:20 did not have antilatent antibodies and could be considered false-positives, we repeated the analysis considering these participants as seronegative and obtained similar results (data not shown).

Conclusions

This study shows a relatively high prevalence of HHV-8 infection in Papua New Guinea; the prevalence does not appear to differ substantially between the capital and remote villages, suggesting that the infection has been endemic for a long time in this region and, as reported for other herpesvirus infections (10), can persist in small, isolated population groups. This similarity between remote rural communities and an urban center does not fully support the conclusions of a study on Amerindians of diverse tribes, which suggested that the limited genetic pool in isolated groups may permit more frequent transmission of a virus with a low prevalence than in heterogeneous populations (11).

Other viral infections, such as HSV-2, EBV, and HCV, were prevalent both in villages and the capital. In particular, HSV-2 was more common in remote villages, but the difference was largely explained by age. EBV prevalence was as high as expected on the basis of previous studies (12). The prevalence of HCV antibodies was higher in Port Moresby than in the villages; however, the difference was not statistically significant. A similar prevalence (4.1%) was found in residents of villages in another part of Western Province (13).

The potential association between HHV-8 and HSV-2 was nonsignificant after the data were adjusted for age. Although seroprevalence of both infections tended to increase with age, the presence of HSV-2-negative and HHV-

Table 1. Seroprevalence of human herpesvirus-8 and other infections, stratified by geographic area, age, and sex

Age (years)	HHV-8[a]		Epstein-Barr virus		Hepatitis C virus		Herpes simplex virus	
	Urban	Rural	Urban	Rural	Urban	Rural	Urban	Rural
<25	13/72 (18.1%)	2/11 (18.2%)	61/66 (92.0%)	11/11 (100.0%)	7/71 (9.9%)	0/11	1/72 (1.4%)	2/11 (18.2%)
25-35	3/12 (25.0%)	3/16 (18.7%)	10/12 (83.3%)	16/16 (100.0%)	1/12 (8.3%)	2/16 (12.5%)	3/12[b] (25.0%)	3/15 (20.0%)
>35	4/10 (40.0%)	12/29 (41.4%)	10/10 (100.0%)	28/29 (96.6%)	2/10 (20.0%)	0/29	3/9[b] (33.3%)	11/28 (39.3%)
Male	14/62 (22.6%)	13/37 (35.1%)	56/61 (91.8%)	36/37 (97.3%)	6/61 (9.8%)	1/37 (2.7%)	5/61 (8.2%)	10/36 (27.8%)
Female	6/32 (18.7%)	4/19 (21.0%)	30/32 (93.7%)	19/19 (100.0%)	4/32 (12.5%)	1/19 (5.3%)	2/32 (6.2%)	6/18 (33.3%)
Total	20/94 (21.3%)	17/56 (30.4%)	86/93 (92.5%)	55/56 (98.2%)	10/93 (10.7%)	2/56 (3.6%)	7/93 (7.5%)	16/54[c] (29.6%)

[a]HHV-8 = human herpesvirus-8.

[b]Chi-square p-value <0.05, comparing age groups in rural and urban strata with reference group: <25 years.

[c]Chi-square p-value <0.05, comparing rural with urban.

Table 2. Serologic correlates of human herpesvirus-8 infection, Papua New Guinea

	HHV-8[a]	PR[b]	p	APR[b]	p
HCV-	35/137 (25.5%)	1		1	
HCV+	1/12 (8.3%)	.33	0.25	0.46	0.34
EBV-	1/8 (12.5%)	1		1	
EBV+	35/141 (24.8%)	1.98	0.47	1.71	0.54
HSV2-	25/124 (20.2%)	1		1	
HSV2 +	10/23 (43.5%)	2.16	0.01	1.64	0.15

[a]HHV-8 = human herpesvirus-8; HCV = hepatitis C virus; EBV = Epstein-Barr virus; HSV2 = herpes simplexvirus-2.
[b]Prevalence ratio, crude and adjusted for age, sex, and urban residence.

8-positive young adults suggests that HHV-8 infection is not necessarily sexually transmitted. In particular, salivary spread, as for EBV, may not be ruled out, as suggested by studies conducted in isolated tribes of the New World, showing high prevalence rates in children (11,14).

Some limitations and possible biases of our study should be mentioned. First, to what extent residents of isolated villages had contacts with others is difficult to quantify. Although government contact with the area was made early in the 20th century, infrastructure and services remain poor, making access difficult. There are no direct connections with this area, which during the rainy season can be reached only by canoe and then on foot, after a 1-hour flight from Port Moresby. To our knowledge, only one person had worked outside the area before retiring to his village of origin. Second, the differing age distribution between study participants recruited in urban and rural areas may represent a possible bias; however, the statistical analysis accounted for these differences. Finally, HHV-8 prevalence might have been overestimated if low antilytic titers were false-positive results; however, the specificity of our assay is supported by the high risk for KS among HIV-infected persons with low antilytic titers (9).

In conclusion, HHV-8 infection appears to be common in remote population groups of Papua New Guinea as well as in Port Moresby, suggesting that the infection has been endemic for a long time among the indigenous population of the country. The detection of a high prevalence of HHV-8 emphasizes the need for clinical monitoring of the study population. The identification of the main modes of transmission for HHV-8 and other infections may help in instituting public health measures to control the infection.

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Dr. Rezza is a medical epidemiologist and director of the AIDS and STD Unit, Laboratory of Epidemiology and Biostatistics, Istituto Superiore di Sanità (Italian National Institute of Health). His research focuses on HIV, HHV-8, and other potentially oncogenic sexually transmitted viruses.

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A Unique *Mycobacterium* Species Isolated from an Epizootic of Striped Bass (*Morone saxatilis*)

Martha W. Rhodes,* Howard Kator,* Shaban Kotob,* Peter van Berkum,† Ilsa Kaattari,* Wolfgang Vogelbein,* Margaret M. Floyd,‡ W. Ray Butler,‡ Frederick D. Quinn,‡ Christopher Ottinger,§ and Emmett Shotts§

*Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia, USA; †U. S. Department of Agriculture, Beltsville, Maryland, USA; ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and §National Fish Health Research Laboratory, Kearneysville, West Virginia, USA

We isolated a *Mycobacterium* sp. resembling *Mycobacterium marinum* and *M. ulcerans* from diseased striped bass *(Morone saxatilis)* during an epizootic of mycobacteriosis in the Chesapeake Bay. This isolate may represent an undescribed *Mycobacterium* species, based on phenotypic characteristics and comparative 16S rRNA gene sequence.

Natural aquatic environments are recognized sources of mycobacteria known to cause disease in both humans and fish. Although *Mycobacterium marinum* is considered the primary causative agent of fish mycobacteriosis, seven *Mycobacterium* species associated with tubercle granulomas in aquarium, cultured, and wild fish populations have been described: *M. abscessus, M. chelonae, M. fortuitum, M. marinum, M. neoaurum, M. scrofulaceum,* and *M. simiae* (1,2). All these species cause disease in humans (3,4). Primary clinical syndromes include skin and soft-tissue infections, cervical lymphadenitis, pulmonary disease, and disseminated infections, the last generally being limited to immunocompromised persons. Human mycobacteriosis following occupational or recreational exposure to the marine environment is frequently associated with trauma such as wounds from handling fish and has been attributed primarily to *M. marinum* (5). Consequently, the discovery of an undescribed *Mycobacterium* species associated with an epizootic of mycobacteriosis in striped bass (*Morone saxatilis*) warrants recognition and additional study.

Mycobacteriosis in fish is a subacute to chronic wasting disease known to affect some 167 freshwater and saltwater species (2). Internal signs of the disease vary according to fish species but typically include granulomas in the spleen, kidney, and liver. External manifestations include scale loss accompanied by hemorrhagic lesions penetrating the musculature in advanced cases. Recently, an ongoing epizootic of mycobacteriosis in striped bass (*Morone saxatilis*) from the Chesapeake Bay was described (Vogelbein W et al., unpub. data). Previous outbreaks of mycobacteriosis in wild striped bass have occurred in Pacific estuaries (6). During the Chesapeake Bay epizootic, we isolated a variety of mycobacteria associated with skin and visceral lesions that included a unique group of slowly growing nonpigmented isolates. We describe one of these isolates, which has specific characteristics similar to those of *M. marinum* and *M. ulcerans*.

Address for correspondence: Martha W. Rhodes, Department of Environmental Sciences, Virginia Institute of Marine Science, College of William and Mary, PO Box 1346, Gloucester Point, VA 23062, USA; fax: 804-684-7186; email: martha@vims.edu

Striped bass (n = 20) we examined included asymptomatic and symptomatic fish with skin ulcerations (Figure) verified histologically to exhibit granulomatous inflammation associated with acid-fast bacilli. All fish were caught in the Chesapeake Bay or one of its tributaries (the James, Potomac, or Rappahannock rivers). Skin and spleen samples from necropsied specimens were processed for routine paraffin histology, sectioned at 5 µm, and stained with hematoxylin and eosin. Selected sections were stained using Ziehl-Neelsen's method for acid-fast bacteria (7). Excised internal tissues (predominately spleen) were homogenized in phosphate buffer using a Ten Broeck tissue grinder and inoculated directly onto culture media or after treatment with one of the following disinfectants (Vogelbein et al., unpub. data): 0.3% Zephiran (Sanofi Winthrop Pharmaceuticals, New York, NY), 2% NaOH, or 2% HCl. Homogenates were inoculated onto Löwenstein-Jensen slants and plates of brain heart infusion agar containing 5% sheep red blood cells and Middlebrook 7H10 agar with albumin-dextrose-catalase enrichment. Initially inoculated media were incubated at 30°C for a minimum of 2 months. Because some isolates exhibited poor growth, a second incubation temperature

Figure. Skin ulcers typical of mycobacteriosis in striped bass (*Morone saxatilis*) from the Chesapeake Bay.

(23°C) was used for primary media inoculated with tissue homogenates.

Purified isolates were characterized phenotypically by traditional methods (8) with incubation at 23°C. Mycolic acids were analyzed by a standardized method for mycobacteria by using reverse-phase high-performance liquid chromatography (HPLC) with UV detection (9,10).

Polymerase chain reaction (PCR) assay and sequence analysis of the 16S rRNA gene were used to characterize one of the slow-growing, nonpigmented mycobacteria, hereafter called isolate M175. This isolate is deposited in the American Type Culture Collection (ATCC), Rockville, MD, as ATCC 700981. The 16S rRNA gene was amplified in 120-µL volumes (11) by using cycle conditions described by van Berkum and Fuhrmann (12). Primers (forward, M16SA, 5'-CGC TGG CGG CGT GCT TA-3' and reverse, M16SB, 5'-ACG GCT ACC TTG TTA C-3') were specifically designed for the amplification of mycobacterial 16S rRNA genes. The PCR buffer (pH 8.5) contained 60 mM Tris-HCl, 15 mM $(NH_4)2SO_4$, and 1.5 mM $MgCl_2$; control reactions without template were included. After purification of PCR products (QIAquick Spin columns, Qiagen Inc., Chatsworth, CA), amplicons were sequenced with a Perkin-Elmer 377 DNA Sequencer in combination with a Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) (11,12).

Granulomatous inflammation was confirmed histologically in spleens of 18 of the 20 fish. Severity of the infection based on the abundance and size of splenic granulomas varied from mild to severe. Skin ulcers were evident in 13 specimens. Granulomatous inflammation was generally associated with acid-fast bacilli in selected stained sections.

Colony development from homogenized tissue was slow, requiring 4 to 6 weeks' incubation at 23°C on the preferred medium, Middlebrook 7H10 agar. Isolate M175 showed little or no growth at 30°C and none at 37°C. Rough nonpigmented colonies were flat with an irregular margin and yielded aggregates of acid-fast nonbranching rods. Isolate M175 was negative for growth on MacConkey agar and Löwenstein-Jensen with 5% NaCl, arylsulfatase, beta-galactosidase, nitrate reductase, semiquantitative catalase, Tween 80 hydrolysis, and Tween opacity. Weak positive reactions for catalase activity after treatment at 68°C and pyrazinamidase after extending incubation to 14 days were observed. Isolate M175 was positive for tellurite reduction, niacin production, and urease. Colonies did not produce pigment after exposure to light for several hours or after prolonged exposure for several days. Based on the aforementioned characteristics, this isolate could not be assigned to an existing species.

The M175 mycolic acid pattern consisted of a single cluster of eight peaks that visually resembled reference patterns (10) for species of the *M. tuberculosis* complex. However, M175 mycolic acid peaks did not superimpose with peaks of *M. tuberculosis* after alignment with the internal size standard. Peak elution times for M175 were suggestive of more polar, shorter, carbon chain-length mycolic acids than those found in *M. tuberculosis* complex species. Comparisons of the M175 pattern with the *Mycobacterium* HPLC mycolic acid database at the Centers for Disease Control and Prevention confirmed a unique pattern suggestive of a new species of mycobacteria.

The sequence of the PCR product of the 16S rRNA gene from *Mycobacterium* isolate M175 was 1,494 nt long. This sequence was deposited in GenBank and was given accession number AY005147. Blast searches of GenBank yielded high sequence similarities of 99.2% to *M. marinum* (13) and *M. ulcerans* (14) and of 98.7% to *M. bovis* (15) and *M. tuberculo-*

Table. Comparison of distinguishing phenotypic features of *Mycobacterium* sp. isolate M175 with genetically (16S rRNA) similar *Mycobacterium* spp. (*M. bovis*, *M. marinum, M. tuberculosis,* and *M. ulcerans*)

Characteristic	*Mycobacterium* sp. (fish isolate M175)	*M. bovis*	*M. marinum*	*M. tuberculosis*	*M. ulcerans*
Optimum growth temperature (°C)	<30	37	30	37	30
Colony morphology	R	R	S	R	S/R
Pigmentation	N	N	P	N	N
Arylsulfatase					
3 days	-	-	V	-	-
14 days	-	-	+	-	+
Niacin	+	-	-/V	+	-/V
Nitrate reduction	-	-	-	+	-
Pyrazinamidase 7 days	-	-	+	+	-
Tween hydrolysis	-	V	+	V	-
Urease	+	+	+	+	-

Source: Data for known species cited from references 17 and 18.
Abbreviations: d = days; N = nonpigmented; P = photochromogenic; R = rough; S = smooth; + = at least 85% strains positive; - = at least 85% negative; V = variable.

sis (16). High sequence similarities between 16S rRNA genes of M175 and other *Mycobacterium* spp. and phenotypic data support the conclusion that M175 belongs within the genus *Mycobacterium*. However, despite the similarities, the 16S gene sequence of M175 differed from *M. ulcerans* by 11 nt (3 insertions and 8 substitutions [one base of the *M. ulcerans* sequence in GenBank is N]) and from *M. marinum* by 10 nt (4 insertions, 6 substitutions [one base of the *M. marinum* sequence in GenBank is N]). Based on sequence differences and contrasting phenotypic characteristics (Table), we conclude that isolate M175 appears to belong to a new, previously undescribed species of *Mycobacterium* (19). Comparative genetic studies of *M. ulcerans* and *M. marinum* based on 16S rRNA sequence analysis have shown very close relationships between these species despite contrasting phenotypic profiles (20-24). The presence of two DNA insertion sequences, IS*2404* and IS*2606*, in *M. ulcerans* but not in *M. marinum* has been used to distinguish the former (22-25).

The public health significance of this unique *Mycobacterium* species is not known. Frequently, mycobacterial disease in fish and cutaneous infections in humans are diagnosed on the basis of clinical presentation and generally attributed to *M. marinum*. Isolation of the causative agent either is not attempted or is unsuccessful, possibly because of loss of viability during specimen decontamination, inappropriate culture conditions, lack of technical experience with mycobacteria, or the prevailing assumption that detection of acid-fast rods is synonymous with a diagnosis of *M. marinum*. Consequently, the extent of environmentally acquired human infections caused by *Mycobacterium* species is not known. Studies to investigate the clinical importance of isolates obtained from persons exposed to marine or estuarine sources would provide data on which to evaluate the public health import of these isolates.

As in the present study, environmental mycobacteria may have lower temperature optima and not grow well on traditional media such as Löwenstein-Jensen. However, a preference for low temperature does not necessarily negate their ability to cause disease in humans, as demonstrated by disseminated infections caused by *M. marinum* and *M. haemophilum* or ulcerative skin disease caused by *M. ulcerans*. An epizootic of mycobacteriosis in striped bass, possibly the most important recreational fish in the Chesapeake Bay, could serve as a reservoir for transmission of mycobacterial infections to humans.

Laboratory challenge studies using striped bass are in progress to evaluate the pathogenicity of isolate M175. Additional research is needed to understand the persistence, distribution, and ecology of these mycobacterial isolates in natural waters, particularly with regard to their transmission to fish. Furthermore, this study also underlines a need to isolate and identify mycobacteria responsible for nontuberculosis infections in humans. This information is essential to determine the extent of human mycobacteriosis associated with occupational and increasingly popular recreational exposure to the natural aquatic environment.

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Ms. Rhodes is a microbiologist in the Department of Environmental Sciences, Virginia Institute of Marine Science. Her research interests focus on public health microbiology related to the estuarine environment.

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Wind-Blown Mosquitoes and Introduction of Japanese Encephalitis into Australia

Scott A. Ritchie* **and Wayne Rochester†**
*Tropical Public Health Unit, Queensland Health, Cairns, Queensland, Australia; and
†University of Queensland, St. Lucia, Queensland, Australia

Backtrack simulation analysis indicates that wind-blown mosquitoes could have traveled from New Guinea to Australia, potentially introducing Japanese encephalitis virus. Large incursions of the virus in 1995 and 1998 were linked with low-pressure systems that sustained strong northerly winds from New Guinea to the Cape York Peninsula.

Japanese encephalitis (JE) is a recently emerged disease in Australia (1). Two major incursions have occurred, in 1995 and 1998; the earlier outbreak caused three human cases on Badu Island in Torres Strait (2,3). In 1996-97, JE virus activity was limited to Saibai Island, the northernmost island in Torres Strait (3). The 1998 incursion was more widespread, with JE activity from the Torres Strait south to the Mitchell River on Cape York Peninsula (3). Human serosurveys in the Western Province of Papua New Guinea, which–along with Irian Jaya–is considered a probable source of the virus in the Australian incursions (4), indicate that JE virus was not confirmed in the region before 1989 (1). Thus, the incursions of JE virus into Australia are novel. The *Culex annulirostris* mosquito is likely the primary vector (5).

JE virus may have been introduced into Australia by infected birds (2), frugivorous bats (J. Mackenzie, pers. comm.), or mosquitoes (2,3). However, the widespread, sudden appearance of JE virus in the Torres Strait and Cape York Peninsula suggests that it was an episodic incursion potentially mediated by mosquitoes (2,3). *Cx. tritaeniorhynchus*, an Asian JE virus vector, is migratory in China (6) and has been collected at altitudes >100 m in India and China (6,7). In New South Wales, Australia, *Cx. annulirostris* has been collected at heights up to 310 m with an estimated flight range of 594 to 648 km, well beyond the 160 km from New Guinea to northernmost Cape York Peninsula (8). Similar incursions of bluetongue virus into northern Australia (9) are thought to be mediated by wind-blown, exotic *Culicoides* midges from Indonesia (10).

The Study

We used computer simulation to investigate whether winds were sufficient to have carried mosquitoes from New Guinea to the Torres Strait and Cape York Peninsula, potentially introducing JE virus into northern Australia. The *Helicoverpa* migration model incorporates wind speed and direction to simulate migratory flights for *Helicoverpa* spp. noctuid moths in Australia (11). We used wind speed and direction generated by the Regional Assimilation and Prognosis System (1995-96) and the Limited Area Prediction System (1996-97 onwards) of the Australian Bureau of Meteorology Research Centre (Melbourne). Backtrack simulations were used to map flight paths of mosquitoes from a focus of JE virus activity back to their potential source. Simulation parameters used were a) flight time: 20:00 Australian Eastern Standard Time; b) flight duration: 0-11 hours; c) flight altitude: 100-400 m; and d) flight speed: 0 meters/second. Flight duration and height were randomly sampled from uniform probability distributions in Monte Carlo simulations of 1,000 replicates (mosquitoes) each. The estimated flight paths are conservative; trajectories would have been longer if we had included the mosquito downwind flight speed (an estimated 5 to 7 km/hr [12]) and a longer flight time.

Backtrack simulations were run from December to March for the 1995-96 and 1997-98 seasons, encompassing the monsoon season, when westerly to northwesterly winds dominate. In 1994-95, simulations were limited to January 16-20 and February 8 to March 31, 1995; flight trajectories on other days were estimated from maps of wind speed and direction provided by the Tropical Area Projection System (Bureau of Meteorology, Darwin, Australia). Simulations were run for Badu Island in the Torres Strait, the northern peninsula area, and the mouth of the Mitchell River on Cape York Peninsula. These sites are 100 km, 160 km, and 675 km from the New Guinea mainland, respectively (Figure 1). Badu Island had JE virus activity in March-April 1995 and February-March 1998, while activity was limited to February-May 1998 in the Cape York Peninsula sites.

The simulations indicate that winds sufficient to transport mosquitoes from New Guinea frequently reach Badu Island (mean 14.2% [Table]). No association with JE activity has been confirmed. In 1994-95, the year of the initial incursion, only eight nights (6.6%) had favorable winds. Conversely, in the 1995-96 and 1996-97 seasons, JE virus activity was limited to islands within 5 km of Papua New Guinea (3) despite favorable winds for transport to Badu Island on 17% of nights (Table). Both Cape York Peninsula locations had fewer nights with favorable winds (Table). Only on December 27, 1997, were winds sufficient to carry mosquitoes from New Guinea to the mouth of the Mitchell River (Figure 2).

Weather conditions before the 1995 and 1998 JE outbreaks suggest that low-pressure systems west of Cape York Peninsula could have carried mosquitoes from New Guinea to the study sites. On January 19, 1995, a large monsoonal

Address for correspondence: Scott Ritchie, Tropical Public Health Unit, Queensland Health, PO Box 1103 Cairns, Queensland 4870 Australia; fax: 61-740-311-440; e-mail: Scott_Ritchie@health.qld.gov.au

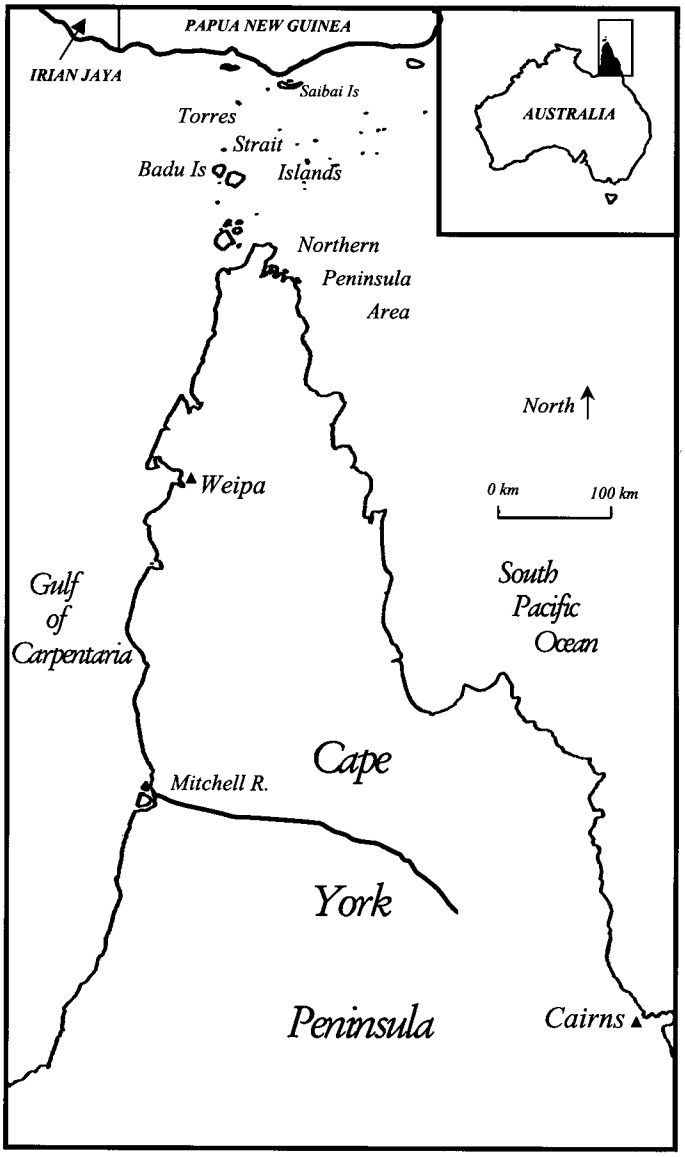

Figure 1. Map showing location of Japanese encephalitis foci (Badu Island in the Torres Strait, the northern peninsula area, and the mouth of the Mitchell River in Cape York) used in backtrack simulations.

low in the Northern Territory sustained 15 to 20 km/hour northerly winds from southern Papua New Guinea across the Torres Strait into northernmost Cape York Peninsula. The weather preceding the widespread JE outbreak in 1998 was especially noteworthy. In December 1997, Tropical Cyclone Sid in the Gulf of Carpentaria west of Cape York Peninsula produced northerly winds of 36 to 72 km/hour, which were capable of carrying mosquitoes from New Guinea to the Northern peninsula area on December 27 (Figure 2). Furthermore, the system persisted in the area, sustaining northwesterly winds capable of carrying mosquitoes from New Guinea to Badu Island and the northern peninsula area for 11 of 14 days and 8 of 14 days, respectively, from December 26, 1997, to January 8, 1998.

The arrival dates of migrating mosquitoes in 1995 and 1997 would have allowed sufficient time for amplification of JE virus in vertebrate hosts and subsequent spillover into humans. In Japan, two cycles of JE viral amplification occur in pigs, with human cases peaking approximately 2 months after pigs are infected (13). Thus, for Badu Island, where human JE cases occurred in late March 1995 and in late February 1998, JE virus was likely introduced in late January 1995 and late December 1997, respectively. These dates correspond to the weather events described above.

Conclusions

Backtrack simulations indicate that winds sufficient to transport mosquitoes from New Guinea to Badu Island occurred frequently (Table). However, many favorable wind conditions were not followed by JE virus activity (e.g., 1995-96 [Table]), suggesting that other factors influence incursions of JE virus.

Several conditions would have to be met to permit a large incursion of windblown, infected mosquitoes from New Guinea into Australia. First, there would have to be a sufficient amount of JE virus at the potential source of migrant mosquitoes. Results of human serologic tests indicate that JE virus has been active in southern Papua New Guinea since 1989 (4), especially in late 1997 and early 1998, when multiple human cases were reported (1). Second, large populations of JE virus-infected mosquitoes must be present. The southern part of New Guinea (approximately 160,000 km^2)

Table. Number of days when winds could have carried mosquitoes from New Guinea to Badu Island in the Torres Strait and to the northern peninsula area and the mouth of the Mitchell River on Cape York Peninsula[a]

Year	No. of days[b]	Badu Island (Torres Strait) days (%)	Northern peninsula area (Cape York) days (%)	Mouth of Mitchell River (Cape York) days (%)
1994-95	121	8 (6.6)	0 (0)	0 (0)
1995-96	122	21 (17.2)	7 (5.7)	0 (0)
1996-97	121	20 (16.5)	8 (6.6)	0 (0)
1997-98	121	20 (16.5)	11 (9.1)	1 (0.8)
Mean	121	17.3 (14.2)	6.5 (5.4)	0.25 (0.2)

[a]Based on backtrack simulations done during the monsoon season (December to March) for the seasons 1994-95 through 1997-98.
[b]Backtrack-simulated flight path with one or more pixels completely within the New Guinea mainland (e.g., Figure 1B).

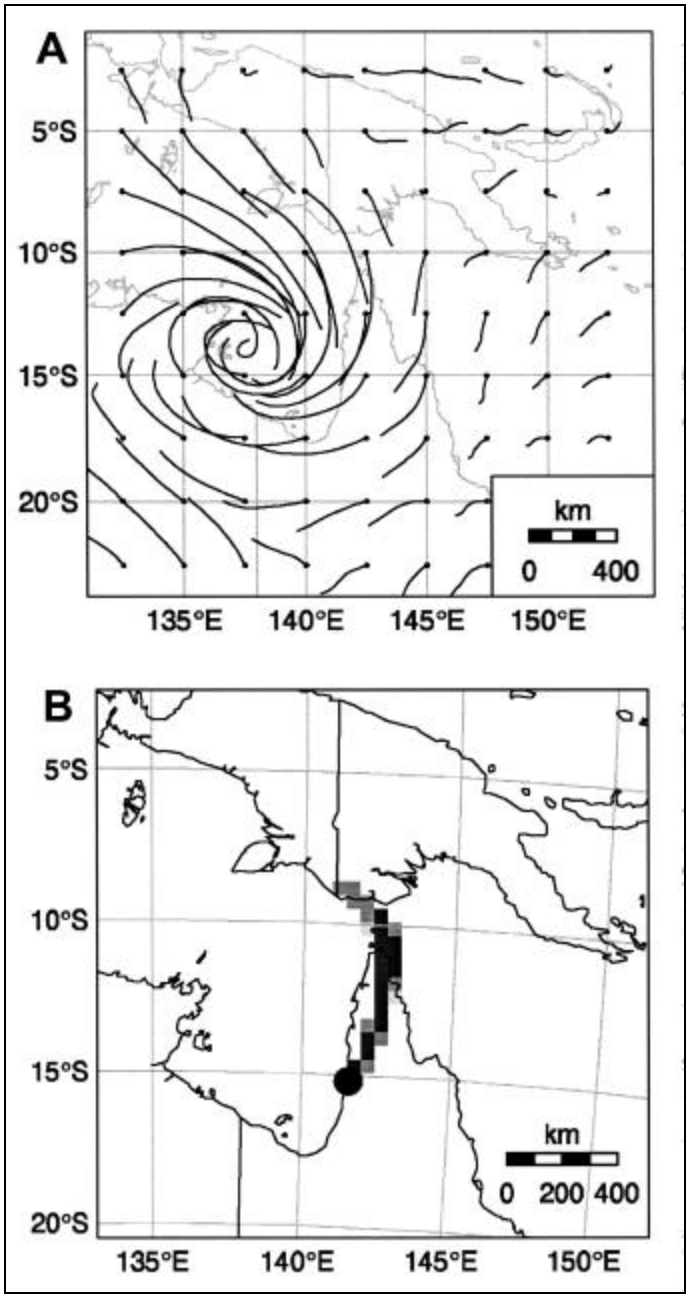

Figure 2. The estimated flight trajectories at 100 m (A) and backtrack simulations (B) of mosquitoes from the Mitchell River for December 27, 1997. Shading represents the number of back trajectory endpoints per km^2 per million simulated mosquito trajectories, with white = 0, light = <10, medium 10 to 20, and dark >20.

is dominated by lowland swamps and lakes. In late 1997 and early 1998, a severe drought created stagnant wetlands, producing high populations of *Cx. annulirostris* (3). In February 1998, 39% of 26 light trap collections in the southeastern part of the Western Province of Papua New Guinea contained >10,000 female *Cx. annulirostris* (C. Johansen, unpub. data), and JE virus was isolated from them. Third, substantial numbers of the *Cx. annulirostris* vector must exploit high winds in the atmospheric boundary layer for long-distance travel. Female *Cx. annulirostris* have been collected at altitudes ≤310 m in western New South Wales at densities comparable with those of mosquitoes collected in India and China (8). Finally, wind speed and direction must be capable of carrying the vector to the area in question.

Our results suggest that the necessary wind conditions occur annually in the Torres Strait but only rarely beyond the northern peninsula area of Cape York Peninsula (Table). Monsoonal winds are typically westerly to west-northwesterly in the Torres Strait; mosquitoes carried from New Guinea by these winds would bypass all but the northernmost Torres Strait islands. The rarity of JE virus incursions deep into Cape York Peninsula indicates that it is an extreme, episodic event. Our simulations link the 1995 and 1998 JE virus incursions with tropical low-pressure systems west of Cape York Peninsula. These large systems can sustain northerly winds capable of carrying mosquitoes from New Guinea or even Badu Island into Cape York Peninsula. In addition, the convergent winds and unstable atmosphere of these low-pressure systems would enhance entrainment of mosquitoes into the upper boundary layer. Associated storms with heavy rain would aid mosquitoes' descent to land. Large-scale migration of insects, including mosquitoes, is often associated with large-scale weather systems with extensive wind fields (14), such as tropical low-pressure systems.

Direct evidence confirms that *Cx. annulirostris* travel from Papua New Guinea into northern Australia. Electrophoretic analysis of *Cx. annulirostris* populations collected from 1996 to 1998 in Papua New Guinea, the Torres Strait, and Cape York indicates substantial gene flow, indicating dispersal between the populations (H. Chapman and S. Ritchie, unpub. data).

Despite evidence that windborne mosquitoes could have introduced JE virus into northern Australia, additional field studies should be conducted. Northerly winds originating from New Guinea can be sampled for *Culex* mosquitoes and other potential vectors. Other incursion mechanisms, such as the movement of viremic birds and fruit bats, need to be investigated, as well as their ability to infect *Culex* mosquitoes.

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Dr. Ritchie is medical entomologist with the Tropical Public Health Unit, Queensland Health, in Cairns, Australia. His main interest is in the ecology and intervention of vector-borne diseases, such as Japanese encephalitis and dengue, in north Queensland, Australia.

Dr. Rochester, Department of Zoology and Entomology, University of Queensland, develops simulation models to predict movements of insect pests in Australia.

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Perceived Etiology of Foodborne Illness Among Public Health Personnel

Timothy F. Jones and Diane Eigsti Gerber
Tennessee Department of Health, Nashville, Tennessee, USA

Few data exist about perceptions regarding the etiology of foodborne illness. Among public health staff throughout Tennessee, the three pathogens most commonly believed to cause foodborne illness in the United States actually account for only 12% of disease. Fewer than 3% of respondents correctly identified the leading cause of foodborne illness.

In the United States, foodborne infections cause approximately 76 million illnesses each year, accounting for 325,000 hospitalizations and 5,000 deaths (1). Foodborne illness has been estimated to cost as much as $23 billion annually in this country (2). The consequences of such illness can range from transient discomfort to meningitis, congenital malformation, and death (3). Changes in eating habits and food preparation behaviors, globalization of the food supply, aging of the population, and other risk factors may be leading to increasing rates of illness (4,5).

Public health and infection control personnel are frequently involved in the identification, investigation, and intervention of foodborne illness outbreaks. In 68% of reported foodborne outbreaks in the United States, the pathogenic cause is not identified (6). An understanding of likely etiology is important for ensuring appropriate management of illness. There are few published data on the knowledge or perceptions of public health personnel regarding the common causes of foodborne disease.

Methods

During April and May 2000, epidemiologists, laboratory staff, and environmentalists from the Tennessee Department of Health presented a series of lectures to public health personnel throughout the state to review the process of investigating foodborne illness outbreaks. Participants included epidemiologists, public health nurses, laboratory staff, and environmentalists.

Before each session, participants were asked the following question: "What are the three most common pathogens causing foodborne illness in the United States?" Verbal instructions included clarification that the question referred to which pathogens were numerically the most frequent causes of illness. Participants ranked their top three answers in writing and submitted them to the course director. Responses were anonymous, although the job category of each respondent was collected. Data were entered and analyzed by using EpiInfo software (7).

Address for correspondence: Timothy F. Jones, Tennessee Department of Health, FoodNet Program, Communicable and Environmental Disease Services, 425 5th Avenue, 4th Floor, Cordell Hull Building, Nashville, TN 37247, USA; fax: 615-741-3857; e-mail: tjones4@mail.state.tn.us

Results

Of 553 attendees, 388 (70%) participants responded to the survey. Respondents included 128 environmentalists, 233 public health nurses, 11 health department physicians, 4 laboratorians, and 12 persons in other positions in the health department. The proportion of participants was representative of the proportion of responders to foodborne illness within the health department.

Ninety percent of persons listed *Salmonella* among the top three most common causes of foodborne illness in the

Table. Percentage of respondents identifying each pathogen as among the top three causes of foodborne illness, and estimated percentage of foodborne illnesses in the United States actually caused by those pathogens

Pathogen	Percentage of respondents listing it among top three causes	Est. percentage of foodborne illness in USA caused by pathogen (1)
Salmonella	90	9.7
Escherichia coli	56	1.3
Staphylococcus	36	1.3
Shigella	32	0.6
Campylobacter	18	14.2
Listeria	16	<0.1
Hepatitis A virus	8	<0.1
Clostridium perfringens	8	1.8
Norwalk-like virus	5	66.7
Viruses[a]	4	67.2
Giardia lamblia	3	1.4
Streptococcus	2	0.4

[a]Respondents who wrote in "viruses" only; does not include those who specified *Norwalk-like virus*.
Est = estimated.

United States; 56% listed *Escherichia coli*; 36% cited *Staphylococcus*; and 32% *Shigella* (Table). Other commonly cited causes of foodborne illness included *Campylobacter, Listeria, Hepatitis A virus*, and *Clostridium*.

Only 5% of respondents listed *Norwalk-like virus* (NLV) among the three most common causes of foodborne illness, and an additional 4% noted "viruses" more generically. Only 4% of respondents listed NLV or viruses as the most common source of foodborne illness.

Results did not vary significantly by job category of the respondents. Public health nurses, environmentalists, and physicians, for example, all listed *Salmonella, E. coli, Staphylococcus*, and *Shigella* as the most common causes of foodborne illness. Persons from all job categories were represented among the 9% of respondents who listed viruses or NLV among the top three causes. No job category was statistically more likely to identify viruses as a common etiology, and in no group was NLV among the five most commonly listed pathogens.

Conclusions

The four pathogens most commonly believed by the survey respondents to be among the major causes of foodborne illness (*Salmonella, E. coli, Staphylococcus*, and *Shigella*) are actually estimated to account for <13% cumulatively of all foodborne disease in the United States (Table). Recent estimates suggest that the most common causes of foodborne illness in the United States, in decreasing order of frequency, are NLV, *Campylobacter, Salmonella, Clostridium perfringens*, and *Giardia* (1). Only 5% of respondents listed NLV among the three most common causes of foodborne illness; this agent is estimated to cause 67% of all foodborne disease in the United States (1). In contrast, *Listeria*, which causes <0.1% of foodborne illness in the country, was believed by 15% of respondents to be among the three most common causes of disease.

The response to a suspected foodborne illness may differ, depending on the likely etiology. Basic methods of case-finding, hypothesis-generating, and investigating exposure histories do not necessarily require knowledge of the frequency of possible pathogens. Other issues, such as stool collection and testing techniques, treatment and follow-up, and preventive recommendations may differ greatly depending on a particular pathogen. If the personnel commonly responsible for recognizing, reporting, and intervening in foodborne illness are unfamiliar with common pathogenic causes of such illnesses, the appropriateness of their responses may be compromised.

Reasons for the discrepancy between perceived and actual etiologies of foodborne illness are unknown. While estimates that two-thirds of foodborne illnesses are caused by caliciviruses may be debated, perceptions of study respondents reflect neither national estimates nor recent experience in Tennessee. Some pathogens incorrectly believed to be common causes of foodborne illness, such as *E. coli* and *Listeria*, cause relatively severe disease, which often generates substantial media attention. Highly publicized outbreaks and severe cases may disproportionately affect perception of a pathogen's incidence. Such factors might be expected to influence public perception more than that of health-care workers, although this study suggests otherwise. There is no evidence that the public health personnel we surveyed have a substantially different understanding than health-care workers elsewhere. Studies on factors that affect both academic knowledge of the causes of foodborne illness, as well as factors such as severity and risk (which likely strongly influence perception of their relative importance), would be of value.

While it is true that the etiology is not identified in a large proportion of foodborne illnesses, lack of knowledge on the part of public health personnel is only one barrier to improving this situation. Lack of resources, competing priorities, the health-seeking behaviors of ill persons, and the activities of clinical and laboratory providers all have important effects on the response to suspected foodborne illness. Despite that, this study suggests that public health personnel on the front lines in responding to foodborne illness have incorrect perceptions of its causes. If this substantial public health threat is to be effectively addressed, appropriately educating the persons relied upon to address the problem is necessary.

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Dr. Jones is an epidemiologist and director of Tennessee's Emerging Infections Program's Foodborne Illness Active Surveillance Network (FoodNet). He was an officer in the Center for Disease Control and Prevention's Epidemic Intelligence Service and currently focuses on foodborne illness and tuberculosis in the Tennessee Department of Health.

Ms. Gerber is a public health nurse and educator, currently working as a surveillance officer and FoodNet coordinator in the Tennessee Department of Health.

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Fluoroquinolone Resistance among *Streptococcus pneumoniae* in Hong Kong Linked to the Spanish 23F Clone

Pak Leung Ho,* Wing Cheong Yam,* Terence K.M. Cheung,* Wilson W.S. Ng,* Tak Lun Que,† Dominic N.C. Tsang,‡ Tak Keung Ng,§ and Wing Hong Seto*

University of Hong Kong, Hong Kong Special Administrative Region (SAR), China; †Tuen Mun Hospital, Hong Kong SAR, China; ‡Queen Elizabeth Hospital, Hong Kong SAR, China; and §Princess Margaret Hospital, Hong Kong SAR, China

Serotypes 6A/B, 19F, and 23F accounted for 73% of 140 mucosal isolates of *Streptococcus pneumoniae* from Hong Kong. In pulsed-field gel electrophoresis analysis, a group of related patterns was shared by 14 of 15 ciprofloxacin-resistant and 12 of 16 ciprofloxacin-susceptible isolates. These strains exhibited capsular switching and were highly similar to the Spanish 23F clone.

Streptococcus pneumoniae, the most important cause of community-acquired pneumonia worldwide, particularly affects young children, elderly persons with chronic cardiopulmonary conditions, and immunosuppressed patients of all ages. Widespread emergence of antimicrobial resistance has become a concern in recent years. In many countries, rates of resistance to penicillin are >40%. Among penicillin-resistant *S. pneumoniae*, 60% to 90% are also resistant to the macrolides, tetracyclines, chloramphenicol, clindamycin, and cotrimoxazole. For this reason, newer fluoroquinolones with expanded activity against gram-positive bacteria have been recommended by the Infectious Disease Society of America as initial treatment of choice for community-acquired pneumonia (1).

Although resistance to the newer fluoroquinolones remains rare in most countries, the percentage of nonsusceptible *S. pneumoniae* has increased from <0.5% for ofloxacin to 5.5% for levofloxacin (MIC ≥4 µg/mL) from 1995 to 1998 in Hong Kong (2). Almost all strains of fluoroquinoline-resistant *S. pneumoniae* were isolated from respiratory tract specimens. Knowledge of the serotype distribution of *S. pneumoniae,* particularly strains with the emerging resistance pattern, is important for development of conjugate vaccines.

We studied the serotype distribution of recent isolates of drug-resistant pneumococci from Hong Kong, including isolates with resistance to the fluoroquinolones. To understand better the emergence of fluoroquinolone-resistant *S. pneumoniae* in this locality, we used pulsed-field gel electrophoresis (PFGE) to compare strains.

The Study

We examined sputum isolates of *S. pneumoniae* obtained from four regional laboratories in Hong Kong during a prospective regional survey in 1998 (2). The four laboratories (A-D) provide microbiology service to seven public hospitals, including a university medical center, four other major medical centers, and two rehabilitation centers. Together they served a population of approximately 3 million in the Hong Kong Island (south and west), Kowloon (central), and the New Territory (south and north) regions of Hong Kong.

All strains were nonduplicate isolates obtained from consecutive clinical samples of hospitalized patients during the second half of 1998. Of 143 isolates obtained, three became nonviable during storage. The numbers of isolates from laboratories A, B, C, and D were 46, 39, 12, and 43, respectively.

MICs for penicillin and erythromycin were determined by the E-test method (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions. MICs for ciprofloxacin, levofloxacin, and trovafloxacin were determined by a standardized broth microdilution procedure with cation-adjusted Mueller-Hinton broth supplemented with 2.5% lysed horse blood (3). All MIC results were interpreted according to National Committee for Clinical Laboratory Standards. For ciprofloxacin, an MIC value of ≥4 µg/mL was regarded as resistant (4). The isolates were serotyped by the quellung reaction (5) with sera of various reactivities (pools A to I, P to T, and selected major groups and serum factors) from the Statens Seruminstitut (Copenhagen, Denmark). All 15 ciprofloxacin-resistant isolates were compared with 16 ciprofloxacin-susceptible isolates, *S. pneumoniae* ATCC 49619, and the well-defined Spanish clones of serotypes 23F and 6B (SP264 ATCC 700669 and GM17 ATCC 700670, respectively), by PFGE after digestion of the genomic DNA with *Sma*I and *Apa*I, respectively (6).

Ciprofloxacin-susceptible isolates of serotypes 6A, 19F, and 23F, matching the serotypes in the ciprofloxacin-resistant isolates, were chosen for PFGE analysis. The 16 ciprofloxacin-susceptible isolates included the remaining 4 isolates of serotype 6A and 6 each of serotypes 19F and 23F, chosen randomly. Of the 16 ciprofloxacin-susceptible isolates, 5 were penicillin sensitive, 3 were intermediate, and 8 were resis-

Address for correspondence: PakLeung Ho, Department of Microbiology, Queen Mary Hospital, University of Hong Kong, Pokfulam Road, Pokfulam, Hong Kong SAR, China; fax: 852-2855-1241; e-mail: plho@hkucc.hku.hk

tant. These ciprofloxacin-susceptible isolates were obtained from all four laboratories (A, 5; B, 4; C, 3; and D, 4). The Fisher exact or chi-square test was used for statistical analysis, with a value of <0.05 indicating statistical significance.

Of the 140 isolates, 18 (12.9%) and 87 (62.1%) were intermediate (MIC 0.11 µg/mL) and resistant (MIC ≥2 µg/mL) to penicillin, respectively. One hundred twelve (80%) of 140 were nonsusceptible to erythromycin (MIC ≥0.5 mg/mL). Fifteen of the 140 isolates were resistant to ciprofloxacin (four isolates from laboratory A, one from laboratory B, two from laboratory C, and eight from laboratory D). The 15 isolates had ciprofloxacin MICs of 4 µg/mL (4/15), 8 mg/mL (2/15), 16 µg/mL (5/15), and 32 µg/mL (4/15). All nine isolates with ciprofloxacin MICs 16 to 32 µg/mL and one with a ciprofloxacin MIC 8 µg/mL were intermediately resistant (2/10) or resistant (8/10) to levofloxacin. One and three isolates with ciprofloxacin MICs of 16 and 32 mg/mL, respectively, were intermediately resistant (1/4) or resistant (3/4) to trovafloxacin. All four trovafloxacin-nonsusceptible isolates were resistant to levofloxacin. All ciprofloxacin-resistant strains were from adults 54 to 88 years of age and were resistant to both penicillin and erythromycin. No isolates were obtained from the children in the 6- to 17-year age group. Penicillin-nonsusceptible strains were common in all other age groups: <2 years (9/11), 2 to 5 years (21/23), 18 to 49 years (9/11), 50 to 64 years (10/11), and ≥65 years (56/84).

Eighteen serotypes or serogroups were identified among the 123 typeable strains (Table). The most common serotypes were 23F (41.4%), 19F (18.6%), and 6B (9.3%). The combined proportions of these three serotypes by age group were as follows: <2 years (9/ 11), 2 to 5 years (20/23), 18 to 49 years (9/11), 50 to 64 years (9/11), and ≥65 years (50/84). Penicillin-nonsusceptible strains were more likely than susceptible ones to belong to these three serotypes (93 of 105 vs. 4 of 35, p<0.001). For the penicillin sensitive, intermediate, and resistant isolates, 10, 4, and 3 isolates were untypeable, respectively. Exclusive of the untypeable isolates, 7 (28%) of 25, 13 (92.9%) of 14, and 84 (100%) of 84 of the penicillin-sensitive, intermediate, and resistant isolates, respectively, were restricted to serogroups 6, 19, and 23. On the basis of identical serotypes, coverage of the recently licensed heptavalent-conjugated vaccine by age groups was 81.8% for those <2 years, 91.3% for 2 to 5 years, 81.8% for 18 to 49 years, 81.8% for 50 to 64 years, and 59.5% for ≥65 years.

PFGE analysis identified seven groups of DNA patterns when either *Sma*I or *Apa*I was used (Figure). The six subtypes of group A (A0-A5) were either identical (A0) to that of the Spanish 23F clone or differed from it by 1 to 4 bands (A1 to A5). Group A patterns were shared by 14 of 15 ciprofloxacin-nonsusceptible (all A1) and 12 of 16 ciprofloxacin-susceptible (3 A0, 3 A1, 3 A2, 1 A3, 1 A4, and 1 A5) isolates. The remaining five distinct profiles (B, C, D, E, F) were identified in each of the remaining five isolates. All strains with group A subtypes were either identical or closely related by *Apa*I analysis.

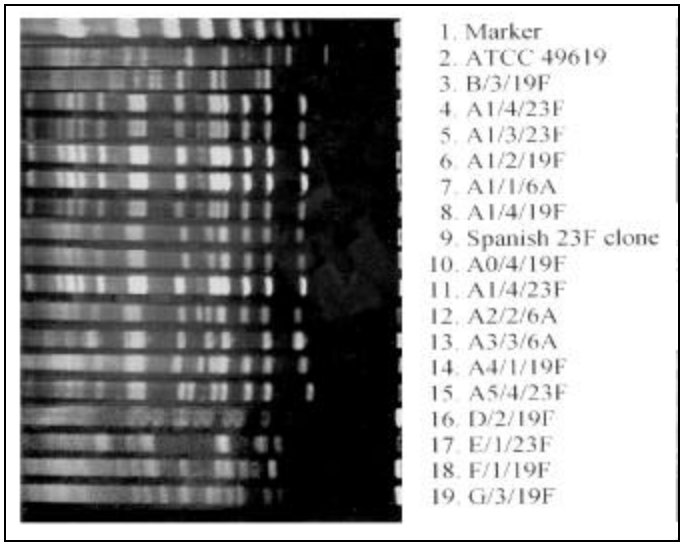

Figure. Pulsed-field gel electrophoresis (PFGE) patterns of *Sma*I-digested genomic DNA of *Streptococcus pneumoniae* isolates. Seven distinct patterns, including A and its subtypes (A0 to A5) and B to G, are shown. Labels indicate the PFGE pattern, hospital source, and serotype. Lane 1: molecular weight markers; lane 2: *S. pneumoniae* ATCC 49619; lanes 3 to 8: ciprofloxacin-resistant isolates; lane 9: Spanish 23F clone (ATCC 700669); lanes 10-19: ciprofloxacin-sensitive isolates.

Conclusions

PFGE analysis showed that most ciprofloxacin-resistant and ciprofloxacin-susceptible *S. pneumoniae* isolates were either identical or closely related to the Spanish 23F clone. This clone is dominant in Hong Kong, where it accounted for approximately 70% of all penicillin-resistant *S. pneumoniae* isolates from 1994 to 1997 (7). Our data suggest that this clone has acquired fluoroquinolone resistance and is already widespread in Hong Kong. In contrast, in Canada and Spain fluoroquinolone-resistant pneumococci are also emerging, but with genetically diverse strains (4). Nonetheless, our finding raises the possibility that this fluoroquinolone-resistant variant of the Spanish 23F clone might spread internationally, as its ancestor did in the past decade (8). Therefore, infection control guidelines should be formulated for screening and isolating patients with fluoroquinolone-resistant *S. pneumoniae*. Furthermore, molecular analysis of fluoroquinolone-resistant strains from other countries compared with those from Hong Kong is also indicated to determine whether this clone has already disseminated outside Hong Kong.

Our data show that the Hong Kong fluoroquinolone-resistant clone is resistant to multiple other antibiotics, including penicillin, erythromycin, and clindamycin (2). This finding implies that the fluoroquinolone-resistant variant could be selected not only by fluoroquinolones but also by other antibiotics. In Sweden, for instance, cotrimoxazole has been shown to select for penicillin resistance in children attending day-care centers (9). Given the wide range of antibiotic classes involved, further emergence of the fluoroquinolone-resistant clone is likely. One potentially sinister situation would be dissemination of the clone to children. Although fluoroquinolone is rarely used in children in Hong Kong, the fluoroquinolone-resistant strain could spread, for example, through household contacts from adults to children. Its spread could readily be facilitated by the frequent misuse of antibiotics for upper respiratory tract infections in children. The therapeutic half-life of fluoroquinolones as treatment for pneumococci is now being challenged. Finally, three different serotypes (6A, 19F, 23F) were expressed by our strains that were closely related in the PFGE analysis. This is likely a result of capsular switching, as reported previously (10).

Table. Distribution of capsular types in 140 strains of mucosal *Streptococcus pneumoniae* with respect to age and resistance to ciprofloxacin

Type	Age group (years)					Ciprofloxacin MIC ≥4 µg/mL		
	<2	2-5	18-49	50-64	≥65	No[a] (n=125) No. (%)	Yes (n=15) No. (%)	No. (%)
19F	4	3	1	4	14	19 (15.2)	7 (46.7)	26 (18.6)
23F	4	12	8	4	30	51 (40.8)	7 (46.7)	58 (41.4)
6A	1	1			3	4 (0.8)	1 (6.7)	5 (3.6)

[a]Number of strains for other serotype or serogroup by age groups: <2 years (1 for 6B and 1 untypeable), 2 to 5 years (5 for 6B, 1 for 9V, and 1 untypeable), 18 to 49 years (1 for 10B and 1 for 35), 50 to 64 years (1 for 6B, 1 for 19C, and 1 for 34), and ≥65 years (6 for 6B, 1 for 7F, 1 for 9A/L, 1 for 10C/F, 1 for 11A, 3 for 13, 1 for 15B, 1 for 19B, 1 for 21, 1 for 22A, 5 for 35, and 15 untypeable). No isolates were obtained from the children in the 6- to 17-year age group. All strains with serotypes or serogroups other than 6A, 19F, and 23F were sensitive to ciprofloxacin.

In conclusion, most of the drug-resistant pneumococci in Hong Kong were of serotypes 6A/B, 19F, and 23F. Fluoroquinolone-resistant strains, which were found only in older adults, were genetically highly similar and probably have arisen by acquisition of fluoroquinolone resistance by the locally dominant Spanish 23F clone.

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Dr. Ho is associate professor in the Department of Microbiology, University of Hong Kong. His interests include infectious diseases, epidemiology, and mechanisms of emerging antimicrobial resistance.

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First Documentation of *Rickettsia conorii* Infection (Strain Indian Tick Typhus) in a Traveler

To the Editor: Spotted fever group rickettsiae are gram-negative intracellular bacilli associated with arthropods, mainly ticks, as vectors. To date, 12 tick-borne rickettsioses are recognized worldwide, seven since 1991 (1). Indian tick typhus (ITT) is a tick-borne rickettsiosis prevalent in India (1). Although the disease has been recognized clinically, cases have been documented only rarely, and then mainly with nonspecific serologic tools, such as the Weil Felix test. We report the first serologically documented case of infection caused by *Rickettsia conorii* (strain ITT) in a French traveler returning from India.

In September 1999, a 25-year-old woman living in France was hospitalized with a 4-day history of fever, headache, vertigo, malaise, and disturbance of vision, followed 3 days later by arthromyalgia and a rash. On the day of admission, she had returned from a 1-month stay in India. She reported a number of bites by unidentified arthropods during her trip. Her temperature was 40°C with relative bradycardia (72/minute). Physical signs included pharyngitis, transient epistaxis, bilateral conjunctivitis, and a maculopapular rash including petechiae, mostly on the trunk and lower limbs but also on her palms and the soles of her feet. There was no inoculation eschar.

Clinical laboratory findings included increased alanine aminotransferase (56 UI/L), lactate dehydrogenase (1,008 UI/L), C-reactive protein (130 mg/L), and erythrocyte sedimentation rate (74 mm/hour). The kaolin cephalin time was 43 seconds (control 34 seconds). Hemoglobin was 10.5 g/dL, and the mean corpuscular volume was 99.7 fL. Repeated blood smears, blood cultures, and a stool bacterial culture disclosed no pathogens. Serologic tests, including assays for rickettsioses, were negative.

The patient received 5 days of empirical doxycycline treatment (200 mg/day), intravenously for the first 2 days because of vomiting. She became afebrile 2 days after therapy was begun. Subsequently, all her symptoms resolved. Ten days later, the immunofluorescence assay for antibodies reactive with spotted fever group rickettsiae showed increased levels of immunoglobulin (Ig) M (1:256) and IgG (1:1024) against *R. conorii* Seven and IgM (1:512) and IgG (1:2048) against *R. conorii* ITT. Serologic findings were completed by Western blot performed with acute-phase serum, which showed a band of approximately 135 kDa against *R. conorii* ITT, but not *R. conorii* Seven. Cross-absorption studies were performed with convalescent-phase sera. Sera were absorbed with *R. conorii* Seven and *R. conorii* ITT antigens and then tested by immunofluorescence assay for remaining antibodies reactive to both antigens. When absorption was performed with *R. conorii* ITT antigens, serologic testing was negative for antibodies to both *R. conorii* Seven and *R. conorii* ITT antigens. However, when absorption was done with *R. conorii* Seven antigens, subsequent serologic testing was negative for antibodies to *R. conorii* Seven, but antibodies to *R. conorii* ITT remained (1:100). Thus, Western blotting and cross-absorption strongly supported that the infection was due to *R. conorii* ITT.

Although ITT was clinically described at the beginning of the century, the etiologic agent has never been isolated from patients in India, nor has a case been diagnosed by strain-specific serologic testing. A spotted fever group rickettsia was isolated in 1950 from a brown dog tick, *Rhipicephalus sanguineus*, collected in India (2) and assumed to be the agent causing ITT. It was designated as *Rickettsia conorii*, the agent of Mediterranean spotted fever, which occurs all around the Mediterranean and is transmitted by the same tick species. However, the disease as it appears in India differs from the common description of Mediterranean spotted fever. The rash is frequently purpuric, and an inoculation eschar at the bite site is rarely found, as in this case. The disease as known in India is mild to moderately severe, although our case may be considered severe (1,3,4).

Strain differences within the species *R. conorii* may account for differences in clinical presentation. Although different isolates of *R. conorii* can be distinguished antigenically (5-7), molecular taxonomic methods demonstrated recently that these rickettsiae are closely related and cluster together (8-10). Thus, the species is considered by many as *R. conorii*, including four serovars: *R. conorii* with three type strains, Seven being the one most commonly identified in our laboratory in isolates from France, Portugal, North Africa (D. Raoult, unpub. data), Kenya, and Morocco (apparently a unique isolate); *R. conorii* Indian tick typhus; *R. conorii* Astrakhan, and *R. conorii* Israel.

Immunofluorescence is the reference diagnostic method for associated rickettsioses, but cross-reactivity among related isolates confounds interpretation of serologic tests. Cross-absorption tests, especially in conjunction with Western blot immunoassays, can be used to determine the rickettsia species involved, as reported in this case. The higher sensitivity of Western blots compared with immunofluorescence has been demonstrated previously in our laboratory; it is frequently positive in acute-phase sera when antibodies cannot be detected by immunofluorescence (11). In this case, we used *R. conorii* Seven as the type strain of *R. conorii* because it is most closely related to *R. conorii* ITT phylogenetically (10). Although these techniques are time-consuming and available only in specialized reference laboratories, they provide data of importance that allow a better understanding of the epidemiology of rickettsioses.

Philippe Parola,*† Florence Fenollar,† Sekene Badiaga,* Philippe Brouqui,*† and Didier Raoult†

*Service des Maladies Infectieuses et Tropicales, CHU Nord, Marseille, France; and †Unité des Rickettsies, Faculté de Médecine, CNRS UMR 6020, Marseille, France

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Multidrug-Resistant *Pseudomonas aeruginosa* Producing PER-1 Extended-Spectrum Serine-ß-Lactamase and VIM-2 Metallo-ß-Lactamase

To the Editor: In *Pseudomonas aeruginosa,* secondary beta-lactamases with extended substrate specificity can be responsible for acquired resistance to the most powerful antipseudomonal beta-lactams, such as expanded-spectrum cephalosporins and carbapenems (1). A number of these enzymes have been described, including extended-spectrum serine-beta-lactamases (ESBLs) of groups 2be and 2d (e.g., PER-1 and various OXA-type enzymes) (2,3) and metallo-beta-lactamases of group 3 (e.g., IMP-1 and the recently described VIM-1 and VIM-2 enzymes) (2,4,5). The secondary ESBLs can degrade penicillins, expanded-spectrum cephalosporins, and monobactams (but not carbapenems) and are often susceptible to serine-beta-lactamase inhibitors (1-3). The secondary metallo-beta-lactamases, on the other hand, are notable for their carbapenemase activity and can degrade virtually all beta-lactams except monobactams, while being resistant to the currently available inhibitors (1,2,5,6).

On March 2000, a multidrug-resistant *P. aeruginosa* (isolate VA-182/00) was isolated in pure culture from a bronchial washing of a 58-year-old patient with multiple myeloma. The patient had been admitted 15 days earlier to the Varese University Hospital with a diagnosis of pneumonia and had been treated with ciprofloxacin (0.5 g twice a day) plus piperacillin (2 g three times a day) for 12 days, and then with imipenem/cilastatin (0.5 g three times a day). No cultures of respiratory tract specimens were done earlier in hospitalization. Multiple myeloma had been diagnosed in 1997, and the patient had been treated with multiple cycles of antiproliferative chemotherapy and had received autologous peripheral blood stem cell transplantation. According to clinical records, *P. aeruginosa* had not been isolated previously during this patient's protracted illness. In vitro susceptibility testing showed that the *P. aeruginosa* isolate was resistant to mezlocillin, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, netilmicyn (MICs, >128 µg/mL), amikacin (MIC, 64 µg/mL), ciprofloxacin and levofloxacin (MICs, >32 µg/mL). Only piperacillin and piperacillin/tazobactam had MIC values slightly lower than the breakpoints for resistance (64 µg/mL and 48/4 µg/mL, respectively), although–considering the normal MICs of piperacillin for susceptible *P. aeruginosa* (2-8 µg/mL)–it was evident that the isolate also had considerable biological resistance to these drugs. A double disk-diffusion test, carried out with standard disks placed 20 mm apart (center-to-center), showed synergy between clavulanate and aztreonam. The treatment was changed to piperacillin/tazobactam (4 g four times a day), and a slow recovery ensued over a 30-day period. The patient died 3 months later following a relapse of the underlying malignancy.

The unusually high carbapenem MICs exhibited by VA-182/00 suggested production of a secondary metallo-beta-lactamase, while the synergy between clavulanate and aztreonam suggested production of a secondary serine ESBL. A crude extract of that isolate, assayed spectrophotometrically (7), exhibited imipenem-hydrolyzing activity (94 nmol/min/mg protein, inhibited by EDTA) as well as aztreonam-hydrolyzing activity (11 nmol/min/mg protein, resistant to EDTA). Analytic isoelectric focusing (IEF) of the extract, followed by development with the nitrocefin chromogenic substrate (7), showed three bands of beta-lactamase activity of pIs 5.4, 5.6, and 6.3, suggesting the presence of at least three different secondary enzymes. A colony-blot hybridization with probes for the bla_{IMP}, bla_{VIM}, and bla_{PER} resistance genes (all of which have been previously detected in *P. aeruginosa* clinical isolates from the same hospital [8,9; Luzzaro F, unpub. data]) yielded positive results with both the bla_{VIM} and the bla_{PER} probes. Amplification of the resistance genes by polymerase chain reaction (PCR) with primers VIM/DIA-f (5'-CAgATTgCCgATggTgTTTgg) and VIM/DIA-r (5'-AggTgggC-CATTCAgCCAgA) for bla_{VIM} genes (4,5) and BLAPER-f (5'-gggACA(g/A)TC(g/C)(g/T)ATgAATgTCA) and BLAPER-r (5'-ggg(C/T)(g/C)gCTTAgATAgTgCTgAT) for bla_{PER} genes (9), yielded amplicons of the expected sizes (522 and 966 bp, respectively). Direct amplicon sequencing identified the two beta-lactamase determinants as bla_{VIM-2} (5) and bla_{PER-1} (10), respectively, a finding consistent with the pIs 5.6 and 5.4 beta-lactamase bands detected in IEF (3,5). Conjugative transfer of the resistance determinants to *Escherichia coli* proved unsuccessful. In a Southern blot analysis of total undigested DNA from VA-182/00, both the bla_{VIM} and bla_{PER} probes apparently hybridized to the chromosomal DNA band; no plasmid bands recognized by either probe were detected. A PCR experiment with primers OXA10-f (5'-ggAA-CAAAgAgTTCTCTgCC) and OXA105-r (5'-TTAgCCAC-CAATgATgCC(C/T)TC), suitable for amplification of bla_{OXA} genes of the OXA-10 group, did not yield an amplicon of the expected size (719 bp), suggesting that the pI 6.3 beta-lactamase band detected by IEF did not correspond to an enzyme of this group.

This is the first observation of a *P. aeruginosa* clinical isolate simultaneously producing a secondary PER-1 ESBL and a secondary metallo-beta-lactamase. The finding, observed in a hospital where both the resistance genes

(bla_{PER-1} and bla_{VIM-2}) had been detected separately among clinical isolates, underscores the possibility of the emergence of new threatening combinations of resistance determinants among nosocomial pathogens. In fact, the recruitment of similar resistance determinants within a single *P. aeruginosa* strain can determine a resistance phenotype to virtually all the available antipseudomonal beta-lactams, an occurrence that can be particularly dramatic when, as in the present case, resistance to beta-lactams is associated with resistance against aminoglycosides and fluoroquinolones. In this case, only piperacillin (which appears to be a relatively poor substrate for both enzymes [3,5]) retained moderate activity in vitro and, administered at high dosage in combination with tazobactam, was apparently effective in vivo. Should a similar resistance phenotype disseminate, it might have strategic implications for the development of new beta-lactamase inhibitors and for selection of beta-lactam compounds to associate wth inhibitors.

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Jean-Denis Docquier,* Francesco Luzzaro,† Gianfranco Amicosante,‡ Antonio Toniolo,† and Gian Maria Rossolini*

*Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Siena, Italy; †Laboratorio di Microbiologia, Ospedale di Circolo e Università dell'Insubria, Varese, Italy; and ‡Dipartimento di Scienze e Tecnologie Biomediche, Università di L'Aquila, L'Aquila, Italy

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Jamestown Canyon Virus: Seroprevalence in Connecticut

To the Editor: *Jamestown Canyon virus* (JCV), a member of the California serogroup, has a wide geographic distribution throughout much of temperate North America. It causes mild febrile illness and, rarely, aseptic meningitis or primary encephalitis (1). JCV has been isolated from mosquitoes each year that surveys have been done in Connecticut, and 28 positive pools from 10 mosquito species were found during 2000 (T. Andreadis, pers. commun.). In contrast, only 14 positive mosquito pools were found to contain *West Nile virus* (WNV), which has recently been introduced into Connecticut (2). JCV has been isolated from *Aedes* mosquitoes in Connecticut, and serologic evidence suggests it is widespread in deer (3,4). No recent seroprevalence surveys have been done in Connecticut, nor have any human cases of infection or disease due to JCV been documented.

We report the results of two seroprevalence surveys done with standard indirect fluorescent assays (IFA) to detect immunoglobulin G antibodies to JCV. One survey examined 1,086 sera collected in 1990 from blood donors. The second survey examined 1,016 sera submitted to the Connecticut State Public Health Laboratory in 1995.

The IFA used JCV-infected baby hamster kidney cells (BHK-21). Infected and uninfected cell suspensions were air dried and fixed onto Teflon-coated, 12-well slides. Prepared slides were stored at -70°C. Sera were tested at a minimum dilution of 1:16. After incubation and washing of the fluorescein-conjugated counterstain, slides were dried and examined by fluorescent microscope (American Optical, Buffalo, NY). The positive human control serum was designated as the 4+ baseline with which the test sera were compared. Selected sera were tested by a serum dilution plaque reduction neutralization test (PRNT) assay with JCV, *La Crosse virus*, and trivittatus virus.

Of the 1,086 sera collected from blood donors in 1990, 164 (15%) were positive by IFA at a minimum dilution of 1:16. Because IFA screening procedures are known to have poor specificity, a subset of 39 IFA-positive and 5 IFA-negative sera was tested by PRNT. None of the IFA-negative sera were positive, while 26 (67%) of the 39 IFA-positive sera were positive for JCV antibodies. Extrapolating the PRNT results to the 164 IFA-positive sera yields an overall positivity rate of 10.1%.

The second serosurvey, performed on 1,016 sera collected in 1995 from apparently healthy patients requesting immune status testing to viruses such as *Varicella zoster* or measles, had 57 IFA-positive specimens. Extrapolating addi-

tional PRNT results from 26 sera, of which 18 (69%) were positive, yields a 3.9% positivity rate.

In addition to our study, with crude seroprevalence rates ranging from 3.9% to 10.1%, another recent study demonstrated JCV antibodies in 2.9% to 13.3% of ill persons in Massachusetts (Tonry J et al., unpub. data). Although the screening results of our first serosurvey (10.1% positive) differed widely from those of the second serosurvey (3.9% positive), even the lower rate indicates substantial levels of human infection in Connecticut.

This report suggests that JCV infection is fairly frequent in Connecticut and that illness may occur, as corroborated by data from neighboring Massachusetts (Tonry J et al., unpub. data) and unpublished laboratory findings from the Connecticut State Public Health Laboratory. The interest in arboviral disease will continue unabated, spurred by the continued occurrence of WNV, and systematic testing for JCV infection may be timely, at least throughout the northeastern United States.

Donald Mayo,* Nick Karabatsos,† Frank J. Scarano,‡ Timothy Brennan,* Daniel Buck,§ Terry Fiorentino,§ John Mennone,§ and San Tran§

*Connecticut Department of Public Health Laboratory, Hartford, Connecticut, USA; †CDC Division of Vector-Borne Infectious Diseases, Fort Collins, Colorado, USA; ‡University of Massachusetts Dartmouth, Dartmouth, Massachusetts, USA; and §Yale University Department of Epidemiology and Public Health, New Haven, Connecticut, USA

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A Newly Discovered Variant of a Hantavirus in *Apodemus peninsulae*, Far Eastern Russia

To the Editor: Hemorrhagic fever with renal syndrome (HFRS) is caused by *Hantaan virus* (HTNV) or *Seoul virus* (SEO) in Asia and *Puumala virus* (PUUV) or *Dobrava virus* (DOBV) in Europe (1). Each of these hantaviruses is predominantly associated with a single rodent species as its primary natural reservoir: HTNV with the striped field mouse *Apodemus agrarius*, SEO with *Rattus norvegicus*, PUUV with the bank vole *Clethrionomys glareolus*, and DOBV with the yellow-necked mouse *Apodemus flavicollis*. An additional rodent reservoir of DOBV, *A. agrarius*, was reported recently (2).

The first HFRS cases (then called "hemorrhagic nephroso-nephritis") were clinically described in the Amur River basin during the 1920s by Russian scientists (3). Serologic studies suggest that numerous hantaviruses are present in humans and rodents in the far east of Asian Russia (4-5). Serologic evidence of hantavirus infection in *A. agrarius*, *A. peninsulae* (Korean field mouse), *R. norvegicus*, *Cl. rufocanus*, *Cl. rutilus*, and *Microtus fortis* has been reported (5). Only *Khabarovsk virus* (KBR), isolated from *M. fortis*, has been characterized in detail, and no association with human disease was established (6).

To genetically characterize hantaviruses in *A. peninsulae*, we studied samples from rodents captured in July and August 1998 in the same region of the forest near Khabarovsk. Lung-tissue samples were screened by enzyme-linked immunosorbent assay for HTNV/SEO/PUUV-related antigen. Samples from four hantavirus-positive rodents were tested by reverse transcription and nested polymerase chain reaction (PCR). Four M-segment PCR products (nt 2639-3000) and two S-segment PCR products (nt 592-945) were produced and directly sequenced (GenBank accession numbers AF332569-AF332573). All sequences were closely related to each other, with nucleotide diversity between strains not exceeding 0.6% for M segments and 1.3% for S segments. Comparative analysis of the M segments showed that hantaviral nucleotide sequences from *A. peninsulae* were very similar to those we identified earlier in HFRS patients (diverging 3.1% to 6.6%), which we term the Amur genotype of HTNV (7). The S-segment sequences of the AMR genotype from human patients were not available for comparison. The nucleotide sequence (the M and S segments, respectively) of the hantavirus detected in *A. peninsulae* diverged substantially from those of other hantaviruses (15% and 19% for HTNV, 21% to 28% for SEO, 22% and 29% for DOBV, 38% and 39% for PUUV, and 36% and 37% for KBR).

Neighbor-joining phylogenetic analysis based on partial sequences of the S segment indicated that the hantaviral sequences from *A. peninsulae* form a separate lineage on the phylogenetic tree, and together with HTNV virus strain 76-118, which originates from *A. agrarius*, constitute a well-supported group. A phylogenetic tree based on partial M segment sequences placed all hantavirus strains originating from *A. peninsulae* or from HFRS patients apart from all HTNV sequences recovered from *A. agrarius* (strain 76-118) and HFRS patients from Korea (strains HoJo, Lee). The taxonomic placement of this hantavirus (Amur genotype) as a distinct hantavirus or a distinct genetic lineage of HTNV remains to be determined. In addition, the finding of distinct DOBV genetic lineages in *A. flavicollis* and *A. agrarius* raises the same question of whether the two DOBV variants represent distinct hantaviruses (2).

A. peninsulae is widely distributed throughout eastern Asia, from Altai and south Siberia to the Russian far east, northeastern and eastern parts of China, and Korea. A survey of hantavirus antigens in rodent populations in the far east of Russia demonstrated the presence of HTNV-like antigen in 8% to 16% of *A. peninsulae* (5). Whether pathogenic AMR genotype of virus exists in *A. peninsulae* throughout far eastern Asia, from Russia to China and Korea, requires further study. Comparing hantaviral genome sequences available from GenBank shows that the M segment nucleotide sequence recovered from an HFRS patient from China (strain H8205, GenBank accession number AB030232) was very similar to the AMR genotype from *A. peninsulae* (94% to

96% identity), suggesting that this hantavirus is also present in *A. peninsulae* in China.

In earlier studies, we found that sera from patients infected by the AMR genotype of hantavirus showed extensive cross-reactivity with HTNV and SEO antigens in immunofluorescent antibody tests (7). Consequently, many HFRS cases previously thought to have been caused by HTNV or SEO may instead have been caused by infection with the hantavirus described here.

Our data represent the first genetic evidence for the AMR genotype of HTNV in *A. peninsulae* and suggest that this rodent species may be a natural reservoir for this pathogenic hantavirus.

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Lyudmila Yashina,* Vasiliy Mishin,* Nina Zdanovskaya,† Connie Schmaljohn,‡ and Leonid Ivanov†

*State Research Center of Virology and Biotechnology "Vector," Koltsovo, Novosibirsk, Russia; †Khabarovsk Antiplaque Station, Khabarovsk, Russia; and ‡U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, USA

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The Cover

Louis J. Steele and Charles F. Goldie
The Arrival of the Maoris in New Zealand (1898)

Oil on canvas, 1380 mm x 2450 mm, Auckland Art Gallery Toi o Tamaki, bequest of Helen Boyd, 1899.

The late 19th-century emergence in New Zealand of the history-painting tradition relates to the European settler (Pakeha) culture's artistic and literary quest for a national identity. By the 1880s the larger towns had established museums and art galleries intent on acquiring important "national" works, as well as art societies that promoted the achievements of local painters. Pakeha writers were crafting new accounts of the distant Maori past, interrogating and embellishing traditional migration legends. Inevitably, painters appropriated this legendary past for their own artistic purposes.

The English-born Louis J. Steele (1843-1918) had trained in Paris during the 1860s and had lived through the Prussian siege and Commune of 1870-71. Cutting a calculatedly bohemian figure in 1880s Auckland, Steele became a leading artist and influential teacher. He encouraged his New Zealand-born protegé Charles F. Goldie (1870-1947) to seek further training with the cosmopolitan Académie Julian in Paris, where he studied from 1893 to 1897. Goldie's training involved the copying of famous works from the Louvre, including Théodore Géricault's early 19th-century history painting The Raft of the Medusa–the compositional inspiration for The Arrival of the Maoris.

Goldie and Steele displayed their painting, which was on a monumental scale by colonial standards, first at their studio and subsequently at the 1899 exhibition of the Auckland Society of Arts. Critics hailed its evocation of the moment in which the desperate mariners sighted their new land, and the "gruesome" and "appalling" evidence of the travelers' privations: "There is a terrible attraction in these naked emaciated figures huddled in all different postures of agony and despair in the canoe" (1). Recent scholarship has linked Pakeha enthusiasm for The Arrival of the Maoris to the 19th-century predilection for shipwreck imagery, as well as to the comforting message that the Maori were themselves immigrants (2).

As early as 1902 there was a report that "Maoris who view the picture in the Art Gallery are indignant at the manner in which it is represented that the natives arrived in New Zealand" (3). Another writer in 1934 described Maori elders' responses to The Arrival of the Maoris and a similar work as follows: "Far from being appreciative, they always regard them with dubious feelings and disdain. To them they are mere creations of the Pakeha mind and not consistent with the traditional records of the matters represented" (4). Maori revulsion towards the painting relates to more than the diminishment of Polynesian maritime prowess, or to the many historical inaccuracies, for the depiction of a desperate band hurtling forward on a broken craft represents a graphic realization of the widespread colonial mythology of the "dying race."

Despite Maori dismay and the vigorous denunciation of ethnologists, The Arrival of the Maoris in New Zealand has secured a firm hold on the mechanisms of perpetual fame. It enjoys a more active reproduction cycle than any other New Zealand historical painting, appearing even within Maori publications. The checkered history of its reception suggests that such paintings of "history," especially colonial appropriations of a Maori past, are likely to remain highly contested.

Roger Blackley
Victoria University of Wellington
New Zealand

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Editorial Policy and Call for Articles

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

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

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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.

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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.

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