EMERGING INFECTIOUS DISEASES

Tracking trends and analyzing new and reemerging infectious disease issues around the world

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Lyme Disease Tuberculosis Vaccines

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Rumors of Disease in the Global Village: Outbreak Verification

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Emerging infectious diseases and the growth of information technology have produced new demands and possibilities for disease surveillance and response. Increasing numbers of outbreak reports must be assessed rapidly so that control efforts can be initiated and unsubstantiated reports can be identified to protect countries from unnecessary economic damage. The World Health Organization has set up a process for timely outbreak verification to convert large amounts of data into accurate information for suitable action. We describe the context and processes of outbreak verification and information dissemination.

Globalization presents new challenges and opportunities in combating diseases likely to cause epidemics. As a result of increased international travel and trade, local events acquire international importance. At the same time, the rapid global expansion of telecommunications and broadened access to news media and the Internet have changed the way society treats information. Reports of disease outbreaks are more widely disseminated and more easily accessible than ever before. However, the quality of information is no longer controlled and may be provided out of context, often causing unnecessary public anxiety and confusion. Rumors that later prove to be unsubstantiated may lead to inappropriate response, causing disruption in travel and trade and economic loss to affected countries.

The World Health Organization (WHO), speaking for 191 member countries, is uniquely positioned to coordinate infectious disease surveillance and response at the global level. WHO receives reports of disease outbreaks around the world from various sources. While some of these reports are warnings of genuine epidemics, others may reflect endemic disease or may be mere rumors.

To investigate and follow up outbreak reports, WHO established an innovative mechanism—outbreak verification—in early 1997. Outbreak verification is a new approach to global disease surveillance (1). Its aim is to improve epidemic disease control by informing key public health professionals about confirmed and unconfirmed outbreaks of international public health importance.

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The Outbreak Verification System

The outbreak verification system follows the general principles of surveillance: systematic collection, collation, analysis, and interpretation of data and dissemination to those who need the information for action (Figure 1). Data derived from an extensive network of information sources are transformed by the outbreak verification team into timely, accurate information about important disease outbreaks.

When the outbreak verification team receives an unconfirmed outbreak report, the relevance to international public health is assessed, and, if appropriate, further information is sought. Once an outbreak is substantiated and considered of public health importance, information is rapidly disseminated to a network of international partners.

Sources of Information

Outbreak verification is based on a broad range of information sources, including national institutes of public health, WHO offices at regional and national level, the United Nations

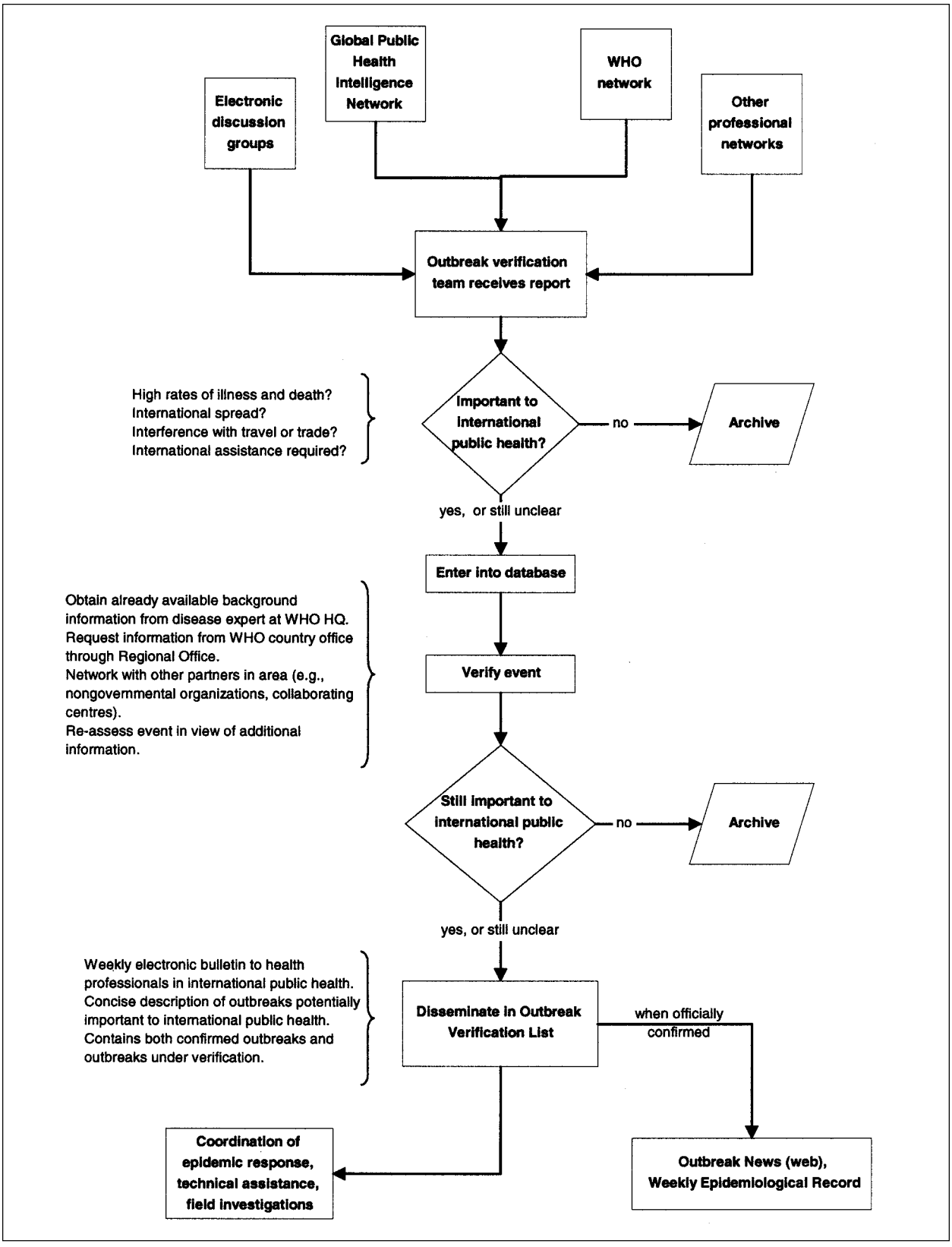

Figure 1. Outbreak verification at the World Health Organization.

system, nongovernmental organizations, WHO collaborating centers, newspapers, television, and radio (2). With the advent of modern communication technologies, many initial outbreak reports now originate in the electronic media and electronic discussion groups. Indeed, the abundance of outbreak-related documents on the World Wide Web presents a challenge: identifying reports of global public health importance.

The tasks of identifying and extracting outbreak reports from the electronic media is mainly performed by the Global Public Health Information Network (GPHIN), an electronic surveillance system developed by Health Canada. GPHIN continuously monitors some 600 sources, including all major news wires, newspapers, and biomedical journals. The system focuses its search on communicable diseases but will soon also cover noncommunicable diseases, food and water safety, environmental health risks, and the health impact of natural disasters (3). The quality of reports retrieved by GPHIN varies considerably, and information may be presented out of context (4).

Other information providers are the Internet and electronic-mail-based discussion groups. Their scope and readership may be worldwide (e.g., ProMed), regional (e.g., PACNET in the Pacific region), or specific (e.g., TravelMed). These groups can be accessed through free and unrestricted subscription. Because they receive outbreak information from many sources, including sources other than the electronic media, they are valuable information providers (5).

Selection of Outbreak Reports for Verification

The verification team first determines if an event is of potential international public health importance. International public health importance has been defined as serious health impact or unexpectedly high rates of illness and death; potential for spread beyond national borders; interference with international travel or trade; or likely need for international assistance in disease control.

Each event is assessed individually on the basis of these criteria. While some diseases will almost always be regarded important for international public health (e.g., Ebola hemorrhagic fever, cholera), others may not, depending on the circumstances.

Process of Verification

Once an event has been assessed as of potential international importance, the process of verification is initiated.

The outbreak verification team establishes the potential importance of the event, on the basis of available background information, endemicity levels, and details of previous outbreaks. This information is then shared by e-mail with designated contacts in WHO regional offices, who seek confirmation of details from health authorities in the countries concerned, usually through the WHO representative. The outbreak verification team may seek additional information from other organizations in the field, such as the International Red Cross, Médecins sans Frontières, and Medical Emergency Relief International.

Upon receipt of feedback, the outbreak verification team determines if the event meets the definition of an outbreak (observed number of cases exceeds expected number of cases in a given population for a given period) and the criteria for international public health importance. Reaching a final decision may require further consultation with the WHO regional office or the country representative or health authorities in-country.

Dissemination of Information

Timely dissemination of outbreak information to those who need to know is a key aspect of the outbreak verification process, and details of outbreaks with potential for international public health importance are disseminated through various channels. Information is shared directly with partners for immediate action (epidemic response) but also routinely with a wider audience through the Outbreak Verification List, the WHO Disease Outbreak News on the World Wide Web, and the Weekly Epidemiological Record (WER).

The Outbreak Verification List is distributed weekly by e-mail to approximately 800 subscribers. The distribution list includes WHO staff worldwide, other UN agencies, national health authorities, field epidemiology training programs, and nongovernmental organizations. Because the Outbreak Verification List is not an official WHO publication, its distribution is limited to subscribers.

The WHO Disease Outbreak News is on the WHO web page and provides the public with information about outbreaks of international

importance. Often events that initially appeared in the Outbreak Verification List are subsequently reported in Outbreak News. Because Outbreak News is in the public domain, only information about officially confirmed outbreaks is disseminated. Outbreak News (http://www.who.int/emc/outbreak_news/index.html) is one of the most frequently accessed sites on the WHO home page.

The third mechanism for communicating outbreak-related information is the WER. This report is published in French and English and issued in print and electronically (http://www.who.int/wer/index.html). It covers epidemiologic information on cases and outbreaks of diseases under the International Health Regulations (yellow fever, plague, cholera) and also on other communicable diseases of public health importance. Recently, an Outbreak News section mirroring the Outbreak News on the web page has been added to the WER.

Outbreak Response

Coordination of timely and effective epidemic response is intrinsically linked to dissemination of information about important disease outbreaks. During the verification process, WHO routinely offers technical assistance for the investigation and control of the event. Such assistance may range from advice (e.g., identifying appropriate laboratory facilities) to deployment of field teams. WHO coordinates the deployment of field teams, drawing from within WHO and among collaborating centers and other international partners.

Effectiveness of Outbreak Verification

From July 1, 1997, to July 1, 1999, the outbreak verification team identified 246 outbreak reports of potential importance for world health and disseminated them in the Outbreak Verification List. Of the 246 outbreaks, 43% occurred in the African region of WHO; 12% each in the regions of the Americas, eastern Mediterranean, and Europe; 11% in the Southeast Asian region; and 9% in the Western Pacific region. Countries subject to complex emergencies were involved in 121 (49%) outbreaks and industrialized countries in 6 (2%) events.

The most common diseases or syndromes disseminated in the Outbreak Verification List were cholera (n = 78), acute hemorrhagic fevers (n = 24), and acute diarrheal diseases (n = 22). In two (0.8%) cases, the Outbreak Verification List disseminated information about events that could not be substantiated later (Figure 2). Seventy-one percent of the initial reports were retrieved from informal or unofficial sources (e.g., the media, electronic discussion groups, nongovernmental organizations), and 29% were provided by official sources (e.g., WHO network, Ministries of Health). Unofficial sources were the

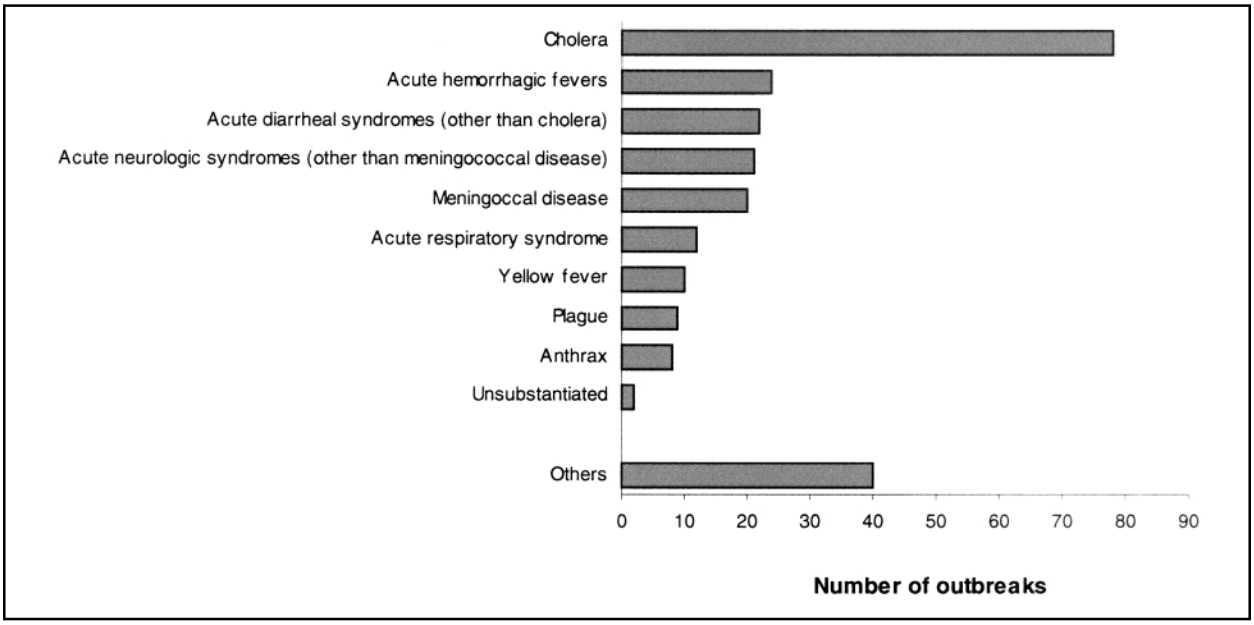

Figure 2. Reports of outbreaks disseminated in Outbreak Verification List, July 1, 1997, to July 1, 1999 (n = 246).

most frequent providers of initial information in all WHO regions and for all diseases, including those subject to the International Health Regulations (cholera, plague, yellow fever).

Information about the date of onset of an outbreak was available in 134 (55%) cases. The median time between reported onset of an outbreak and the outbreak verification team's receipt of the first report was 18 days (from 1 to 215 days). This interval was similar for official and unofficial sources but varied considerably for different diseases: 13 to 15 days (median) for acute hemorrhagic fevers, anthrax, and cholera; 20 to 35 days (median) for yellow fever and plague; and >50 days (median) for acute respiratory syndrome and meningococcal disease. Most reports were verified within a few days and important events usually within <48 hours. The median time between receipt of a first report and appearance of the event in the weekly Outbreak Verification List was 3 days (0 to 69 days).

In addition to the 246 disseminated outbreak reports, 69 events were verified from July 1, 1997, to July 1, 1999, but were not reported in the Outbreak Verification List. Follow up was undertaken because initial reports suggested international public health importance. Of the 69 events, 58 (84%) were excluded from the Outbreak Verification List because they did not meet the criteria for outbreaks or for international public health importance. Four (6%) reports were unsubstantiated, including two reports of smallpox, one of yellow fever, and one of viral hemorrhagic fever. In seven (10%) events, follow up could not be completed, and the verification process remained inconclusive. The 69 excluded events did not differ from the 246 disseminated outbreaks with regard to their distribution by WHO region, initial source of information, or type of disease or syndrome. A reassessment of the 62 verified events did not identify any outbreaks that should have been classified retrospectively as of international importance.

Whenever the outbreak verification team invokes a verification process, assistance to the country in which the event takes place is offered directly by WHO headquarters or through the WHO regional and country offices. Past examples of such assistance include supply of essential materials to outbreak sites, transport of laboratory specimens from the field to appropriate diagnostic facilities, organization of vaccination programs, training of field staff as part of outbreak control measures, or deployment of field teams for disease control. Recent examples of direct assistance by WHO and its partners in field investigations include support for Rift Valley fever in Kenya and Somalia (6), monkeypox in the Democratic Republic of the Congo (7), avian influenza (H5N1) in Hong Kong, Special Administrative Region of China, Ebola hemorrhagic fever in Gabon (8), relapsing fever and acute respiratory infections in southern Sudan, influenza in Afghanistan, and Marburg virus infection in the Democratic Republic of the Congo.

Conclusions

Outbreak verification is a new approach to global disease surveillance. Its aim is to improve epidemic disease control by providing accurate and timely information about important disease outbreaks. While the outbreak verification concept has remained unchanged since its start in early 1997, its daily application continues to evolve as more data are gathered and more experience is gained.

Currently, most outbreak reports are received from the media, and field personnel are mainly contacted for assistance with verifying reported events. This approach is subject to information bias, which results from the uneven dispersal and use of modern technology throughout the world. Also, different languages are not equally represented in the news media or addressed by electronic search engines. While these shortcomings are partly offset by the information received directly from the WHO network, a more active dialogue should be established with field personnel. Receiving primary information directly from the field will lead to earlier detection of important events and events that escape identification. Although thought to be small, the number of important outbreaks recognized only locally is unknown.

The number of outbreak reports selected for verification is small compared with the number of reports received by the outbreak verification team. While the criteria for selecting outbreak reports for verification have been established, their application requires an individual assessment of each single event. Some see in this selection process a lack of transparency and argue that the reader is the best judge of what to believe. This may be the case for those who have time, good information networks, and access to advanced communication technology. However,

most international public health workers have none of these and are poorly informed about such events. WHO therefore considers that sharing filtered information is valuable. In a recent survey among the Outbreak Verification List recipients, 72% percent of the respondents stated that the list was very useful or indispensable to their work, and 70% cited the list as their first source of information about a particular event.

Applying the selection criteria is also difficult if available information is insufficient to determine if an event should be classified as an outbreak (number of cases in excess of expected numbers). This problem arises particularly when dealing with endemic diseases in the absence of established epidemic thresholds. The Outbreak Verification List addresses the issue by mentioning events with clear implications for international public health that are not regarded as outbreaks in a separate Notes section. The Outbreak Verification List shares relevant and often sensitive information with public health professionals while the verification process is still under way. Although this has led on rare occasions (<1%) to the dissemination of information about unsubstantiated events, the Outbreak Verification List usually provides timely and accurate information about important disease outbreaks.

Because of its confidential nature, the Outbreak Verification List is not in the public domain, and some argue that WHO is not timely in addressing the information needs of the public about epidemics (4). However, WHO communicates information as soon as it is verified. In some instances, this takes time, but the delay prevents release of inaccurate information.

Industrialized countries feature infrequently in the Outbreak Verification List because it is assumed that they can deal with outbreak situations. This is, of course, not always true and leads to an overrepresentation of developing countries in the Outbreak Verification List. However, most outbreaks in developing countries are contributed by nations with complex emergencies. While the reporting may accurately reflect the breakdown of the public health and social infrastructures, it may also contain an element of overreporting due to heightened media attention associated with complex emergencies.

As a new concept, early outbreak verification efforts focused mainly on the development of process indicators (information gathering, verification, information dissemination). More outcome-oriented indicators need to be addressed to assess the outbreak verification impact at country level and within WHO. While providing public health professionals with timely and accurate information about important disease outbreaks improves epidemic preparedness and response, this has not been quantified. Possible outcome indicators could include the time interval between first report and the commencement of investigation and control efforts or the proportion of outbreaks with laboratory confirmation. Additional tasks to be addressed in the future are more detailed analyses, including electronic and print mapping to provide both baseline (endemic) and outbreak information, and standardized reports to regions and countries.

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Malaria on the Move: Human Population Movement and Malaria Transmission

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Reports of malaria are increasing in many countries and in areas thought free of the disease. One of the factors contributing to the reemergence of malaria is human migration. People move for a number of reasons, including environmental deterioration, economic necessity, conflicts, and natural disasters. These factors are most likely to affect the poor, many of whom live in or near malarious areas. Identifying and understanding the influence of these population movements can improve prevention measures and malaria control programs.

Malaria, the world's most prevalent vector-borne disease, is endemic in 92 countries, with pockets of transmission in an additional eight countries (1). Approximately 41% of the world's population is at risk, and each year 300 million to 500 million clinical cases of malaria, >90% of them in Africa, are reported. Worldwide, approximately 2 million deaths per year can be attributed to malaria, half of these in children under 5 years of age.

Historically, population movement has contributed to the spread of disease (2). Failure to consider this factor contributed to failure of malaria eradication campaigns in the 1950s and 1960s (3). The movement of infected people from areas where malaria was still endemic to areas where the disease had been eradicated led to resurgence of the disease. However, population movement can precipitate or increase malaria transmission in other ways as well. As people move, they can increase their risk for acquiring the disease through the ways in which they change the environment and through the technology they introduce, for example, through deforestation and irrigation systems (4). Such activities can create more favorable habitats for *Anopheles* mosquitoes; at the same time, workers may have increased exposure to the vector. Furthermore, people can inadvertently transport infectious mosquitoes to malaria-free areas, reintroducing disease. Population movement is also increasingly implicated in the spread of drug resistance in malaria (5).

The unprecedented increase in mobility in the last few decades has led to greater concern about the relationship between mobility and malaria. There are a number of reasons for increased mobility. First, sophisticated forms of transport now permit the swift movement of people over huge distances. Air travel has increased by almost 7% a year in the last 20 years and is predicted to increase by >5% a year during the next 20 years (6). Second, in the developing world a rapidly increasing population is putting pressure on scarce resources, leading to major population redistribution. This particularly involves the movement from rural to urban areas. Third, natural disasters such as droughts and floods have created approximately 25 million environmental refugees (7). Finally, conflict, often a result of population pressures and environmental degradation, displaces vast numbers of people. We examine the impact of population movement on malaria transmission.

A Typology of Population Movement

The decision-making process leading to population movement can best be understood in the light of "push and pull" forces (8). When their needs can no longer be met in a particular environment, people move elsewhere. The "push factor" could be environmental degradation, population pressure on land, droughts, famines, conflict, or loss or lack of employment. When people are satisfied with their situation but

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believe that a move elsewhere will provide new and attractive opportunities, a "pull factor" is involved. This pull factor could be better political, economic, or social opportunities or improved living conditions. Push and pull factors can operate simultaneously; for example, people can be pushed by environmental deterioration and scarce resources and pulled by the economic opportunities offered by development projects.

Population movements can be differentiated by their temporal and spatial dimensions. Temporal dimensions include circulation and migration. Circulation encompasses a variety of movements, usually short-term and cyclical and involving no longstanding change of residence. Migration involves a permanent change of residence (9). Circulation can be subdivided into daily, periodic, seasonal, and long term (Table) (9). Daily circulation involves leaving a place of residence for up to 24 hours. Periodic circulation may vary from 1 night to 1 year, although it is usually shorter than seasonal circulation. Seasonal circulation is a type of periodic circulation in which the period is defined by marked seasonality in the physical or economic environment. This type of circulation involves persons or groups who are absent from their permanent homes during a season or seasons of the year. Long-term circulation, defined as absence from home for longer than a year, affects groups such as wage laborers and traders, who maintain close social and economic ties with their home area and intend to return.

In terms of spatial dimensions, the movements to and from malarious areas are of epidemiologic importance. People who move can be categorized as either active transmitters or passive acquirers (2). Active transmitters harbor the parasite and transmit the disease when they move to areas of low or sporadic transmission. Passive acquirers are exposed to the disease through movement from one environment to another; they may have low-level immunity or may be nonimmune, which increases their risk for disease.

Based on the above definitions, a typology can be devised that identifies categories of population movement. Different activities can be associated with these categories, and these activities, in turn, can be associated with differing risks for malaria transmission. All types of population movement can be accommodated in this typology, and people may exhibit more than one type of mobility.

Population Movement and Malaria

In developing countries, activities involving population movement include urbanization, colonization, labor related to agriculture and mining, and conflict. In industrialized countries, the impact of population movements on malaria risk is mainly related to intercontinental travel.

In some regions, malaria risk may increase as a result of a combination of different forms of mobility, as well as other factors unrelated to population movements. For example, in the African highlands, many of the issues described below act concurrently (10). The categorization of the various examples below simplifies a complex issue but is useful for indicating major processes related to mobility and malaria risk.

Urbanization

The world's urban population is growing at four times the rate of the rural population (11). Urban pull is prevalent throughout the developing world, with rural-to-urban migration taking place faster than ever before (12). Sub-Saharan Africa is the most rapidly urbanizing region in the world (13), and the urban population in India has doubled in the last 2 decades (14). When accompanied by adequate housing and sanitation, urbanization can lead to a decrease in malaria through reductions in human-vector contact and vector breeding sites. However, in developing countries, rapid, unregulated urbanization often leads to an increase in or resumption of malaria transmission because of poor housing and sanitation, lack of proper drainage of surface

Table. A typology of population movement (adapted from [2])

Circulation				
Daily	Periodic	Seasonal	Long-term	Migration
Commuting	Trading	Fishing	Laboring	Urbanization
Trading	Pilgrimage	Pastoralism	Colonization	Refugees
Cultivation	Mining	Laboring		Colonization
Wood-cutting	Tourism			

water, and use of unprotected water reservoirs that increase human-vector contact and vector breeding.

Although water pollution in urban areas usually leads to decreases in vector populations, some vectors, such as *An. arabiensis* in the forest belt of West Africa, may adapt to breeding in polluted waters (15). In Asia, *An. stephensi* is proving adaptable to urban conditions, and in India it is a well-established vector of urban malaria. In urban areas of India, water is not supplied regularly and is stored in houses, providing extensive breeding places for *An. stephensi* in overhead tanks and cisterns. In periurban areas, where 25% to 40% of the urban population live in poor housing without proper water supply and drainage, another vector, *An. culcifacies,* also transmits malaria (14).

In India, the fact that the National Malaria Eradication Program concentrated only on rural areas, ignoring the problem of urban malaria, was one of the factors leading to a resurgence of malaria in the 1970s (14). Several types of population movement contributed to malaria transmission in India. First, circulation from stable rural malaria areas to unstable urban areas had firmly established malaria transmission in urban areas. Then, after the National Malaria Eradication Program, rural areas became free of endemic malaria but were receptive, so circulation from urban areas back to rural areas reintroduced malaria transmission. Changes in vector behavior (exophilic and exophagic behavior limiting the effectiveness of spraying), vector resistance to insecticides, and increasing drug resistance, especially in *Plasmodium falciparum* (14), also played a role. Population movement also contributed to drug resistance, with people of different immune status moving from endemic- to nonendemic-disease areas, accelerating transmission of resistant strains.

Colonization of New Territory

Through the interaction of a number of factors, the colonization of unpopulated or sparsely populated areas may be accompanied by an increase in malaria. Settlers, who have low-level immunity or are nonimmune, may migrate into a disease-endemic area, spreading the disease. Initially, housing tends to be basic, leading to close human-vector contact. Moreover, housing is often near rivers or lakes to facilitate water collection, increasing the exposure of humans to mosquitoes. Activities to develop an area, such as deforestation and irrigation, can increase the number of vector breeding sites, contributing to an increase in malaria. Colonization may be accompanied by major building projects, such as dams, canals, highways, or mining activities—referred to as the tropical aggregation of labor—which can further enhance malaria transmission.

Reemergence of malaria through mobility related to colonization occurred in Brazil. Malaria had been practically eradicated from most areas of the Amazon region by the national malaria campaign in the 1950s and 1960s (16). Since the 1960s, however, the incidence of malaria has increased dramatically because of massive population movements to colonize new territory. New highways were built in the 1960s, linking the Amazon region to the rest of the country and attracting laborers to work on road construction. In the 1970s, many more people were attracted to the region by agricultural settlements and hydroelectric projects. Finally, in the 1980s, the discovery of gold led to a greater influx of people, along with the establishment of hundreds of mines throughout the region. The population of Rondônia State, which received the greatest number of migrants, increased from 113,000 in 1970 to 1,200,000 in 1990. Malaria cases in Rondônia increased from 20,000 to 174,000 in the same period. In Brazil as a whole, approximately 50,000 cases of malaria were reported in 1970; by 1990, reports had increased to 577,520, representing 10% of the world's reported cases outside Africa (17). Of this total, >98% were recorded in the Amazon region.

The types of population movement involved in the colonization of the Amazons are migration and long-term circulation from malaria-free areas of Brazil to the malaria-endemic Amazon region. The people involved are nonimmune passive acquirers who on becoming infected can become active transmitters. If these active transmitters return to their initial place of residence in a malaria-free but highly receptive area, they can reintroduce the parasite and initiate an outbreak of malaria. For example, in 1985, 26 new active foci of malaria were recorded in Brazilian states outside the Amazon region (16). Settlers in the Amazon region are highly mobile, moving with daily, periodic, and seasonal circulation from settlements in unstable disease-endemic regions to hyperendemic-disease regions of

the rainforests. This mobility keeps settlements unstable and at high risk for epidemics through the constant flow of parasitemic laborers (17,18).

Agricultural Labor

Swaziland provides an example of how agricultural labor has changed the spread of malaria. In the 1950s, control measures (DDT spraying) were successfully implemented in the lowveld so that agricultural development could take place, and by 1959, malaria had been all but eradicated from Swaziland (19). However, agricultural developments in the 1950s involving an irrigation project for the cultivation of sugar cane created conditions favorable for malaria. Vector density increased, along with a high frequency of feeding on humans, as no domestic or wild animals were around the project area to serve as alternative hosts. This resurgence of malaria was catalyzed by the reintroduction of parasite carriers in the form of migrant workers from disease-endemic areas of Mozambique, who were involved in migration or long-term circulation to work on the sugar estates in the 1960s and early 1970s (19).

In Colombia, the annual parasite index (defined as the ratio between the number of cases reported and the population at risk) has increased threefold since the 1960s (20). This increase seems to be related to the migration of nonimmune people to areas such as the Naya basin, where malaria is endemic, and to the circulation of groups within the Naya basin. The circulation is predominantly seasonal, related to agriculture. People descend from hills and terraces, where malaria risk is minimal, to the malarious delta zone to cultivate and harvest their crops. In doing so, they are exposed to the anopheline population of the area and are at high risk for malaria. A large number of people are involved in this circulation (approximately 60% of the area's population is mobile for approximately 4 months of the year), and this population density, combined with the large vector population, maintains transmission at high levels (20).

Refugees

The number of officially recognized refugees has steadily increased, from approximately 5 million in 1980 to >20 million in late 1994 (21). In addition, an estimated 25 million people have fled their homes but remain internally displaced in their countries of origin (22). The displacement of large numbers of people and their circulation can favor malaria transmission. If refugees are nonimmune, they could travel through or to malarious regions and acquire the infection, and if they are infectious, they could disseminate the disease to other areas.

Malaria is one of the most commonly reported causes of death among refugees and has caused high rates of both illness and death among refugees and displaced persons in disease-endemic countries, such as Thailand, Sudan, Somalia, Burundi, Rwanda, and the Democratic Republic of Congo (23). In recent outbreak among Burundian refugees at a refugee camp in northwestern Tanzania, deaths from malaria and anemia in children under 5 years of age have increased 10-fold since the outbreak, reflecting the lack of immunity in this age group (24). In the Sahel region of Africa, where civil wars and conflicts have occurred for many decades and large numbers of displaced people live in resettlement or refugee camps often located in lowland disease-endemic areas, epidemics are common (25).

As a result of 15 years of continuous war, which displaced hundreds of thousands of people, Luanda, the capital of Angola, underwent an unprecedented population increase in the 1980s. This population movement resulted in a shift in malaria endemicity in Luanda from hypoendemic to mesoendemic level within 5 years (26). As a cause of child deaths, malaria moved from sixth to first place. Increasing parasite resistance to chloroquine also became a major problem. This situation arose because of the enormous influx of displaced people of low socioeconomic status into an environment with stagnant water reservoirs. The population movements that increased malaria transmission in Luanda were long-term circulation and migration from stable rural areas to an unstable urban area.

Besides movements of large numbers of people, wars and civil unrest tend to favor malaria transmission. The disruptive effect of war on agriculture and water management can increase vector breeding sites; the destruction of housing can increase human-vector contact; the destruction of cattle can prompt zoophilic vectors to become anthropophilic if their usual food supply is disrupted (27); and control measures can be seriously diminished if health-care facilities are reduced or unavailable.

Intercontinental Travel

The intercontinental transfer of malaria can occur through the introduction of an infective vector into a nonendemic-disease area, as in so-called airport malaria, or through the movement of a parasitemic person to a nonendemic-disease area, as in imported malaria. Although the incidence of these cases is low, they account for most malaria transmission in industrialized countries.

Airport Malaria

Airport malaria is defined as malaria acquired through the bite of an infected tropical anopheline mosquito by persons whose geographic history excludes exposure to this vector in its natural habitat (28). The vector is usually introduced into a nonendemic-disease country on an international flight. For example, random searches of airplanes at Gatwick Airport (London) found that 12 of 67 airplanes from tropical countries contained mosquitoes (29). After a mosquito leaves the aircraft, it may survive long enough to take a blood meal and transmit the disease, usually in the vicinity of an airport. In temperate climates, temperature and humidity can be favorable in the summer for the mosquito not only to survive but also to move around and perhaps lay eggs. With the enormous and continuing increase in air traffic, cases of airport malaria may increase. Several such cases are described below.

During a hot summer in 1994, six cases of airport malaria were identified in and around Roissy-Charles-de-Gaulle Airport (30). Four of the patients were airport workers, and the others lived in Villeparisis, approximately 7.5 km away. Anopheline mosquitoes were thought to have traveled in the cars of airport workers who lived next door to two of the patients. In 1989, two cases of *P. falciparum* malaria were identified in Italy in two persons who lived in Geneva (31). Another five cases of airport malaria were reported in Geneva in the summer of 1989 (32). High minimum temperatures were thought to have allowed the survival of infected anophelines introduced by aircraft. In Britain, two cases of *P. falciparum* malaria were observed in persons living 10 km and 15 km from Gatwick Airport (33). Hot, humid weather in Britain may have facilitated the survival of an imported mosquito.

Imported Malaria

Throughout the world, many countries are reporting an increasing number of cases of imported malaria (Figure) because of the great increase in long-distance travel in recent decades. For example, cases imported from Africa to the United Kingdom rose from 803 in 1987 to 1,165 in 1993, and the ratio of all imported cases of *falciparum* to *vivax* malaria rose from 0.76 in 1984 to 1.52 in 1993 (36).

Recently, a woman in Italy was infected with malaria through a bite from a local species, *An. labranchiae* (37). This species was a common malaria vector in Italy until the country was declared malaria free in 1970. Local breeding sites, including isolated pools in dried-up irrigation channels, were identified, and the mosquito responsible is thought to have acquired the parasite after biting a parasitemic girl who had acquired malaria in India. Airport malaria was ruled out because of the distance from the nearest airport. This may be the first case of malaria introduced to Europe in 20 years and demonstrates the hazards of population movement (the parasite had been introduced from India) combined with human activities (providing vector breeding sites).

In the United States, recent outbreaks of presumed local mosquito-borne transmission have been reported in California, with migration from a disease-endemic area (38). The people involved were migrant workers from malaria-endemic areas. An outbreak in 1986 involved 28 cases (26 in Mexican migrant workers) of *P. vivax* during a 3-month period (39). The epidemic curve indicated secondary spread, which confirmed local mosquito-borne transmission.

In the early 1990s, outbreaks were identified in neighborhoods of Houston with many immigrants from countries with malaria

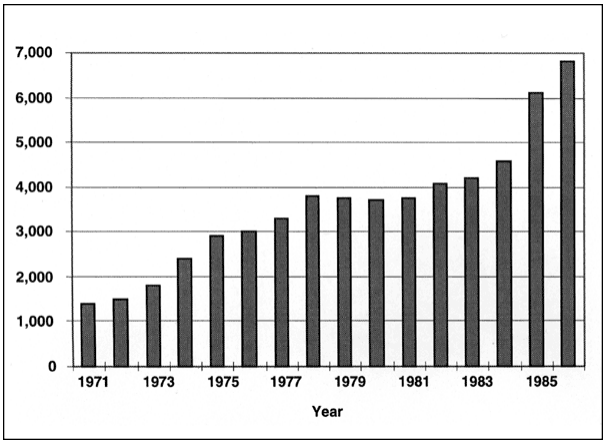

Figure. Imported cases of malaria in Europe (34,35).

transmission. These outbreaks occurred when the weather was hot and humid and thus conducive to the completion of the sporogonic cycle and the survival of female anophelines (38). Given that climate change could lead to more favorable conditions for vector survival in Europe and the United States (40), the increase in incidence in both airport and imported malaria is cause for concern. If temperatures increase, uninfected introduced or local mosquitoes could survive long enough after taking a blood meal from a parasitemic person for the completion of the sporogonic cycle of the parasite, thus enabling transmission.

Conclusions

Population movements that either place people at risk for malaria or cause them to pose a risk to others cannot be stopped. However, prevention measures can address the causes of these movements which, in developing countries, are often prompted by need rather than choice; if living conditions and opportunities improved in their place of residence, people would not be forced to move. In cases where movements are unavoidable, people should be made aware of the risks and have adequate access to treatment. Epidemics are more likely to take place in areas where health care may be poor or lacking. Areas at risk for epidemics through the influx of infected people should be identified to avoid or control epidemics. Particular attention should be paid to urban areas, given the increasing number of cases of urban malaria and the ongoing trend toward uncontrolled urbanization.

The risk for increased malaria transmission through economic development of an area should be analyzed thoroughly before development begins. For example, sound environmental management can restrict vector breeding and reduce human-vector contact. Such management could include proper maintenance of irrigation systems and adequate water supply and sanitation facilities for workers. Personal vector-control measures, such as bed nets, and antimalarial drugs should be used.

Regarding the risks that airport and imported malaria pose for industrialized countries, measures should be taken to ensure that cases of malaria are promptly diagnosed and treated. Strengthened surveillance would help prevent the reintroduction of malaria transmission by local mosquitoes, which could acquire the infection by biting persons with airport or imported malaria. Given the economic resources and quality of health care in industrialized countries, the likelihood is low that isolated outbreaks of malaria will lead to reestablishment of transmission.

The relationship between malaria transmission and population movement is complex, but future attempts to eradicate or control malaria will be futile if they are not based on understanding of this link. Because the current magnitude and diversity of population movements are unprecedented, this issue is worthy of attention.

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The *bdr* Gene Families of the Lyme Disease and Relapsing Fever Spirochetes: Potential Influence on Biology, Pathogenesis, and Evolution

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Species of the genus *Borrelia* cause human and animal infections, including Lyme disease, relapsing fever, and epizootic bovine abortion. The borrelial genome is unique among bacterial genomes in that it is composed of a linear chromosome and a series of linear and circular plasmids. The plasmids exhibit significant genetic redundancy and carry 175 paralogous gene families, most of unknown function. Homologous alleles on different plasmids could influence the organization and evolution of the *Borrelia* genome by serving as foci for interplasmid homologous recombination. The plasmid-carried *Borrelia direct repeat (bdr*) gene family encodes polymorphic, acidic proteins with putative phosphorylation sites and transmembrane domains. These proteins may play regulatory roles in *Borrelia*. We describe recent progress in the characterization of the *Borrelia bdr* genes and discuss the possible influence of this gene family on the biology, pathogenesis, and evolution of the *Borrelia* genome.

Species of the genus *Borrelia* cause human and animal infections (1). In North America, Lyme disease and endemic relapsing fever pose the greatest threat to human health and have received the most attention of the borrelial diseases. Approximately 14,000 cases of Lyme disease are reported in the United States each year; however, the actual number of cases may be 10-fold higher (2). Lyme disease was not recognized as a distinct clinical entity in North America until the 1970s (3). The causative agent, a previously uncharacterized spirochete transmitted through the bite of infected ticks of the *Ixodes ricinus* complex (*I. scapularis* in the Northeast and Midwest and *I. pacificus* on the West Coast) (4,5), was classified in the genus *Borrelia* and named *B. burgdorferi.* With the emergence of Lyme disease and the identification of its etiologic agent, *Borrelia* research focused on the development of reliable Lyme disease diagnostic assays and vaccines, and the phenotypic and genotypic diversity of *Borrelia* was thoroughly analyzed. Through modern molecular taxonomic techniques, several newly described species of *Borrelia* have emerged as possible causative agents of Lyme disease or at least as agents genetically related to *B. burgdorferi* (6-15). The *B. burgdorferi* sensu lato complex is composed of the following species: *B. turdae, B. tanukii, B. bissettii, B. valaisiana, B. lusitaniae, B. bissettii, B. andersonii, B. japonica, B. garinii*, and *B. afzelii.* Of these, *B. burgdorferi*, *B. garinii,* and *B. afzelii* are the dominant species associated with infection in humans.

Relapsing fever has been studied not only for its impact on human health but also as a model system for antigenic variation. There are two general forms of relapsing fever, epidemic (louse borne—*Pediculus humanus*) and endemic (tick borne—*Ornithodoros* spp.) (1). Epidemic relapsing fever tends to be associated with poor living conditions and social disruption (famine and war)

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and is rare in the United States. Endemic relapsing fever is more prevalent, predominantly in the western regions. Three closely related *Borrelia* species, *B. hermsii, B. turicatae,* and *B. parkeri*, are associated with this disease. Hallmark features of relapsing fever include cyclic fever and spirochetemia. The molecular basis for these features can be attributed to the differential production of dominant variable surface antigens of the Vmp protein families (16). The 40 or so plasmid-carried *vmp* related genes in the *B. hermsii* genome are expressed only one at a time. A single expression locus exists, and genes not at this site lack a promoter element and are therefore not transcribed (17). The expressed Vmp becomes a primary target of a vigorous humoral immune response that kills most of the spirochetal population. However, at a frequency of approximately of 1 x 10^{-3} to 1 x 10^{-4} per generation, the identity of the expressed Vmp changes (18) through gene conversion (19). The net effect of this nonreciprocal event is to replace the gene located in the expression locus with one that was previously silent. Production of a new antigenically distinct Vmp allows evasion of the humoral immune response. This ongoing change in Vmp synthesis allows the relapsing fever spirochete population to reestablish itself in the host, thus leading to spirochetemia and the relapse of fever. Antigenic variation systems have also been identified in the Lyme disease spirochetes; however, they appear to exert a more subtle effect (20).

While clinical relapsing fever and Lyme disease differ from each other in many ways, their causative agents share many similarities at both the biologic and genetic levels. At the biologic level, they are host associated and undergo similar environmental transitions in the course of cycling between mammals and arthropods. In view of the distinctly different characteristics of these environments, the spirochetes must be able to adapt rapidly. Evidence suggests that the relapsing fever and Lyme disease spirochetes use related proteins to adapt to or carry out similar functions in changing environments. For example, homologs of the plasmid-carried *ospC* gene of the Lyme disease spirochetes are carried by several other *Borrelia* species, including the relapsing fever spirochetes (21). Both *ospC* and its relapsing fever spirochete homolog (*vmp33*) are selectively expressed during the early stages of infection, which suggests that they play a common functional role (22,23). The *B. burgdorferi* Rep or Bdr protein family is also distributed genuswide. Members of this polymorphic protein family possess highly conserved putative functional motifs and structural properties, which suggests that they may also carry out an important genuswide role (24,25).

The *Borrelia* Genome

At the molecular level, a unique feature of *Borrelia* is the unusual organization and structure of their genome. Unlike most bacteria, which carry their genetic material in the form of a single, circular DNA molecule, *Borrelia* have a segmented genome (26-28). Most genetic elements carried by these bacteria are linear with covalently closed termini or telomeres (27). The telomeres are characterized by short hairpin loops of DNA (29). If heat denatured, these linear molecules relax to form a single-stranded circular molecule. If reannealed, they base-pair upon themselves to form a double-stranded linear molecule that by physical necessity possesses a short single-stranded hairpin loop at each telomere. Genetic elements of this structure are rare in bacteria and are reminiscent of certain viral genomes. In *B. burgdorferi* (isolate B31), the largest of the linear genomic elements is the 911-kb chromosome (30). The chromosome carries 853 putative ORFs, most of which are thought to encode housekeeping functions. The remaining 12 linear and 8 circular genetic elements are plasmids. The plasmids might best be thought of as mini-chromosomes, since as a group they are indispensable in situ and may carry genes encoding proteins involved in housekeeping functions (31). In addition, they may further deviate from the true definition of a plasmid in that their replication may not be independent and may instead be tightly coordinated with the replication of the chromosome (32,33).

Nearly 50% of the plasmid-carried ORFs lack homology with known sequences, which suggests that their encoded proteins may define the unique biologic and pathogenetic aspects of *Borrelia* (30). Several of the proteins derived from these plasmid-carried genes of unknown function are antigenic or selectively expressed during infection, which indicates that they function in the mammalian environment (20,34-37). A striking feature of the plasmid-carried ORFs is that they are organized into 175 paralogous gene families of two or more members (30). Hence, the

DNA content of the plasmids is highly redundant. Since the maintenance of DNA is energetically expensive, it is likely that this redundant DNA is of biologic importance to *Borrelia*. The paralogous gene families of *Borrelia* have been the focus of intensive research as they are thought to play important roles in pathogenesis and to influence genome organization and evolution (20,30,35,38-40).

Identification of *Borrelia Direct Repeat (bdr)* Related Genes

The *bdr* gene family is a large, polymorphic, plasmid-carried, paralogous gene family of unknown function that was originally identified in *B. burgdorferi* (41,42). Members of this gene family have been characterized in several *Borrelia* species and isolates (Table 1) and have

Table 1. *Borrelia* species carrying *bdr*-related genes or expressing proteins immunoreactive with anti-Bdr antisera

Species	Associated disease	Arthropod vector	*Bdr*-related information
B. burgdorferi	Lyme disease, endemic worldwide	*I. scapularis, I. ricinus, I. pacificus*	All *bdr*-gene family members (18 total) total) have been identified in isolate B31G (30), *bdr*-alleles that are organized into 3 subfamilies (D,E,F) (25), the genes are carried on variably sized linear and circular plasmids (30)
B. afzelii	Lyme disease, Eurasia	*I. ricinus, I. persulcatus*	Single *bdr* gene has been sequenced (43); several *Bdr*-related proteins have been detected by immunoblot analysis (this report)
B. garinii	Lyme disease, Eurasia	*I. ricinus*, *I. persulcatus*	Bdr proteins detected by immunoblot analyses only (this report)
B. tanukii	Not associated with human disease	*I. tanuki*	Bdr proteins detected by immunoblot analyses only (this report)
B. turdae	Not associated with human disease	*I. turdus*	Bdr proteins detected by immunoblot analyses only (this report)
B. bissettii	Not associated with human disease	*I. pacificus, I. scapularis, I. spinipalpis*	Bdr proteins detected by immunoblot analyses only (this report)
B. andersonii	Not associated with human disease	*I. dentatus*	*bdr* genes detected by hybridization and Bdr-related proteins by immunoblot analyses (this report, data not shown)
B. valaisiana	Not associated with human disease	*I. columnae, I. ricinus*	Bdr proteins detected by immunoblot analyses only (this report)
B. japonica	Not associated with human disease	*I. ovatus*	Bdr proteins detected by immunoblot analyses only (this report)
B. hermsii	Endemic relapsing fever, United States	*Onithodoros hermsii*	Numerous *bdr* genes have been described and are carried on both linear and circular plasmids (25,46); several Bdr proteins have been detected by immunoblot analyses (this report) (44)
B. parkeri	Endemic relapsing fever, United States	*Onithodoros parkeri*	Two *bdr*-related genes have been sequenced (25), and others have been detected by hybridization with genes residing on both linear and circular plasmids (46); several Bdr proteins detected by immunoblot analyses (this report)
B. turicatae	Endemic relapsing fever, United States	*O. turicata*	At least nine *bdr*-related genes have been described and are present on linear plasmids ranging from 25 to 220 kb in size (24,46); several Bdr proteins have been detected by immunoblotting (this report) (24,44)
B. miyamotoi	Relapsing fever?	*I. persulcatus*	Bdr proteins detected by immunoblotting only (this report)
B. coriaceae	Epizootic bovine abortion, United States	*O. coriaceus*	*bdr*-related genes and proteins detected by hybridization (46) or immunoblotting (this report)
B. anserina	Avian spirochetosis, United States	*Argas persicus*	*bdr*-related sequences have been detected by hybridization (46); Bdr-related proteins were not detected in in vitro cultivated bacteria (this report)

been assigned various gene names (25,41-44) (Table 2). We have adopted the *bdr* designation in the context of a nomenclature system (25), summarized below. Genes belonging to the *bdr* gene family were first identified through the analysis of repeated DNA sequences in *B. burgdorferi* sensu lato complex isolates (41,42). Seven nonidentical but closely related copies of a plasmid-carried repeated element were identified in *B. burgdorferi* 297 (42). Three additional copies of this repeated sequence were further identified in *B. burgdorferi* 297 (45). These loci carry several ORFs that were designated as *rep+, rep-*, LPA, LPB (the LP genes have recently been redesignated as *mlp* for multicopy lipoprotein [45]), *rev,* and the *orfABCD* operon (note: ORFs A and B have been redesignated as *blyA* and *blyB*). Some of these genes, particularly *rep* and *mlp,* exhibit allelic variation and encode polymorphic proteins, the functions of which are under investigation. Focusing specifically on the *rep* or *bdr* genes, the *rep* designation was originally

Table 2. *Borrelia* Bdr homology groups and gene nomenclature

Bdr subfamily designation	Species/revised gene designation	Accession or TIGR number	Previous gene names	Ref.
Subfamily A				
B. turicatae OZ-1	$bdrA_1$	AF062395	*repA*	(46)
B. turicatae OZ-1	$bdrA_2, A_3, A_4$	AF128445-AF128447	none	(25)
B. hermsii YOR-1	$bdrA_1, A_2, A_3$	AF143473-AF143475	none	(25)
B. hermsii HS1	$bdrA_1, A_2$	AF143457-AF143458	none	(25)
B. hermsii MAN	$bdrA_1, A_2$	AF143465, AF143467	none	(25)
B. parkeri	$bdrA_1$	AF143455	none	(25)
Subfamily B				
B. turicatae OZ-1	$bdrB_1, B_2, B_3, B_4, B_5$	AF128448-AF128452	none	(24)
B. hermsii MAN	$bdrB_1, B_2, B_3$	AF143463, AF143464, AF143466	none	(25)
Subfamily C				
B. parkeri	$bdrC_1$	AF143455	none	(25)
B. hermsii MAN	$bdrC_1, C_2, C_3, C_4, C_5$	AF143468-AF143472	none	(25)
B. hermsii HS1	$bdrC_1, C_2, C_3, C_4$	AF143459-AF143462	none	(25)
B. hermsii YOR-1	$bdrC_1$	AF143476	none	(25)
B. parkeri	$bdrC_2$	AF143456	none	(25)
Subfamily D				
B. burgdorferi B31G	$bdrD_1, D_2, D_3,$	BBL35, BBM34, BBO34	*bdrO, bdrK, bdrM*	(30)
B. burgdorferi B31G	$bdrD_4, D_5, D_6$	BBP34, BBQ42, BBS37	*bdrA, bdrV, bdrE*	(30)
B. burgdorferi B31	$bdrD_7$	X87201	ORF-E (lp50 allele)	(41)
B. burgdorferi B31	$bdrD_8$	X87127	ORF-E (cp30.5 allele)	(41)
B. burgdorferi B31	$bdrD_9$	U42599	ORF-E (cp18 allele)	(41)
B. burgdorferi B31	$bdrD_{10}$	BBN34	*bdrQ*	(30)
B. burgdorferi B31	$bdrD_{11}$	BBR35	*bdrG*	(30)
Subfamily E				
B. burgdorferi B31G	$bdrE_1, E_2, E_3$	BBL27, BBN27, BBO27	*bdrP,* none, *bdrN*	(30)
B. burgdorferi B31G	$bdrE_4, E_5, E_6$	BBR27, BBS29, BBQ34	*bdrH, bdrF, bdrW*	(30)
B. burgdorferi 297	$bdrE_1, E_2$	U45421, U45422	*rep+2.9-1, rep+2.9-2*	(42)
B. burgdorferi 297	$bdrE_3, E_4$	U45423, U45424	*rep+2.9-3, rep+2.9-4*	(42)
B. burgdorferi 297	$bdrE_5$	U45425	*rep+2.9-5*	(42)
B. burgdorferi 297	$bdrE_6$	AF046998	*rep+2.9-8*	(45)
B. burgdorferi 297	$bdrE_7$	AF046999	*rep+2.9-9*	(45)
Subfamily F				
B. afzelii DK1	$bdrF_1$	Y08143	*p21*	(43)
B. burgdorferi B31G	$bdrF_1, F_2, F_3$	BBF03, BBG33, BBH13	*bdrS, bdrT, bdrU*	(30)

chosen to reflect a central repeat motif carrying domains in the deduced amino acid sequences. The + and - designations were assigned to indicate that the overlapping *rep*+ and *rep*- genes are located on opposing DNA strands. Plasmid-carried repeated DNA sequences were also identified in *B. burgdorferi* B31 and found to carry either all or a subset of seven ORFs, designated A through G (41). Of relevance to this discussion are the ORF-E sequences that are *rep* or *bdr* homologs. A *bdr*-related gene was also identified in *B. afzelii* DK1 and designated as *p21* (43). *B. afzelii* causes Lyme disease in Europe and Asia. The *rep*+, ORF-E, and *p21* designations have recently been replaced with *bdr* gene designations (24,25,44).

To assess and compare the composition and complexity of the *bdr* gene family among species and isolates of the *B. burgdorferi* sensu lato complex, restriction fragment length polymorphism (RFLP) patterns were determined (Appendix). Genomic DNA digested with *Xba1* was Southern blotted and probed with an oligonucleotide targeting the *bdr* genes (Figure 1). A variable number of hybridizing bands of different size were detected. These analyses demonstrate that extensive *bdr* gene families are carried by *B. burgdorferi* sensu lato complex isolates and that the RFLP patterns vary at the inter- and intraspecies level. Hybridization analyses of other *Borrelia* species showed that they also carry *bdr*-related gene families (24,25,46). *bdr*-related genes have been detected by hybridization in *B. turicatae, B. hermsii*, *B. parkeri*, *B. coriaceae,* and *B. anserina* (25,46). Isolates of these species also exhibit substantial variation in their *bdr* RFLP patterns at the intraspecies level. Table 1 lists the *Borrelia* species that carry *bdr*-related genes and indicates the methods by which these genes or proteins were detected.

Sequences flanking some *bdr* alleles also appear to be distributed genus wide. Some *bdr* alleles of *B. turicatae, B. parkeri,* and *B. hermsii* are flanked by genes that are homologs of genes carried by the Lyme disease spirochetes (24,25). As a specific example, the *B. turicatae* $bdrA_1$ gene is flanked by ORFs that are homologs of the BBG34 and BBG30 genes of *B. burgdorferi* (24,25). In the Lyme disease spirochetes, BBG34 is part of a three-member paralogous gene family, while BBG30 is a single-copy gene (30). Located between BBG30 and BBG34 is BBG33, a member of the *bdr* gene family (recently redesignated as $bdrF_2$) (25). Although these divergent *Borrelia* species carry related genes, their organization differs (24), which indicates that rearrangement has taken place in the ancestral plasmid that carried these homologs. Figure 2 compares the

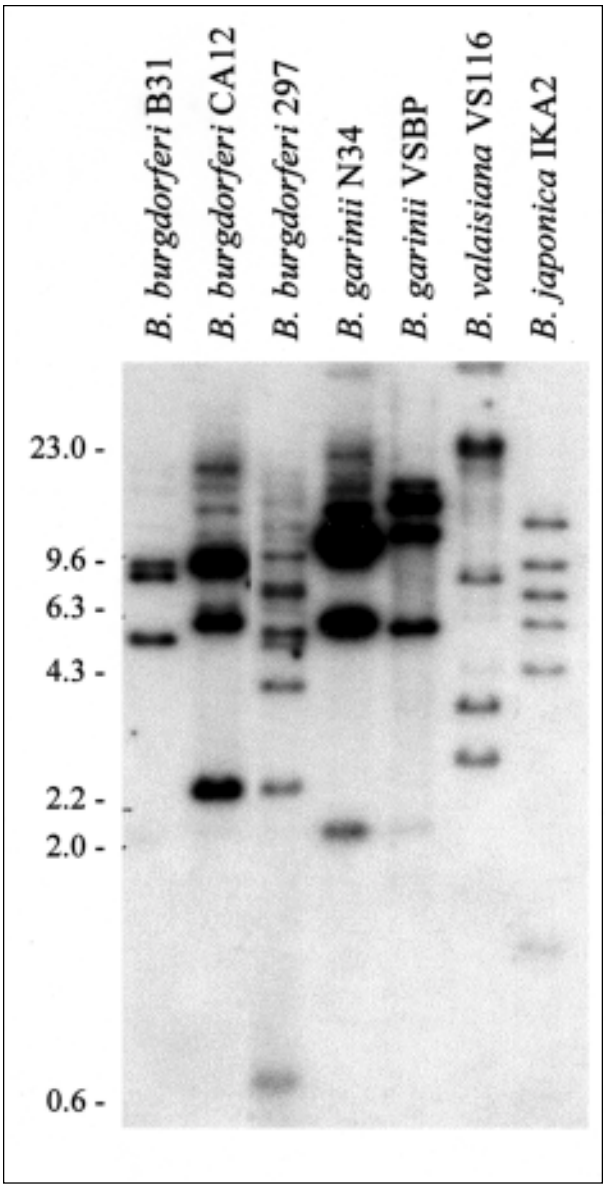

Figure 1. Restriction fragment length polymorphism pattern analysis of the *rep* or *bdr* genes of the Lyme disease spirochetes. Total DNA, isolated from *Borrelia* cultures, was digested with *Xba1,* fractionated by electrophoresis, and transferred onto membranes for hybridization. Hybridization was performed by the bdrAB-R1 oligonucleotide (46). The species and isolates analyzed are indicated above each lane. MW markers in kb are indicated.

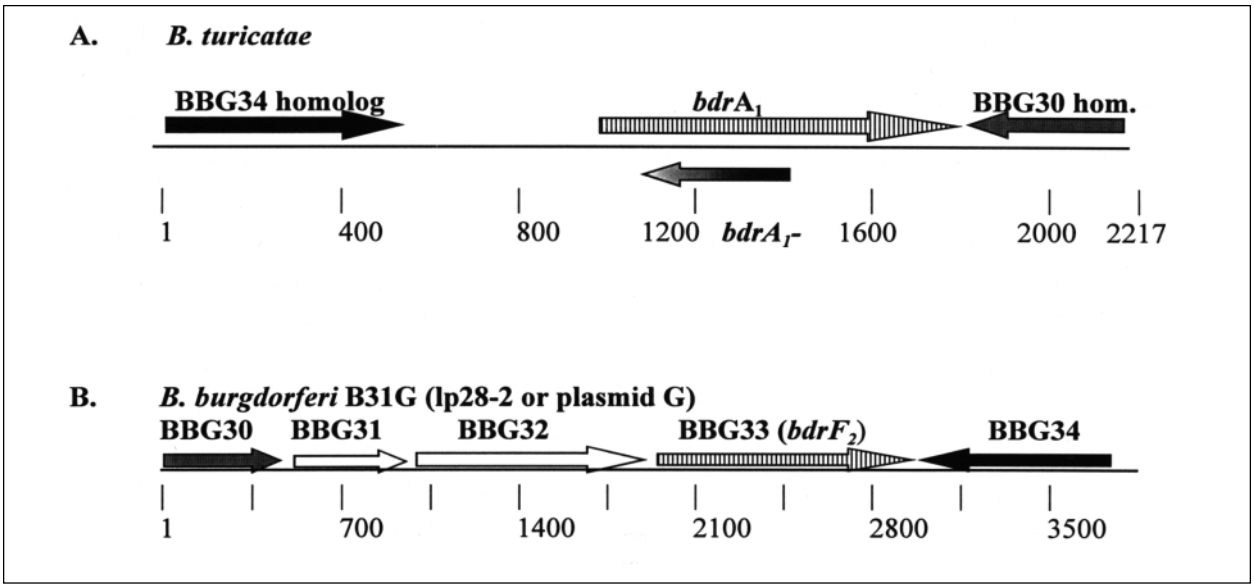

Figure 2. General organization of two *bdr* loci in *Borrelia turicatae* and *B. burgdorferi.* The gene arrangement depicted for *B. turicatae* was determined through cloning and sequence analysis of a 2,217 base-pair *XbaI* restriction fragment. The arrangement for the *bdr*-carrying locus of *B. burgdorferi* was previously determined through the sequencing of the *B. burgdorferi* B31 genome (30). The arrows indicate the direction of transcription. Genes exhibiting homology are indicated by similar shading or hatch marks. Genes indicated by unfilled arrows are not homologous. The numbering is indicated for scale and is not indicative of the positioning of these genes on the plasmids that carry them.

organization of two *bdr* loci from *B. turicatae* and *B. burgdorferi.*

Evolutionary Analyses of *bdr*-Related Sequences: Revised Nomenclature for the Bdr-Related Proteins

To simplify the complicated nomenclature of *bdr*-related genes, a *bdr* nomenclature system has been developed that assigns gene names on the basis of phylogenetic relationships inferred from comparative analysis of genetically stable regions of the *bdr* genes (25). This system, which is applicable genuswide, allows for a ready assessment of relationships among *bdr* paralogs and orthologs. The rationale for this system stemmed from the results of a comprehensive evolutionary analysis of >50 *bdr*-related sequences from five *Borrelia* species that demonstrated that *bdr* sequences are organized into six distinct subfamilies, designated A through F (25). Subfamilies are not necessarily species specific; some contain *bdr* alleles from different *Borrelia* species (25). Since members of a given subfamily are closely related to one another with identity values for the N terminal domain being >95%, each member is assigned the same gene name designation, and paralogs are distinguished by a numerical subscript. In *B. turicatae* OZ-1, two *bdr* subfamilies, *bdrA* and *bdrB,* contain at least four and five members, respectively (24). Members of the *bdrA* subfamily are designated $bdrA_1$, $bdrA_2$, $bdrA_3$, and $bdrA_4$, while members of the *bdrB* family are designated $bdrB_1$ through $bdrB_5$. This revised Bdr nomenclature scheme was modeled after that proposed for bacterial polysaccharide synthesis genes (47) and is in accordance with the nomenclature guidelines established by Demerec (48).

The subfamily affiliation of *bdr* genes can be readily determined through comparative sequence analyses of the amino acid segment preceding the polymorphic repeat motif region of these proteins (described in detail below) (25). Relationship assessments based on the genetically stable N terminal domain (vs. complete sequences) are preferable because the calculated evolutionary distances and clustering relationships are not artificially skewed by the variable number of repeat motifs present in the repeat motif domain. Since the genetically unstable repeat motif domain comprises as much as 50% of the total coding sequence in some alleles, it can

have a substatial impact on inferred relationships. In addition, extensive sequence variation in the carboxyl termini of the Bdr proteins at the inter-species level makes it difficult to align this domain with confidence, which further influences the inferred relationships.

bdr evolutionary analyses show that *Borrelia* species carry members of at least two *bdr* subfamilies (25,44). In fact, *B. burgdorferi* carries three distinct subfamilies. Multiple Bdr subfamilies in diverse *Borrelia* species suggest that there has been selective pressure to maintain multiple *bdr* alleles and *bdr* genetic diversity. This genetic diversity may increase the functional diversity of the Bdr proteins.

Molecular Features and Physical Properties of the Bdr Proteins

While early analyses of *Borrelia bdr* genes demonstrated their multicopy nature (41,42,46), the full extent of the complexity of the *bdr* gene family in the Lyme disease spirochetes was not fully recognized until the *B. burgdorferi* genome sequence was determined (30). *B. burgdorferi* B31 was found to carry 17 distinct *bdr*-related genes (and one truncated variant) distributed among different linear and circular plasmids. *B. turicatae,* which carries at least nine different *bdr* alleles, carries these genes exclusively on linear plasmids (24,25,46). Other relapsing fever spirochete species (*B. parkeri* and *B. hermsii*) are similar to the Lyme disease bacteria in that they carry *bdr* genes on both linear and circular plasmids (25). In the Lyme disease spirochetes each of the 32-kb circular plasmids, with the exception of plasmids M and P, carry two different *bdr* genes separated by seven or eight ORFs. Each of these circular plasmids carries one *bdrD* subfamily member and one *bdrE* subfamily member. The maintenance of genes belonging to different subfamilies on a single plasmid is consistent with the possibility that each carries out a different function. In contrast, in the Lyme disease spirochetes, the *bdrF* subfamily members are localized to linear plasmids with only a single *bdr* gene per plasmid. These observations suggest that there has been selective pressure to maintain the association of specific subfamilies with specific types of plasmids. Less is known about the *bdr*-carrying plasmids and the organization of the *bdr* genes and subfamilies in the relapsing fever borreliae. However, as in the Lyme disease spirochetes, in *B. turicatae* most *bdr*-carrying plasmids carry two *bdr* genes, one from subfamily *bdrA* and one from subfamily *bdrB* (24).

The sequence of more than 50 *bdr* alleles from five different *Borrelia* species has been determined (Table 2) (24,25,41-43,46). These extensive comparative sequence analyses led to the identification of conserved features that provide insight into the possible biologic roles of the Bdr proteins. For example, all *bdr* alleles carry centrally located repeat motif domains (Figure 3). Although conserved in sequence, these domains vary in length among alleles as a result of varying numbers of the repeat motif. The core tripeptide

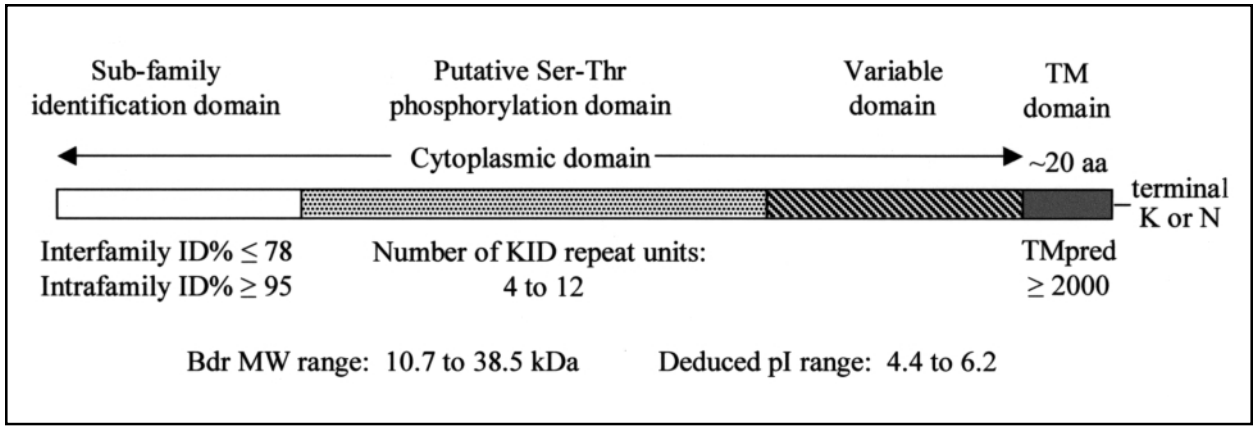

Figure 3. Key features and putative functional domains of the Bdr proteins. The schematic depicts a prototype Bdr protein with the characteristics of each domain indicated. The abbreviation, ID%, is for percentage amino acid identity at either the inter- or intra-family level as indicated in the figure. Standard amino acid abbreviations are used in the figure to denote the conserved C-terminal lysine (K) or asparagine (N) residues, which are thought to be exposed in the periplasm and the cytoplasmically located core tripeptide of the repeat motif (lysine-isoleucine-aspartic acid; KID).

the repeat is the sequence KID. The repeat motifs encode consensus casein kinase 2 phosphorylation (CK2P) motifs of the sequence T/SKID/E (43). While it may appear somewhat paradoxical for bacteria to carry casein kinases, casein kinase is a descriptive term broadly applied to at least two classes of ubiquitous protein kinases for which the substrates may include various enzymes and noncatalytic proteins involved in important cellular regulatory functions (49). Most proteins phosphorylated by CK2-like kinases are highly acidic, as are the *Borrelia* Bdr proteins (isoelectric points between 5 and 6). The phosphorylation site in CK2P motifs is either the Ser or Thr residue of the motif. Although histidine kinases have been known to exist in some bacteria, it has been widely held that bacteria lack Ser - Thr kinases. However, Ser - Thr kinases have recently been identified in several bacterial species, including *Myxococcus*, *Anabeana, Freymella, Yersinia,* and *Streptomyces* (50). Most importantly, analysis of the *B. burgdorferi* genome sequence identified a putative Ser - Thr kinase designated BB0648 (30,50). This ORF carries a domain that exhibits homology with the active site of Ser - Thr kinases. *B. burgdorferi* also carries a homolog of the PPM family of eucaryotic protein Ser - Thr phosphatases (30,50). The presence of these genes in *B. burgdorferi* suggests that the *Borrelia* possess the machinery necessary for Ser - Thr phosphorylation and dephosphorylation.

Another important conserved feature identified through sequence analyses is the hydrophobic carboxyl terminal domain of approximately 20 amino acids. Computer analyses conducted with the TMpred program indicate that this domain has a high propensity to form a transmembrane helix (24,25). The Tmpred values for the 20 aa C-terminal domains are 2,000 to 2,600. A value of 500 or greater is considered significant (24,25). Comparison of the Bdr putative transmembrane domain sequences from the Lyme disease spirochetes with those from the relapsing fever spirochetes indicates that, while there is conservation in physical properties, there is essentially no conservation of primary sequence. However, sequence conservation does exist at the subfamily level (24,25). Since the Bdr proteins lack an obvious export signal, membrane association would most likely be with the spirochetal inner membrane, with the rest of the protein, which is hydrophilic, extending into the cytoplasm. The terminal residue of the protein is in almost all cases a positively charged amino acid (lysine or asparagine). This residue could extend into the periplasm and serve to anchor the Bdr proteins to other cellular components, such as the peptidoglycan.

Immunologic Analyses of the Bdr Proteins

The presence of multiple *bdr* alleles and *bdr* subfamilies within isogeneic populations has prompted speculation that there may be differential expression at either the subfamily or individual allele level, possibly in response to environmental stimuli (46). Limited studies of *bdr* expression and production, based on either mRNA detection or immunoblot analyses, have been performed. Porcella et al. (42) used Northern hybridization to determine if expression of *B. burgdorferi bdr*-related genes occurs during cultivation in the laboratory under standard culture conditions (33°C in BSK media). *Bdr* transcripts were not detected by this approach. Similarly, in an earlier analysis, we also conducted Northern hybridization experiments to assess *bdr* expression (46). We detected expression of *B. turicatae* OZ1 *bdrA* subfamily members in bacteria cultivated under standard laboratory growth conditions (46). However, when reverse transcriptase (RT)-PCR methods were applied, transcription of a single *bdrA* allele was detected (46). *B. turicatae* OZ-1 was later demonstrated to carry at least nine *bdr* alleles, four of which belong to the *bdrA* subfamily. Analysis of the sequence of these alleles showed that all four should have been readily amplified by the RT-PCR primer set because of the conservation of the primer binding sites (24). The lack of detection of transcript derived from these alleles suggested that only a subset of the *bdr* A subfamily alleles is expressed. This raised the possibility that other *bdr* alleles are either nonfunctional genes or their expression requires different environmental stimuli. The transcriptional expression of the *bdrB* subfamily has not been specifically assessed. Thorough transcriptional analyses using allele-specific probes and primers are an important step, since they allow specific assessment of the expression of individual *bdr* alleles under differing environmental conditions. In addition, analyses of the upstream DNA sequences of individual *bdr* alleles and their genomic location may elucidate the molecular basis for *bdr* transcriptional regulation.

Immunologic analyses have provided a somewhat different overall picture regarding Bdr production. Immunologic analyses described in this report and elsewhere (44) demonstrate that several members of the *bdr* gene family are expressed during in vitro cultivation. We conducted a comprehensive analysis of the expression of Bdr proteins among *Borrelia* species. When antisera raised against recombinant *B. afzelii* $BdrF_1$ (24) were used in immunoblot analyses, several immunoreactive proteins were detected in cell lysates of all *Borrelia* species tested (Figure 4). The only exception was *B. anserina,* a causative agent of avian spirochetosis. Although *bdr*-related sequences have been detected in *B. anserina* by hybridization techniques (46), immunoreactive proteins were not detected in immunoblot analyses. Additional analyses are required to determine if this indicates absence of translational expression or the lack of epitope conservation in this species. In any event, the fact that immunoreactive bands were not detected in this species attests to the specificity of the anti-Bdr antisera. As a further demonstration of the specificity of the antisera and to highlight the fact that the Bdr proteins are unique to *Borrelia*, a cell lysate of *Leptospira interrogans* was included in the immunoblot analyses. Immunoreactivity with proteins in the Bdr size range was not observed with the anti-Bdr antisera in this spirochete species. *Borrelia* species that expressed immunoreactive proteins included *B. garinii, B. burgdorferi, B. turdae, B. tanukii, B. japonica, B. valaisiana, B. afzelii, B. coriaceae, B. bissettii, B. miyamotoi, B. parkeri, B. hermsii,* and *B. turicatae* (Table 1). Particularly striking was the extensive variation in the number and molecular weight of the immunoreactive proteins expressed, with up to 12 distinct Bdr proteins detected. Variation in expression patterns was observed at both the inter- and intraspecies level. Analysis of three *B. burgdorferi* isolates (B31G, cN40, and CA12) demonstrated variability in both the size and number of expressed Bdr proteins. Isolate B31G has been demonstrated by genomic sequencing to carry 18 distinct *bdr* alleles. Immunoblot analyses show that not all alleles are expressed during in vitro cultivation; therefore, some alleles may be differentially regulated.

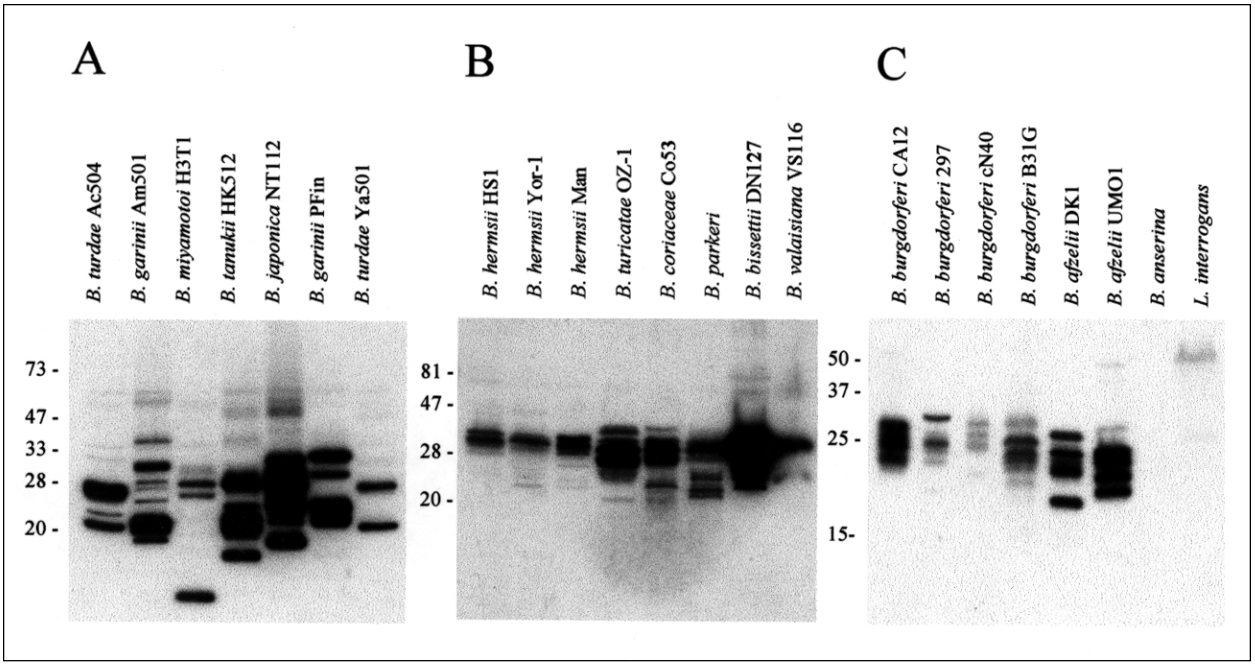

Figure 4. Immunoblot analyses demonstrating the variation in Bdr protein expression in *Borrelia* species and isolates. Bacteria were cultivated and prepared for analysis as described in the methods. Proteins were fractionated by SDS-PAGE, immunoblotted and screened with anti-$BdrF_{1\text{-}B.afzelii\ DK1}$ antisera. The species and isolates analyzed are indicated above each lane in panels A, B and C. The migration positions of the protein standards are indicated in each panel.

The broad immunoreactivity of the antisera with diverse *Borrelia* species indicates that some epitopes are conserved genuswide. In view of the sequence divergence in the N and C terminal domains of the Bdr proteins derived from different subfamilies, it is likely that the cross-reactive epitopes reside in the conserved repeat motif region. Consistent with this, computer analyses of the repeat domain of all determined Bdr protein sequences predict them to be alpha helical and to have a surface exposed on the protein and a positive Jameson-Wolf antigenic index (24,25,44). The conservation and synthesis of these polymorphic proteins in such a diverse group of *Borrelia* species suggest that they play an important role in *Borrelia* biology genuswide.

The Bdr Proteins and *Borrelia* Biology: An Overview

Bdr genes and extensive *bdr* gene families have now been identified and characterized in several diverse *Borrelia* species (24,25,42-44,46). Comparative sequence analyses, which have identified conserved putative functional domains, have provided the basis for the development of hypotheses regarding Bdr function and cellular location. The Bdr proteins, which lack known consensus export signals, are likely anchored to the cytoplasmic membrane through their conserved, hydrophobic, putative transmembrane spanning domain. The C-terminal positively charged amino acid may be exposed to the periplasm, where it may interact with other cellular components that may include the peptidoglycan. The repeat motif domain, which is predicted by computer analyses to be hydrophilic and surface exposed on the protein, likely extends into the cytoplasm. The conserved repeat motif domain that carries the putative Ser - Thr phosphorylation motifs may then be accessible for phosphorylation or to interact with other cytoplasmic proteins or DNA to form a membrane anchored complex. As with numerous other proteins, phosphorylation and dephosphorylation could play a regulatory role, perhaps in signaling or sensing.

Multiple polymorphic *bdr* alleles may increase the functional range and diversity of the Bdr proteins. Functional partitioning among Bdr proteins could offer a possible explanation of why *Borrelia* expend such biologic energy to maintain these genes in large gene families and express variants of these proteins. The homology among *bdr* alleles may also allow or lead to the continual modification of these genes through homologous recombination. In fact, the variable nature of the repeat motif region, which is clearly not evolutionarily stable, has likely arisen from slipped-strand mispairing, recombination, or rearrangement. In view of the extensive genetic redundancy of the plasmid component of the *Borrelia* genome, recombination in and among related sequences on different plasmids could affect the organization and evolution of the genome and ultimately host-pathogen interaction. Inter- or intra-plasmid exchange of DNA sequences could provide a mechanistic basis for the extensive genetic variability that has been widely described for *Borrelia* plasmids (28,29,51-59). In spite of the apparent necessity for at least most of the plasmids for survival, as inferred from their ubiquitous distribution among *Borrelia* isolates, these bacteria are able to tolerate remarkable genomic variability. Diversity in the plasmids and the genes they carry may actually be exploited as a tool for phenotypic diversity and rapid environmental adaptation.

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Appendix

Bacterial Cultivation, DNA Isolation, and Southern Hybridization Analyses

Isolates belonging to the *Borrelia burgdorferi* sensu lato complex were cultivated in complete BSK-H media (Sigma) at 33°C. To cultivate the relapsing fever borreliae and other *Borrelia* species, the complete BSK-H media were supplemented with additional rabbit sera (Sigma) to a final concentration of 12% (vol/vol). Bacteria were harvested by centrifugation and washed with phosphate buffered saline (pH 7.0), and DNA was extracted (25). For Southern hybridization analyses, 5 □g of DNA from each isolate was digested under standard conditions with *Xba1* and fractionated by electrophoresis in 0.8% GTG agarose gels. (The DNA was transferred onto membranes for hybridization by vacuum blotting using the VacuGene system as described by the manufacturer (Pharmacia). All other Southern hybridization methods were as previously described (39).

Immunoblot Analyses

Bacterial cultures were grown and harvested as described above. One OD600 equivalent of cells was pelleted and resuspended in 100 □l of standard SDS-sample buffer with reducing agents. The cell lysates (7 □l) were fractionated by electrophoresis in 15% SDS-PAGE gels and electroblotted onto Immobilon P membranes (38). The immunoblots were blocked overnight in blocking buffer (1X PBS, 0.2% Tween, 0.002% NaCl, and 5% nonfat dry milk) and then incubated with a 1:1,000 antisera dilutions. ImmunoPure Goat anti-mouse IgG (H+L) peroxidase conjugate served as the secondary antibody. The secondary antibody was incubated with the blots for 1 hour at room temperature at a 1:40,000-fold dilution and then the blots were washed three times with wash buffer. For chemiluminescent detection, the Supersignal West Pico Stable Peroxide solution and the Supersignal West Pico Luminol/Enhancer solution were used. Both reagents were from Pierce Chemical Company, Rockford, IL and were used as described by the manufacturer. The immunoblots were exposed to film for time frames of 5 to 30 seconds.

Vaccines for Mucosal Immunity to Combat Emerging Infectious Diseases

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The mucosal immune system consists of molecules, cells, and organized lymphoid structures intended to provide immunity to pathogens that impinge upon mucosal surfaces. Mucosal infection by intracellular pathogens results in the induction of cell-mediated immunity, as manifested by CD4-positive ($CD4^+$) T helper-type 1 cells, as well as $CD8^+$ cytotoxic T-lymphocytes. These responses are normally accompanied by the synthesis of secretory immunoglobulin A (S-IgA) antibodies, which provide an important first line of defense against invasion of deeper tissues by these pathogens. New-generation live, attenuated viral vaccines, such as the cold-adapted, recombinant nasal influenza and oral rotavirus vaccines, optimize this form of mucosal immune protection. Despite these advances, new and reemerging infectious diseases are tipping the balance in favor of the parasite; continued mucosal vaccine development will be needed to effectively combat these new threats.

Behavioral changes in the human host may be contributing to the emergence of new diseases. Perhaps emerging pathogens become resistant to antibiotics or (through genetic recombination) become more resistant to host defenses. Recombination events or lack of exposure can result in loss of immunity of the population to the pathogen, as has been well documented with influenza virus. Recombination events increase the infection rate by the emerging pathogen and, in the case of influenza virus, occasionally result in pandemics. A large number of emerging pathogens are mucosally transmitted and must cross mucosal barriers to infect the host. Thus, our ultimate defense against new and reemerging infectious disease will require effective mucosal vaccines.

Mucosal surfaces are prominent in the gastrointestinal, urogenital, and respiratory tracts and provide portals of entry for pathogens. Increased sanitation and hygiene, the use of antibiotics, and childhood vaccination have enormously decreased the death rate from infectious diseases over the last century (1). Thus, infectious agents not controlled by antibiotics and improved sanitation and hygiene measures would most likely be prevalent under current conditions. Pathogens in this category include respiratory viruses, for example, influenza virus. Another group includes sexually transmitted disease (STD) pathogens, for example, HIV. The fact that a) pneumonia and influenza virus and b) HIV infection were listed as the leading causes of death (ranked number 6 and 8, respectively) by infectious agents in the United States in 1997 confirms this notion (1). However, other viral and bacterial STDs are of major concern.

The best defense against these predominantly mucosal pathogens would be vaccines, preferably mucosal vaccines capable of inducing both systemic and mucosal immunity. Although numerous strategies exist for the induction of mucosal immune responses, we will focus on the use of live attenuated vectors, such as *Salmonella typhi* and adenovirus. In a recent National Institutes of Health news release (April 8, 1999) from the Institute of Medicine report on domestic vaccine priorities for the future, which compared costs and health benefits, influenza was listed as one of seven diseases requiring an effective vaccine. The induction of protective mucosal immunity to influenza virus has made considerable progress with the development of cold-adapted influenza strains, which are in phase-3 clinical trials. Mucosal immunity, which is important for long-term protection, forms a first

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line of defense against mucosally transmitted pathogens such as influenza. Mucosal defense against pathogens consists of both innate barriers, such as mucous, epithelium, and innate immune mechanisms, and adaptive host immunity, which at mucosal surfaces consists predominantly of $CD4^+$ T cells, secretory immunoglobulin A (S-IgA), and antigen-specific cytotoxic T-lymphocytes (CTLs). This review will focus on the antigen-specific mucosal immune system.

Emerging Pathogens

The major obstacle in combating emerging infectious diseases is the lack of more effective antibiotics and vaccines. Misuse of antibiotics has led to antibiotic-resistant pathogens, further intensifying the need for mucosal vaccine development—a cost-effective disease-prevention tool. The Department of Vaccines and Other Biologicals within the World Health Organization (WHO) has defined several priority vaccines for accelerated introduction; they include pneumococcal, *Haemophilus influenzae* type b, rotavirus, and hepatitis B. Other vaccine goals within WHO are eradication of polio, reduction of measles cases by 90%, and elimination of neonatal tetanus (2). For most pathogens targeted by WHO for vaccines, induction of mucosal immunity appears most appropriate based on the routes of infection. Thus, a better understanding of the mucosal immune system is needed before effective mucosal vaccines can be developed.

The Mucosal Immune System

Mucosal inductive sites in humans, such as the Peyers patches in the intestinal tract and the nasal-associated lymphoreticular tissue in the oropharyngeal cavity, stand as sentinels to the intestinal and respiratory tracts and represent the major sites where mucosal immune responses are initiated (Figure 1). Common features of these inductive sites are microfold or M cells.

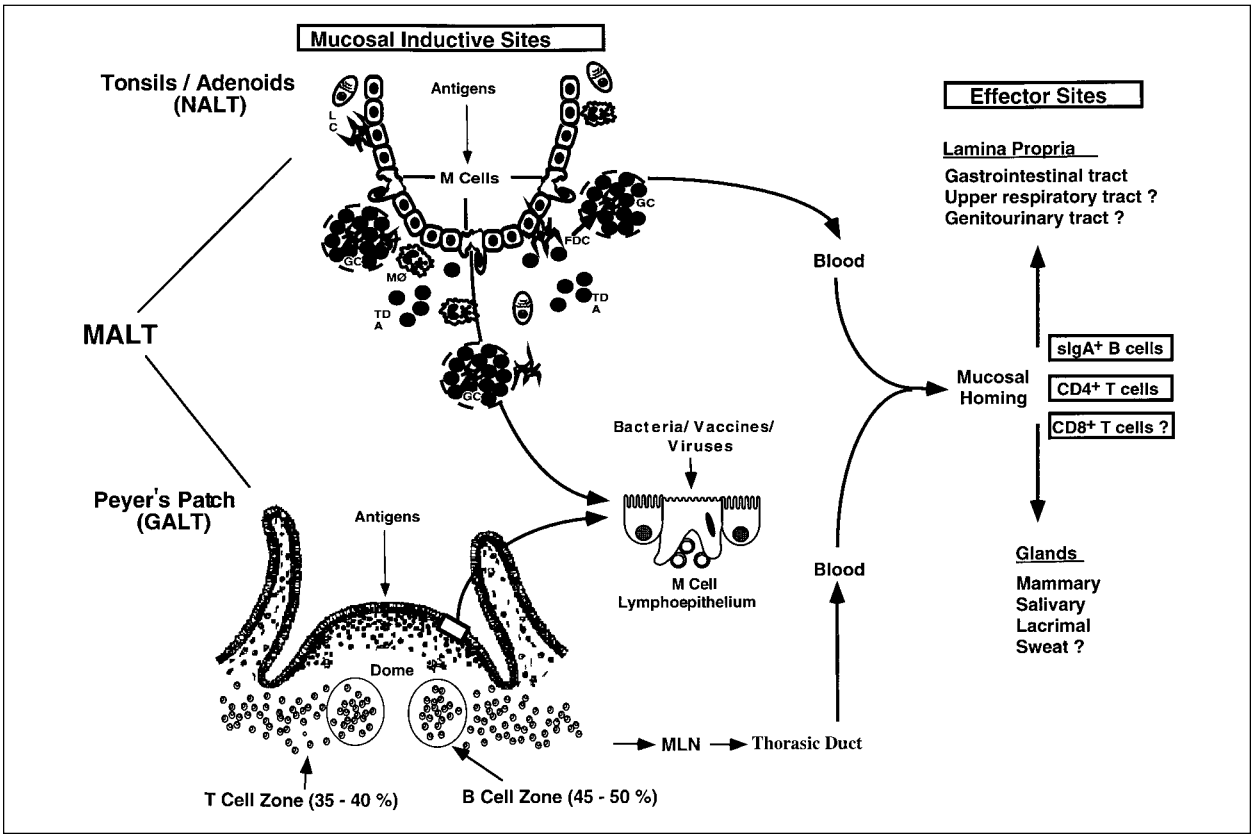

Figure 1. M cells and the induction of mucosal immunity. M cells are present in mucosal inductive sites in both the intestinal and upper respiratory tract, specifically in Peyer's patches and the nasal-associated lymphoid tissue, the tonsils and adenoids. M cells are thought to play an important role in antigen processing and possibly the induction of antigen-specific mucosal immunity in mucosal effector sites. Tissues followed by question marks are presumed sites since limited data are available on these tissues.

Although the precise function of M cells has not yet been established, recent studies indicate that they are involved in uptake, transport, processing, and possibly presentation of microbial antigens (3,4). The interaction of epithelial cells with T and B lymphocytes induces epithelial cells to differentiate into M cells in vitro (5), indicating the importance of lymphocyte-epithelial cell interactions for maintaining M cells in the follicle-associated epithelium of the Peyer's patches. These lymphocyte- M cell interactions can occur in the pocket of the M cells and are mediated through thin cellular extensions, indicating that cell-cell interactions are an intricate part of the M-cell function and that they may facilitate transfer of luminal antigens, viruses, bacteria, and other protein components (3). The ability of the M cell to transport particulates from the lumen across the epithelial barrier has been exploited by some pathogens to facilitate entry into the host, as has been demonstrated for invasive strains of *Salmonella* (6) and reovirus (7) (Figure 1). Identification of bacterial and viral virulence factors associated with targeting M cells, such as the sigma protein from reovirus, may allow development of mucosal vaccines and vectors to deliver vaccine components directly into mucosal inductive sites.

T-Cell and Cytokine Involvement in B-Cell Isotype Commitment to IgA

Although the variables involved in the switching of B cells to polymeric IgA (pIgA)-producing plasma cells have been studied, many questions remain. In recent years, gene-deleted or knockout mice have contributed to a better understanding of the role of specific cells, cytokines, and surface molecules involved in IgA isotype switching. Presumably, isotype switching occurs in mucosal inductive sites, while IgA production by plasma cells occurs in mucosal effector sites, separating the IgA switching and IgA secretion by B cells into different immune compartments (8). Each of these stages requires specific signals, such as costimulatory molecules, cytokines, and T-helper cells, to give rise to antigen-specific S-IgA Abs in mucosal effector sites.

Neither Th1- nor Th2-type cytokines contributed significantly to the switching of B cells to surface IgA positive ($sIgA^+$) B cells (Figure 2).

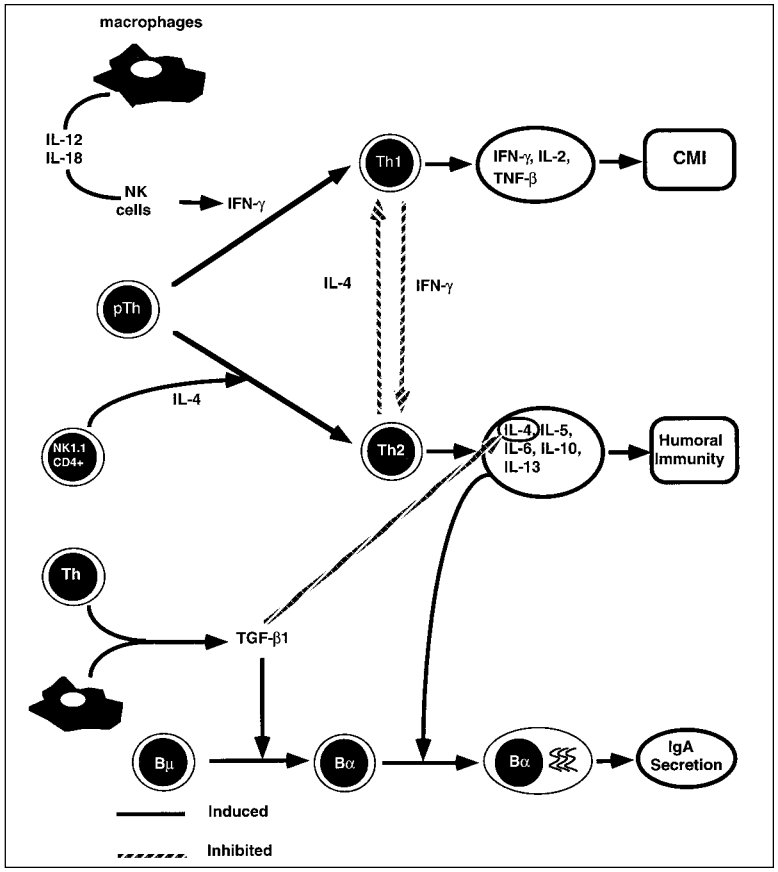

Figure 2. Differentiation and regulation of T-helper subsets and the immune response in the mucosal compartments. Encounter of pathogen-derived antigen or vaccine antigen will stimulate T-helper cells to secrete cytokines. Depending on the stimulus, a Th1 or Th2 cell response is induced. For example, intracellular pathogens will induce production of IL-12/IL-18 by macrophages, activating IFN-γð production by NK cells and inducing differentiation to a Th1-mediated immune response, which supports CMI and production of complement-fixing antibodies, presumably by production of cytokines such as IFN-γð, IL-2, and TNF-βð. A Th2 response can be observed upon infection with parasites or upon vaccine administration; this response is characterized by production of cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13 which support humoral immunity. However, for induction of a S-IgA, TGF-βð1 is required to enable B cells to switch to IgA. TGF-βð1 production is associated with inhibition of IL-4 production by Th2 cells inhibiting IgE production.

This process required the presence of transforming growth factor βð1 (TGF–βð1), which can activate the switch of B cells to the IgA isotype (9). TGF-βð1 induces a small proportion (<2%) of B cells to switch to IgA in activated B-cell cultures (9,10). However, TGF-βð1, when used in combination with additional signals, increased TGF-βð1-induced switching in 10% to 20% of B cells and approached IgA^+ B-cell levels observed in Peyer's patches (11). Thus, multiple activation signals contribute to the switch to IgA, i.e., B-cell activation by cross-linking the B-cell antigen receptor, CD40-CD40L interactions to promote switching, TGF-βð1 by directing the switch to IgA, and Th2-type cytokines by increasing the number of post-switch IgA^+ B cells and their differentiation into IgA-secreting plasma cells. In addition, activated T cells and dendritic cells from the Peyer's patches were more effective in switching $sIgM^+$ $sIgA^-$ B cells to IgA-producing cells than were T cells and dendritic cells derived from the spleen (12). This suggests that mucosal inductive sites contain specialized T cells or dendritic cells beneficial for B cells to differentiate into IgA-producing cells.

T-cell helper functions play important roles in generating antigen-specific humoral and cell-mediated immunity in both systemic and mucosal compartments. The importance of $CD4^+$ T cells for generating protective immunity is illustrated by the lack of these cells in AIDS patients. The differentiation of Th0 cells into either Th1 or Th2 is driven by cytokines such as interleukin 12 (IL-12), interferon γð (IFN-γð), and IL-4, respectively. For example, intracellular pathogens, such as viruses and intracellular bacteria, induce production of IL-12 or IL-18 by activated macrophages, presumably after ingestion of the particulate pathogen, inducing IFN-γð production in natural killer (NK) cells, which in turn drives the differentiation of Th0 cells toward a Th1 phenotype producing IFN-γð, IL-2, and tumor necrosis factor βð (TNF-βð)ð (Figure 2). Murine Th1-type responses are associated with cell-mediated immunity, such as delayed-type hypersensitivity and IgG2a antibody responses (8). Th0 cells are differentiated into Th2-type cells when soluble exogenous antigen is administered, triggering $CD4^+$, $NK1.1^+$ T cells to produce IL-4. The Th2 cell produces more IL-4, expanding Th2-cells, which support the associated immune response. Th2 cells secrete cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. The production of IL-4 supports IgG1 subclass and IgE production, but other antibodies such as IgG2b and IgA, are also produced during a Th2-dominated response (8).

It is not known whether Th1 or Th2 cells are beneficial for optimal S-IgA production. Historically, Th2-type cytokines were considered major helpers for antibody responses. For example, S-IgA Ab responses were supported by mucosal adjuvants such as cholera toxin, which induced polarized Th2 cell responses (13). However, S-IgA Ab responses may also be induced through Th1-dominated responses, as observed with intracellular pathogens such as *Salmonella* in the gastrointestinal tract (14) or influenza virus in the upper respiratory tract (15). Thus, either Th1 or Th2 cells or a combination of these cell types can support antigen-specific S-IgA Ab responses. In this respect, Th2-type cytokines play a role in terminal differentiation of B cells, that are already committed to IgA (16-18), while the Th1-type cytokine IFN-γð has been implicated in the induction of the polymeric Ig receptor (pIgR) needed for transport of S-IgA (19). Cross-inhibition of Th1 and Th2 cell-directed IgG2a and IgE production was mediated through IFN-γð and IL-4, respectively (Figure 2) (20,21).

Mucosal S-IgA Antibody Responses

The hallmark of the mucosal immune system is the production of S-IgA. S-IgA results from transcytosis of pIgA across the epithelium through binding to the pIgR. S-IgA is released from the pIgR by cleavage of the receptor, resulting in pIgA covalently associated with a substantial part of the pIgR, i.e., the secretory component (22). This complex, referred to as S-IgA, seems to be more resistant to proteolysis in external secretions. Additional roles for S-IgA in protection are suggested by its reduction of influenza virus attachment and its prevention of internalization of virus into baby hamster kidney cells. In contrast, the action of monomeric IgA is indistinguishable from that reported for IgG and is less efficient than S-IgA for inhibition of influenza virus entry (23). In addition, pIgA, as opposed to IgG or monomeric IgA, neutralizes virus intracellularly, as first was shown with Sendai virus (24). Furthermore, transport of pIgA containing immune complexes across epithelial cells expressing the pIgR is another defense mechanism of the mucosal immune system against pathogen entry (25).

These characteristics of pIgA are beneficial in preventing infection and inflammation at epithelial surfaces. The transport of pIgA across epithelial cells allows active elimination of immune complexes at mucosal sites and even virus inside epithelial cells. Evidence that these observations were not an artifact of the in vitro system was provided by the murine backpack model of rotavirus-specific monoclonal antibodies (mAb) to VP4 and VP6 proteins. In this model, nonneutralizing VP6-specific IgA mAb were protective, but not when administered directly to the gastrointestinal tract, indicating that IgA transcytosis played a prominent role in effective immune exclusion (26). Thus, virus-specific, intra-epithelial IgA can inhibit viral entry and replication.

Cell-Mediated Immunity at Mucosal Surfaces

Although S-IgA has been shown to be an important effector molecule to protect mucosal surfaces, the contribution to mucosal protection by the cellular immune system should not be underestimated. The strategic advantage of cell-mediated versus antibody-mediated immune responses is that T cells can recognize peptides derived from core proteins of the pathogen, such as influenza virus. Core proteins are usually expressed and presented much earlier during infection than proteins targeted for neutralizing antibodies, such as HA and NA of influenza virus. Subsequently, cell-mediated immunity (CMI) occurs before the induction of antibodies and forms an early line of defense. Although antibodies to core proteins are also formed later in the immune response, their exact role in protective immunity is not clear. Besides supporting humoral immunity, $CD4^+$ T-helper cells function in CMI as producers of cytokines, which mediate delayed-type hypersensitivity and support CTLs and which as such are critical components of the CMI responses to intracellular pathogens. For example, major histocompatibility complex (MHC)-restricted CTL responses are supported by Th1 cells. Cytotoxic cells can be classified based on antigen specificity and MHC restriction, i.e., nonspecific cytotoxic cells and antigen-specific, MHC-restricted CTLs. The first kind is composed of various cell types, including NK cells and antibody-dependent cytotoxicity, and functions very early in the immune response (day 1 to 3), and these cells are detected throughout the mucosal immune system. Presumably, they decrease pathogen load in the early stage of the immune responses, while antigen-specific responses are still being established. The second type, antigen-specific CTL, achieved optimal activity a little later than nonspecific CTL, i.e., at day 3 to 5 of the immune response before antibody production.

Both antigen-specific and nonspecific cytotoxic cell types can control growth of intracellular pathogens by two distinct mechanisms (Figure 3).

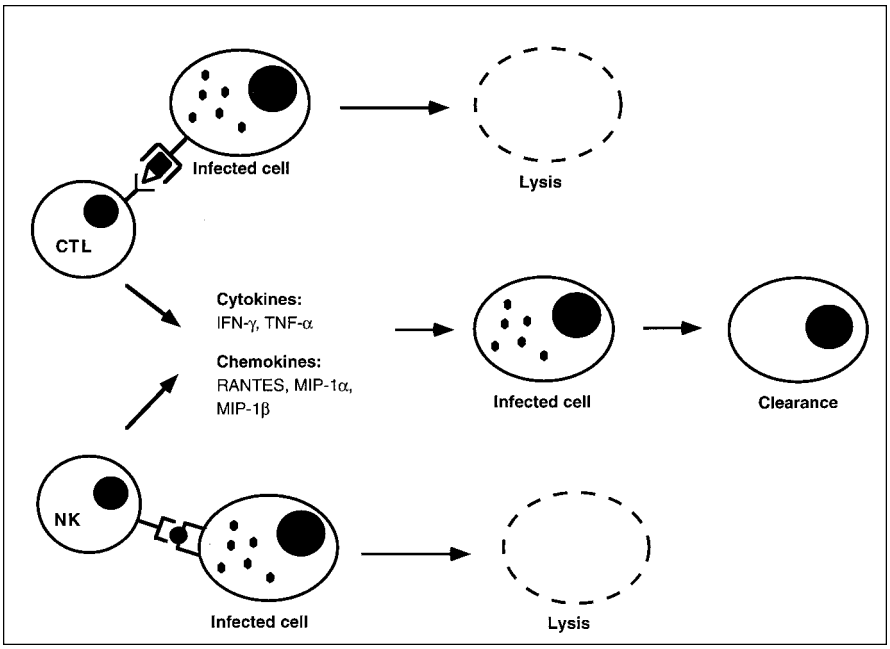

Figure 3. Pathways of intracellular pathogen clearance from infected cells by cytotoxic cells. Intracellular pathogen-derived antigens complexed to MHC class I molecules are recognized by CTLs, while NK cells recognize the absence or suppressed levels of MHC class I molecules on infected cells. Activated cytotoxic cells deliver apoptotic signals through Fas ligand and perforin to infected cells. They also secrete cytokines (IFN-γð, TNF-αð) and chemokines (Rantes, MIP-1αð, MIP-1βð) to inhibit or suppress intracellular pathogen replication.

First, they can respond to the infection by secreting a number of cytokines such as IFN-γδ and TNF-αδ (27) or chemokines such as Rantes, macrophage-inflammatory protein-1αδ (MIP-1αδ)δ, and MIP-1βδ (28,29). These soluble factors inhibit growth of intracellular pathogens such as viruses without destroying the host cell. Second, cytotoxic cells can effectively and efficiently recognize and lyse infected cells and prevent multiplication of viruses. Little is known about the induction, compartmentalization, and homing pattern of cytotoxic cells. Their presence in the mucosal compartment upon infection reflects their importance for protection against pathogens at mucosal surfaces.

Antigen-Specific CTLs

CTLs play an important role in the elimination of cells infected with various intracellular pathogens by recognizing pathogen-specific antigen/MHC complexes. Antigen-specific CTLs inhibit further spread of pathogens and help to terminate infections. Compartmentalization of pathogen-specific CTL responses has been reported and located at the site of initial infection. For example, CTLs preferentially compartmentalize in mucosa-associated lymphoreticular tissues after pulmonary or intestinal infection.

CTLs in the Intestinal Tract

Presentation of rotavirus by the intestinal mucosal surface was not required for induction of virus-specific cytotoxic intra-epithelial lymphocytes in the intestinal tract (30). In addition, the site at which rotavirus is first presented to the immune system will determine the site where rotavirus-specific CTL precursors (pCTL) first appear; however, regardless of the route of inoculation, rotavirus-specific pCTL can be found throughout the lymphoid system 21 days after the initial infection (31). Adoptive transfer of splenic lymphocytes from immunized animals protected suckling mice against murine rotavirus-induced gastroenteritis in the absence of rotavirus-specific neutralizing antibodies, indicating that antigen-specific CTLs protect against mucosal pathogens in the intestinal tract (32). Thus, thymus-derived αδβδ T cells can migrate to the intestinal epithelium after antigen-specific activation and protect the host against subsequent challenge. This notion is supported by findings that systemic immunization with attenuated macaque-specific Simian immunodeficiency virus induced virus-specific CTL responses in gut-associated lymph nodes and limited superinfection following mucosal challenge (33).

CTLs in the Respiratory and Urogenital Tracts

The distribution of CTLs following influenza virus infection in different mucosal compartments indicates that lymph nodes draining mucosal surfaces function as reservoirs for memory T cells. Mediastinal lymph nodes, the draining lymph nodes of the lungs, are considered the site where antigen presentation to T cells initially occurs before clonal expansion. Subsequently, T cells migrate to effector sites (mesenchyma of the lungs and airways) to interact with infected cells (34,35). Thus, compartmentalization of memory CTL responses to mucosa-associated lymphoreticular tissue may be related to the initial site of virus infection. This notion was confirmed by the observation that induction of protective antiviral memory CTL in mucosal tissues depends on region-specific mucosal immunization (36). HIV-specific CTL have been detected in the cervix (37) or semen (38) of HIV-infected persons. These studies indicate that the initial site of antigen exposure and induction of antigen-specific CTL responses in the urogenital tract are associated. Further evidence for this notion comes from the use of MHC class 1 tetramer technology, by which antigen-specific quantitation of $CD8^+$ T cells can be performed. Upon intranasal influenza administration, most antigen-specific, IFN-γδ-producing effector $CD8^+$ T cells were located in bronchial lavages and both effector (eCTL) and memory CTL (mCTL) occurred at a much higher frequency than initially thought based on limiting dilution assays (39). Since respiratory virus infection induces enlargement of the mediastinal lymph nodes early in the immune response and since these lymph nodes contain a relative small number of mCTL after initial exposure, a strong recruitment from circulating T cells occurs, or alternatively, clonal expansion of the resident mCTL takes place (39).

The presence of CTLs in mucosal compartments may contribute to the control of, and recovery from, infection by intracellular pathogens at mucosal surfaces. Since different pathogens have distinct infection routes or different localization in the host, compartmentalization of

protective, antigen-specific CTLs may vary, based on the specific pathogen. In general, mucosal infection induces primarily antigen-specific CTLs in the mucosal compartment and mucosa-associated lymphoid organs to control pathogens at the portal of entry, i.e., the mucosal surfaces.

The Common Mucosal Immune System

Antigenic exposure at mucosal sites activates mucosal B and T-lymphocytes to emigrate from the inductive site and home to various mucosal effector sites. The common mucosal immune system involves homing of antigen-specific lymphocytes to mucosal effector sites other than the site where initial antigen exposure occurred. This pathway has almost exclusively been documented for S-IgA antibody responses at mucosal surfaces mediated by B cells, but similar events are assumed to take place with T cells. Different immunization routes, such as oral, rectal, and intranasal, can induce generalized mucosal immune responses. However, oral immunization induced a more restricted mucosal response, as reflected by a more restricted homing receptor profile than nasal immunization. Specifically, after systemic immunization the predominant homing receptor on antibody secreting cells is the L selectin, after oral immunization the αð4βð7 integrin, and after nasal immunization a large portion expressed both the L selectin and the αð4βð7 integrin. The fact that nasal immunization induced antibodies in a broader range of tissues, such as saliva and the urogenital tract, than oral immunization reflects the more restricted nature of oral immunization (8).

Circumstantial evidence indicates the existence of a common mucosal immune system for cell-mediated immunity (44). The data available indicate that antigen-specific CTL responses at mucosal surfaces are dictated by induction of CTL locally and are not due to migration from distant sites. CTL do normally migrate to the systemic compartment. It could be hypothesized that the presence of antigen-specific CTL in the systemic compartment would allow for quick, protective responses at any mucosal site, but more research is needed to confirm this hypothesis. Such research is crucial, since limited CTL activity at mucosal surfaces could be a built-in mechanism to protect the mucosal epithelium from damage, a notion supported by the observation that pCTL in immunologically privileged sites fail to differentiate into fully functional CTL, unless exposed to antigen (40). This concept could have a major influence on future vaccine development. If mucosal antigen-specific memmory CTL responses are observed only after mucosal immunization, optimal protection against pathogens would require the use of mucosal vaccine. However, systemically induced CTL can generate an antigen-specific mucosal CTL response; in addition, systemic immunization can be used for cell-mediated protection at mucosal surfaces.

Mucosal Vaccines

Although mucosal application of vaccines is attractive for many reasons, only a few mucosal vaccines, mostly oral, have been approved for human use. These vaccines include poliovirus, *Salmonella typhi*, and the recently approved tetravalent rotavirus vaccine, RotaShield, consisting of reassorted rhesus-human rotaviruses. The latter vaccine has recently been associated with intussusception, a type of bowel obstruction, and its use has been suspended to await a more detailed analysis of this major problem. Human approved mucosal vaccines so far involve live attenuated pathogens, and for this reason oral poliovirus vaccine is recommended after receiving the injected inactivated virus, since a limited number of polio cases occur after immunization with the live attenuated virus. Since the live virus induces better, longer-lasting protection, it is given after some level of systemic immunity has been achieved, to limit possible problems. Another oral vaccine is the typhoid fever vaccine, which consists of attenuated *S. typhi* strain Ty21a. The cold-adapted influenza virus (CAIV), which is in advanced clinical trials, is the first mucosal vaccine given nasally to humans and has been shown to generate protective immune responses (41). Thus, CAIV are promising vehicles for generating protective immunity to influenza in children. The use of CAIV may also resolve some of the problems observed during the recent outbreak of the Hong Kong virus. Due to its relatedness with A/Chicken/Hong Kong/258/97 (H5N1) virus, the production of this virus for vaccine purposes was severely hampered because of its lethal effect on chicken eggs. The use of CAIV, which is readily produced by reassortment, might overcome this problem and allow production of high-titer virus for vaccine purposes.

An alternative approach is the use of DNA vaccines. Plasmid DNA was used in clinical trials

to induce protection against several pathogens, including hepatitis B virus, herpes simplex virus, HIV, malaria, and influenza (42). However, in all cases induction of antibodies and CTL in the systemic but not the mucosal compartment was reported. Although some progress has been made in inducing mucosal immunity in laboratory animals with DNA vaccines by using cationic lipids or other delivery vehicles, as well as immunostimulatory CpG dinucleotide motifs, no reports exist on the induction of mucosal immunity by DNA vaccines in humans. Unmethylated CpG dinucleotides are immunostimulatory, especially when presented in a 6 base-pair motif in which the central CpG is flanked by two 5' purines and two 3' pyrimidines.

Another promising avenue for mucosal vaccines is the use of bacterial adhesins. Mucosal antibodies to these proteins block the pathogen's ability to penetrate mucosal barriers. Adhesins are very attractive options because of the highly conserved nature of these proteins due to their association with conserved host receptor proteins. The pilus-associated adhesin FimH from uropathogenic *E. coli* binding to mannose-oligosaccharides is a vaccine target. Mucosally administered vaccines containing FimH are in clinical trials that will assess their efficacy compared with parenterally administered vaccines. Furthermore, the recently approved acellular pertussis vaccine also contains adhesins, i.e., the filamentous hemagglutinin and pertactin, which recognize sulphated sugars on glycoconjugates and the integrin-binding protein motif Arg-Gly-Asp, respectively. This indicates that adhesin-specific immunity might be a successful approach for generating mucosal protection against pathogens (43). The importance of blocking the initial attachment and entry into the host cell has been recognized for some time for viruses such as influenza, but the use of this approach for bacteria, still in its infancy, has enormous potential for mucosal vaccines.

Future Directions

The mucosal immune system is a complex and redundant system that generates large amounts S-IgA as well as cell-mediated immunity at mucosal surfaces to prevent pathogen infiltration and inflammation. The mucosal immune system should be most efficient in providing protection against pathogens and generating longer-lasting protection through using attenuated pathogens for vaccines purposes. The only mucosal vaccines approved for humans are attenuated pathogens. Future mucosal vaccines will also involve vaccine strategies other than attenuated pathogens. For example, DNA vaccines or subunit vaccines, such as bacterial adhesins, in combination with potent mucosal adjuvants (such as QS-21, a saponin derived from the bark of the South American tree *Quillaja saponia Molina*, mutant enterotoxins, unmethylated CpG motifs, or cytokines such as IL-12) or mucosal delivery systems, such as microspheres, will have the potential to be the next generation of vaccines inducing mucosal protection to pathogens in humans.

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Competence of American Robins as Reservoir Hosts for Lyme Disease Spirochetes

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To explore the competence of American robins as a reservoir for Lyme disease spirochetes, we determined the susceptibility of these birds to tickborne spirochetes and their subsequent infectivity for larval vector ticks. Robins acquired infection and became infectious to almost all xenodiagnostic ticks soon after exposure to infected nymphal ticks. Although infectivity waned after 2 months, the robins remained susceptible to reinfection, became infectious again, and permitted repeated feeding by vector ticks. In addition, spirochetes passaged through birds retained infectivity for mammalian hosts. American robins become as infectious for vector ticks as do reservoir mice, but infectivity in robins wanes more rapidly.

Lyme disease spirochetes (*Borrelia burgdorferi* s.l.) perpetuate in cycles involving rodent reservoir hosts, such as white-footed mice (*Peromyscus leucopus*) in eastern North America (1) and *Apodemus* spp. mice in Eurasia (2). Such hosts readily become infectious to vector ticks and appear to remain so lifelong. They serve as natural reservoirs of infection because numerous subadult vector ticks parasitize them, they are abundant, and they remain in constant residence in tick-infested sites. Rats (*Rattus norvegicus* and *R. rattus*) (3,4) and European dormice (*Glis glis* and *Eliomys quercinus*) (5,6) are similarly competent and locally important hosts to these pathogens. Although hares (*Lepidus timidus*) support infection with Lyme disease spirochetes in Europe (7), the competence of rabbits for these spirochetes has not been proven. A related spirochete, *B. andersonii*, appears to be specific to American rabbits (8,9). Ungulates, however, are not competent as hosts for Lyme disease spirochetes. Deer in intensely enzootic sites fail to infect vector ticks (10,11), and cattle and sheep exposed to infected vector ticks never become infectious (12). Lyme disease spirochetes infect diverse mammals, but not all of them serve as competent hosts.

Certain birds may also contribute to the transmission of Lyme disease spirochetes. Numerous vector ticks parasitize birds in nature, including spirochete-infected larval ticks that presumably acquired infection from these birds (13-19). Seabirds appear to maintain such a cycle on Arctic islands (20). In an enzootic site, American robins (*Turdus migratorius*) are considered to be an avian candidate as a reservoir of infection because they are locally abundant, forage in ground and brush vegetation, and are frequently infested by vector ticks (21). Catbirds (*Dumetella carolinensis*) in enzootic sites, however, appear not to infect vector ticks (22). Although spirochetes have been isolated from naturally infected European blackbirds (*Turdus merula*) (15), a laboratory study failed to demonstrate reservoir competence of these birds (23); the reason for this discrepancy remains unclear. Nymphal vector ticks occasionally become infected after feeding on spirochete-exposed pheasants (*Phasianus colchicus*); these birds, however, cannot contribute to transmission because larval ticks seem not to feed on them, either in the laboratory or in nature (24,25).

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Chickens (*Gallus gallus*) become infectious, but only transiently and only when they are about a week old (26). Spirochetes inoculated by syringe can be detected in various tissues of canaries (*Serinus canaria*), bobwhite quail (*Colinus virginianus*), and Japanese quail (*Coturnix coturnix*) (27-29). The competence of candidate reservoir birds as hosts for tickborne spirochetes has not been thoroughly evaluated.

Tickborne Lyme disease spirochetes may readily infect certain birds, and these birds may subsequently infect numerous vector ticks. Therefore, we determined the susceptibility of American robins to tickborne spirochetes and their subsequent infectivity for larval deer ticks (*Ixodes dammini*, which differ from *I. scapularis* [30]). American robins are suitable candidate hosts because they are infested naturally by numerous vector ticks. We evaluated the reservoir competence of captive robins and determined whether they could subsequently be reinfested and reinfected by vector ticks and whether spirochetes passaged through birds remain infective to rodents.

Materials and Methods

American robins were captured in mist nets in Brookline, MA (U.S. Fish and Wildlife Scientific Collecting Permit# MB719506-0), and maintained at 20°C with a photoperiod of 16:8 hrs (L:D) (Animal Welfare Assurance# A-3431-01). To establish that these birds had not already been infected by Lyme disease spirochetes, nymphs derived from xenodiagnostic larval deer ticks that had engorged on them were examined by dark-field microscopy. No spirochete-infected ticks were found. The birds were held in captivity for 6 months before they were exposed to infected ticks.

The colony of laboratory-raised deer ticks was originally isolated from ticks captured in Ipswich, MA, and was maintained by feeding adults on rabbits and subadults on outbred laboratory mice. To infect ticks, larvae were fed on outbred laboratory mice infected with the N40 strain of *B. burgdorferi* s.s. The resulting infected nymphal ticks were used to infect the robins. Xenodiagnosis was used to determine whether host animals had become infected by spirochetes; larval ticks were permitted to feed on them and the resulting nymphs were examined for spirochetes by dark-field microscopy. The larval ticks used for the xenodiagnosis were in their third generation of continuous laboratory breeding and had not previously been exposed to spirochete-infected hosts. Engorged ticks were held at 20±2°C over supersaturated $MgSO_4$ in sealed desiccator jars with 16 hours of light per day. Hatched or molted ticks remained in screened vials until they were placed on hosts 2-4 months later.

To infest the robins with subadult ticks, each bird was restrained while ticks were brushed onto its head and neck. Each bird was then placed in a cotton bag suspended over water and kept in the dark for approximately 3 hours to limit their activity. The birds were then caged individually over pans of water in cages with wire-mesh floors and fronts (Safeguard Products, New Holland, PA). The contents of the pans were inspected twice a day, and detached ticks were removed promptly. The birds were exposed three times to nymphal ticks to test their susceptibility to repeated feeding (Figure 1). The first and last of these infestations also served to infect them. Xenodiagnostic larval ticks were permitted to feed on these robins nine times during the study.

Figure 1. American robins with attached nymphal deer ticks (*Ixodes dammini*).

Results

To evaluate the reservoir competence of birds for Lyme disease spirochetes, we exposed each of four American robins to the bites of 12 spirochete-infected nymphal deer ticks and determined the duration of their subsequent infectivity for larval deer ticks. The presence of spirochetes in these infecting nymphs was verified by dark-field microscopic examination of the gut contents of the resulting adults; virtually all (97%) contained spirochetes. Birds were exposed initially to

xenodiagnostic larvae at the same time they were exposed to the infecting nymphs. Xenodiagnosis was repeated after two days and at intervals for six months. Although larval ticks feeding simultaneously with the infected nymphs failed to acquire spirochetes, two birds infected xenodiagnostic larvae within 6 days after exposure to infected nymphs (Figure 2). All four birds were infectious to xenodiagnostic ticks that

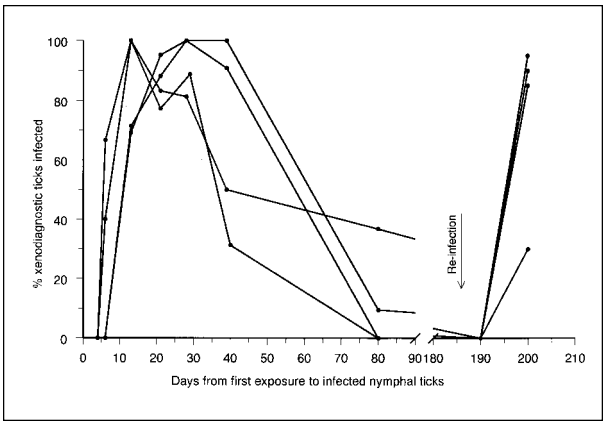

Figure 2. Infectivity to larval vector ticks of four American robins exposed to nymphal deer ticks infected with Lyme disease spirochetes on days 0 and 186. Each observation was recorded on the day on which each xenodiagnostic test was complete.

detached 12 days after exposure and infected 88% (±6.6) of ticks for at least another 3 weeks (Figure). Infectivity waned by 2 months and disappeared by 6 months. Almost all the ticks became infected that fed 2 and 4 weeks after the birds were infected (Table 1). American robins are fully but transiently competent as hosts for Lyme disease spirochetes.

We then determined whether American robins tolerate reinfection by tickborne spirochetes. Six months after they were initially infected, when vector ticks no longer acquired spirochetes from them, eight infected nymphs were permitted to engorge on each bird. All four robins regained infectivity for ticks within the next 2 weeks (Figure 2). Virtually all xenodiagnostic larval ticks that fed on three of these reinfected birds acquired spirochetes, and a third of them did so on the fourth bird. Although spirochete-infected robins only transiently infect vector ticks, the birds remain tolerant to reinfection and become infectious again.

Table 1. Infectivity to larval deer ticks of four American robins infected with tickborne Lyme disease spirochetes

Days from exposure until xenodiagnosis[a]	Xenodiagnostic ticks No. examined	% infected	± SE[b]
0-5	16	0	0
6-9	28	28.6	16.5
12-15	52	84.6	7.0
20-23	78	85.9	6.0
28-30	62	91.9	6.7
39-41	50	68.0	20.3
79-82	65	13.8	10.2
189-191[c]	32	0	0
199-201	80	75.0	15.2

[a]Each interval indicates the days on which xenodiagnostic ticks detached from their hosts.
[b]SE = standard error
[c]Birds were exposed again to infected nymphal ticks on day 186.

The ability of nymphal deer ticks to reinfest American robins was evaluated by recording their feeding success. On initial exposure, 96% of 48 nymphs feeding on the four birds engorged successfully; 98% of 40 nymphs engorged 3 weeks later; and 82% of 32 nymphs engorged 6 months later, when the birds were exposed for the third time. The robins appear to freely tolerate repeated feeding by nymphal deer ticks.

To determine whether spirochetes passaged through birds retain infectivity for mice, we permitted nymphs that had acquired infection as larvae from birds to feed on mice. The ticks that were used had acquired spirochetes 3 weeks after their avian hosts had been infected. Each of four outbred laboratory mice was exposed to six of the resulting nymphs. Two weeks later, 10 xenodiagnostic larval ticks were permitted to engorge on each of these mice. All but one of the 40 resulting nymphal ticks acquired spirochetes (Table 2). Spirochetes, therefore, remain infectious to mammals after a tickborne passage through birds.

Discussion

A competent reservoir host for the agent of Lyme disease readily acquires infection from vector ticks, permits spirochetes to proliferate, and readily infects vector ticks. In white-footed mice, infection generally becomes established after a single feeding by an infectious tick, and more than half retain infection for approximately 6 months (31). As many as three-quarters of the

Table 2. Infectivity for laboratory mice of Lyme disease spirochetes that had previously infected American robins

	Avian host					Murine host			
	Infecting nymphs		Xenodiagnostic ticks[a]			Infecting nymphs		Xenodiagnostic ticks	
Bird no.	No. ticks	% engorged	No. examined	% infected	Mouse no.	No. ticks	% engorged	No. examined	% infected
1	12	83.3	18	83.3	1	6	100	10	90
2	12	100	21	95.2	2	6	83.3	10	100
3	12	100	22	77.3	3	6	100	10	100
4	12	100	17	88.2	4	6	83.3	10	100

[a]Xenodiagnostic ticks that had engorged on each bird were used to infect the corresponding mouse.

larval ticks that feed on such mice acquire infection, which subsequently wanes. Hamsters are similarly infectious (32). The various murids, glirids, voles, and sciurids that have been tested appear as reservoir-competent for spirochetes as are cricetids (2,5,6,33). Although certain genospecies of the Lyme disease spirochetes are said to be more mouse-adapted than others (34), no experimental evidence is available to support this concept. Rodents, in general, are readily infected by Lyme disease spirochetes, and most remain infectious to vector ticks for at least 6 months.

The standard of proof that has been applied to rodent reservoirs of spirochetal infection has not previously been applied to candidate avian reservoirs. Certain birds, including quail and canaries, can readily be infected by syringe-inoculated spirochetes. Spirochetal DNA generally becomes detectable in the viscera (27-29), and viable spirochetes can be cultured from these tissues. Tickborne spirochetes infect pheasants (24) and week-old chickens (26). About a third of these chicks infect ticks, but they remain infectious for only a week. Adult pheasants infect about a quarter of vector ticks that engorge on them. European blackbirds appear not to be susceptible to tickborne infection (23). American robins, however, appear far more reservoir-competent. Robins bitten by spirochete-infected nymphal ticks subsequently infect larval ticks, and virtually all larvae become infected after feeding on these birds throughout the following month. Infectivity declines, however, and few ticks acquire infection from these birds after approximately 3 months. Certain, but not all, bird species can readily be infected by Lyme disease spirochetes and subsequently become highly but transiently infectious.

A competent reservoir host tolerates feeding by both subadult stages of vector ticks. Although nymphs feed readily on pheasants, larvae seldom attach to these birds, either in nature or in the laboratory (24). About as many nymphal as larval deer ticks attach naturally to American robins (21). Several times as many larvae as nymphs, however, feed on white-footed mice in nature (35). Nymphal vector ticks attach more readily to rats, dormice, or the American reservoir host (*P. leucopus*) than to European mice (*Apodemus* spp.) or voles (2,3,5,31). Perhaps particular subadult stages of vector ticks are more readily attracted to certain vertebrate animals than to others.

To be competent as reservoir hosts for Lyme disease spirochetes, an animal must tolerate repeated feedings by vector ticks. White-footed or laboratory mice serve as hosts to deer ticks almost as readily during their fifth exposure as their first (36). Voles are much less tolerant (37), and rabbits (unpub. obs.) and guinea pigs (38) seldom tolerate more than one episode of attachment. Vector ticks feed repeatedly on American robins as successfully as on the natural rodent reservoirs of the Lyme disease spirochete.

Spirochete-infected larval ticks frequently are taken from birds in Lyme disease-enzootic areas. Of 20 species of North American passerine birds that were parasitized by larval vector ticks, 45% hosted spirochete-infected larvae (13,18). Spirochetes infected 10% to 40% of these batches of infected ticks. In Europe, 81% of 16 bird species harbored infected ticks; of these cohorts, 11% to 75% of the larval ticks were infected (17). Because Lyme disease spirochetes infect <1% of questing larval vector ticks captured in nature and because inherited infection is exceedingly rare (39,40), these larval ticks most likely acquired spirochetes from their avian hosts. Nevertheless, the avian host-range of Lyme disease spirochetes remains to be defined.

An effective reservoir of infection for a pathogen would generate for each primary

infection at least as many secondary infections. The contribution of various alternative reservoir hosts for Lyme disease spirochetes, however, can be compared by estimating the proportion of infections in nymphal vector ticks that derive from a particular vertebrate population. This calculation includes estimates of feeding density of vector ticks on the candidate reservoir host, its local density, and its prevalence of infection (2,5). At least twice as many vector ticks appear to acquire infection from a population of edible dormice (*Glis glis*) as from other rodents locally abundant in Central Europe (5). Similar evidence implicates white-footed mice in eastern North America (1). Information that would permit comparable estimates of the reservoir significance of local populations of birds, however, remains unavailable. Thus, it is still uncertain whether a bird, although reservoir-competent for the spirochete, may serve as an important reservoir in nature.

Because the prevalence of Lyme disease spirochetes in American robins has not yet been determined for a representative enzootic site, the importance of these birds as reservoir hosts remains speculative. Robins may contribute to the force of transmission of the agent of Lyme disease, because these locally abundant birds are reservoir-competent and may be infested by numerous larval ticks. Of the larvae that feed on peridomestic birds in North American enzootic sites, approximately three-quarters appear to engorge on robins (21). Although they may be about as abundant as white-footed mice, robins appear to forage more frequently on lawns than do mice (21). The contribution of robins to the peridomestic risk for Lyme disease may depend on the ability of engorged larval ticks detaching from the birds to develop in such habitat. Questing nymphal ticks appear less prevalent on lawns than on other vegetation in residential enzootic sites (41). Birds are more vagile than mice, which might dilute risk by diffusing infected ticks into sites in which transmission is unlikely. Alternatively, their vagility might seed new foci of transmission, if ticks detach in sites that are suitable for their development. Certain migratory passerines have been associated with long-distance dispersal of vector ticks (17-19). Robins may contribute to the emergence of Lyme disease in previously unaffected sites to the extent that the season of their migration overlaps with that of the activity of subadult vector ticks. Our finding that American robins are reservoir-competent for Lyme disease spirochetes warrants further epizootiologic studies, including estimates of the prevalence of spirochetes in these birds in nature.

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Dr. Richter is a postdoctoral fellow conducting joint research in the laboratories of Dr. Matuschka at the Charité Medical School, Humboldt-Universität zu Berlin, and Dr. Spielman, at the Harvard School of Public Health. Her research interests focus on the immunologic and molecular interface of the host-vector-pathogen relationship in the epizootiology of Lyme disease.

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Vibrio cholerae O139 in Calcutta, 1992-1998: Incidence, Antibiograms, and Genotypes

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We report results of surveillance for cholera caused by *Vibrio cholerae* O139 from September 1992, when it was first identified, to December 1998. *V. cholerae* O139 dominated as the causative agent of cholera in Calcutta during 1992-93 and 1996-97, while the O1 strains dominated during the rest of the period. Dramatic shifts in patterns of resistance to cotrimoxazole, neomycin, and streptomycin were observed. Molecular epidemiologic studies showed clonal diversity among the O139 strains and continuous emergence of new epidemic clones, reflected by changes in the structure, organization, and location of the CTX prophages in the *V. cholerae* O139 chromosome.

Vibrio cholerae, the gram-negative organism that causes cholera, is well defined on the basis of biochemical tests and DNA homology studies (1), but the serogroups of the species differ in their pathogenic potential. Of the 193 recognized "O" serogroups of *V. cholerae* (2), only O1 and O139 cause epidemic and pandemic cholera. *V. cholerae* O139 was first identified in September 1992 in southern India (3) and rapidly spread to all cholera-endemic areas in India (4) and neighboring countries (5). In February 1994, a new clone of *V. cholerae* O1 El Tor biotype (6) replaced the O139 serogroup as the dominant serogroup causing cholera in Calcutta (7). After a 33-month quiescent period, a new clone of *V. cholerae* O139 (8,9) appeared in August 1996 in Calcutta (10) and was the dominant serogroup until September 1997. This new clone has also spread to other parts of India (11).

The National Institute of Cholera and Enteric Diseases, Calcutta, conducts continuous surveillance for cholera in Calcutta in particular, and in India in general. During September-October 1998, we observed an increase in the incidence of O139 cholera, prompting study of these O139 strains. We report the findings of surveillance performed from September 1992 to December 1998 in India, which enabled us to track and catalog changes in the O139 strains since its identification.

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The Study

Hospital Surveillance and Bacteriology

Stool specimens were obtained from patients admitted to the Infectious Diseases Hospital, Calcutta, the only hospital that admits cholera patients from the city and its suburbs. Since 1995, on two randomly selected days per week, every fifth patient has been enrolled from all patients with diarrhea or dysentery, with or without other complaints, visiting the emergency department of the Infectious Diseases Hospital, Calcutta. Before 1995, only patients with diarrhea admitted to the Infectious Diseases Hospital, Calcutta, from Monday to Friday between 9 a.m. and 1 p.m. were enrolled. Methods of collection, transport, and bacteriologic examination of stool samples and identification and serotyping of *V. cholerae* have been described (7). The National

Institute of Cholera and Enteric Diseases, Calcutta, is India's national reference laboratory for cholera, and strains from throughout the country are sent here for confirmation and phage typing. Strains received are characterized by an array of tests (4). In this study, representative strains of *V. cholerae* O139 isolated from hospitalized patients in Calcutta from 1992 to 1998 and 15 *V. cholerae* O139 strains from other parts of India (eastern, central, northern, and southern India) isolated in 1998 were randomly selected for molecular characterization.

Antimicrobial Susceptibility

The *V. cholerae* O139 strains were examined for resistance to ampicillin (10 μg), chloramphenicol (30 μg), cotrimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (100 μg), gentamycin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg), and tetracycline (30 μg) by using antibiotic-impregnated commercial disks (Hi Media, Mumbai, India). Characterization of the strains as susceptible or resistant was based on the size of inhibition zones around each disk, according to the manufacturer's instructions, which followed World Health Organization recommendations (12). Strains showing an intermediate zone of inhibition were interpreted as resistant on the basis of previous minimum inhibitory concentration studies conducted with *V. cholerae* (13).

Polymerase Chain Reaction (PCR) Assay

PCR assays (both multiple and single) were used to screen the O139 strains for five important virulence genes. The first PCR assay used three pairs of oligonucleotide primers for virulence genes *ctxA* (301 bp), *tcpA* (classical; 617 bp), and *tcpA* (El Tor; 471 bp), and the second and third PCR assays used primer pairs for the virulence genes *zot* and *ace*, respectively (Table 1) (15). The composition of the reaction mix and cycling conditions for amplification have been described (16).

Amplification was done by using an automated thermal cycler (Biometra Gottingen, Germany). *V. cholerae* strain VC20 (El Tor, Ogawa), 569B (classical, Inaba) and *Escherichia coli* DH5-α strains were used as positive and negative controls. Amplified products were electrophoresed in a 2% agarose gel (SRL, Bombay, India) in 1x TAE buffer along with standard molecular weight markers (MWM), stained with ethidium bromide (Sigma), and recorded with a video documentation system (Pharmacia Biotech, San Francisco, CA).

Table 1. Oligonucleotide primer sequences used in polymerase chain reaction assays of *Vibrio cholerae* O139

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Ref.
*ctx*A	CTCAGACGGGATTTGTTAGGCACG TCTATCTCTGTAGCCCCTATTACG	301	14
*tcp*A (El Tor)	GAAGAAGTTTGTAAAAGAAGAACAC GAAGGACCTTCTTTCACGTTG	471	14
*tcp*A (classical)	CACGATAAGAAAACCGGTCAAGAG ACCAAATGCAACGCCGAATGGAG	617	16
ace	GCTTATGATGGACACCCTTTA TTTAACGCTCGCAGGGC	282	15,[a]
zot	GGGCGAGAAAGGACGC CCTTGTAGCGGTAGCTCG	834	[a]

[a]Referred to in this study.

Pulsed-Field Gel Electrophoresis (PFGE)

Genomic DNA of various strains of *V. cholerae* was prepared in agarose plugs (17). For complete digestion of the DNA, 50U of *Not*I enzyme was used. PFGE of inserts was done by the contour-clamped homogeneous electric field method on a CHEF-mapper (Bio-Rad) with 0.5x TBE Buffer [44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA (pH 8.0)] for 40.24 hours. A DNA size standard (λ-ladder; Bio-Rad) was used as molecular weight standard. A model 1,000 minichiller (Bio-Rad) was used to maintain the temperature of buffer at 14°C. Run conditions were generated by the auto-algorithm mode of a CHEF Mapper PFGE system with a size range of 20-300 kb. Gels were stained in distilled water containing 1.0 μg ethidium bromide per ml for 30 minutes, rinsed several times, and photographed. After electrophoresis, ethidium bromide staining and photography, transfer of DNA from gel to Hybond N^+ membrane (Amersham International PLC, Buckinghamshire, England) and Southern blot hybridization with an O139-specific probe were done as described (17).

The DNA adjacent to Tn5*lac* insertion in MO10 (an O139 strain from the Madras outbreak) rendered the strain O139 negative in agglutinability with O139-specific antiserum. The O139 probe used in this study was prepared as described earlier (18).

Restriction Fragment Length Polymorphism (RFLP) of *ctxA* Gene and O139 Specific Gene

A *ctxA* probe consisting of a 540-bp *Xba*I-*Cla*I fragment of ctxA cloned in pKTN901 with *Eco*RI

linkers was used (19). A modification of the method of Murray and Thompson (20) was used for DNA extraction for *ctx* RFLP (16). The transfer of DNA from gel to Hybond N^+ membrane (Amersham) and hybridization with probes was done (16) with the ECL Nucleic Acid Detection System (Amersham). The membranes were then washed and exposed to Kodak Biomax film (Eastman Kodak Co., Rochester, NY) and developed according to manufacturer's instructions.

Results

The O139 serogroup dominated as the major cholera-causing serogroup in 1993 and from September 1996 to September 1997, while the O1 serogroup dominated during the remaining part of the study period (Figures 1, 2). The rate of isolation of O139 serogroup was lower in 1998 than in any other year since 1992, the year it was identified (Table 2). The seasonality in incidence of the O139 serogroup showed an interesting trend: apart from 1993 and 1994, more O139 cholera cases were recorded during the latter half of the year, coinciding with the second peak of cholera cases (Figures 1, 2). However, the peak incidence of cholera caused by the O1 serogroup was generally observed during June-July, except for 1993 and 1997, when the O1 peak occurred during October and September, respectively (Figures 1, 2). The O139 strains were the dominant cholera-causing serogroup during these years (Table 2; Figures 1, 2).

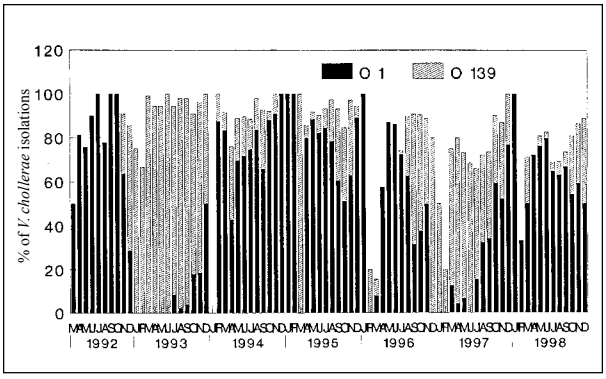

Figure 2. Monthly isolation profile of the percentage of *V. cholerae* O1 and O139 serogroups from patients hospitalized with acute secretory diarrhea at the Infectious Diseases Hospital, Calcutta, India, from March 1992 to December 1998.

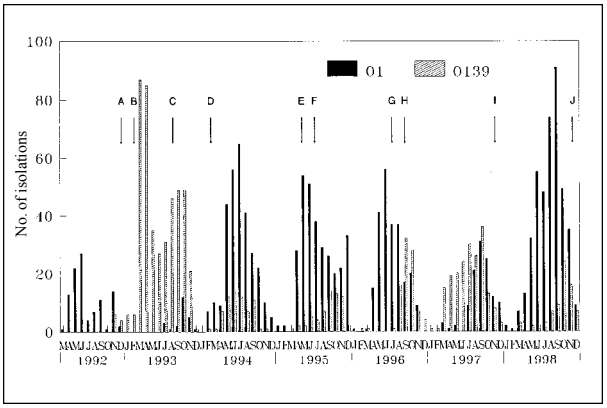

Figure 1. Monthly isolation profile of the *Vibrio cholerae* O1 and O139 serogroups from patients hospitalized with acute secretory diarrhea at the Infectious Diseases Hospital, Calcutta, India, from March 1992 to December 1998. Arrows denote the month in which changes in *V. cholerae* strains were noted: A, appearance of *V. cholerae* O139 in Calcutta in November 1992; B, displacement of *V. cholerae* O1 by *V. cholerae* O139 in January 1993; C, reappearance of *V. cholerae* O1; D, domination of *V. cholerae* O1 over *V. cholerae* O139; E, isolation of nontoxigenic *V. cholerae* O139 in April 1995; F, appearance for the first time of cotrimoxazole-susceptible strains of *V. cholerae* O139; G, appearance of cotrimoxazole-susceptible and neomycin-resistant strains of *V. cholerae* O139; H, domination of *V. cholerae* O139 over *V. cholerae* O1; I, replacement of *V. cholerae* O139 by *V. cholerae* O1 as the major cholera causing serogroup; J, appearance of cotrimoxazole- and streptomycin-susceptible strains of *V. cholerae* O139.

The antibiotic resistance patterns of randomly selected O139 strains isolated from Calcutta during 1992-1998 (Figure 3) showed dramatic changes in resistance to cotrimoxazole, with all the 1992, 1993, and 1994 O139 strains being resistant, while most of the strains isolated during 1997 and 1998 were sensitive. Likewise, resistance to neomycin and streptomycin also increased until 1996, then declined. O139 strains isolated throughout the study were resistant to furazolidone and most were resistant to ampicillin. The dominant drug-resistance patterns among O139 strains isolated in Calcutta from 1993 to 1998 were A C Co Fz S (30%), A C Co Fz S (48.1%), A Co Fz S (49.1%), A Fz N S (96%), A Fz N S (32%), and A Fz (98.3%).

PCR studies with 106 representative O139 strains showed that all but one strain (CO853)

Table 2. Isolation rates of *Vibrio cholerae* O139 from patients with acute secretory diarrhea admitted to the Infectious Diseases Hospital, Calcutta, 1992–1998

Year	No. of samples screened	No. (%) of samples positive for *V. cholerae*			
		Total positive	O1	O139	Non-O1
1992	359	131 (36.5)	102 (28.4)	10 (2.8)	19 (5.3)
1993	890	491 (55.2)	24 (2.7)	443 (49.8)	24 (2.7)
1994	748	400 (53.5)	296 (39.7)	67 (8.9)	37 (4.9)
1995	873	399 (45.7)	305 (34.9)	62 (7.1)	32 (3.7)
1996	1746	399 (22.9)	234 (13.4)	62 (3.6)	103 (5.9)
1997	1643	397 (24.2)	114 (6.9)	196 (11.9)	87 (5.3)
1998	2118	642 (30.3)	416 (19.7)	73 (3.4)	153 (7.2)

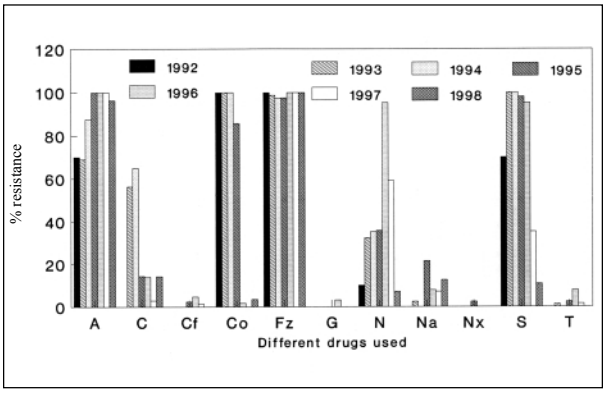

Figure 3. Antibiotic resistance pattern of the *V. cholerae* O139 strains. Abbreviations: A, ampicillin; C, chloramphenicol; Cf, ciprofloxacin; Co, cotrimoxazole; Fz, furazolidone; G, gentamycin; N, neomycin; Na, nalidixic acid; Nx, norfloxacin; S, streptomycin; T, tetracycline.

were positive for the 471-bp *tcpA* (El Tor) amplicon, indicating that almost all have the organelle required for intestinal colonization. The presence of 301-bp *ctxA*, 843-bp *zot*, and 282-bp *ace* amplicons in all strains except CO853 indicates that all the *tcpA*-positive strains have an intact CTX prophage.

The PFGE profile of three randomly selected O139 strains, sharing the unique $CTX^{ImmCalcutta}$ prophage and isolated from Calcutta during 1996 and 1997, has a banding pattern similar to that of the reference strain MO45 (ATCC 51394) isolated during 1992 in Madras (Figure 4A). The band patterns exhibited by all the O139 strains differed from that of the classical and El Tor biotype representative strains of *V. cholerae* O1, 569B, 2164-88, and CO840. However, the pattern of the reference O139 strain MO45 was identical to that of the O1 strain MO1, which was described as the progenitor strain of the O139 serogroup (21). The Southern blot of the PFGE DNA fragments with O139-specific probe showed that the probe hybridized at the same position in all the O139 strains. However, the control O1 strains and the progenitor strain MO1 did not hybridize with the probe (Figure 4B).

RFLP was then conducted to determine any differences in number, location, and arrangement of the CTX prophage in the genome of O139 strains. As the CTX prophage of the 1992-93 and 1996 O139 strains is well characterized (8,11,16,22), we took only one representative strain from 1992 (SG24) and 1996 (AM258) (8,11,22). Five O139 strains (PG265, PG269, PG316, PG337, and PG351) isolated during September 1998 (when an upsurge in O139 strains was observed) were included in the study. RFLP of the CTX prophage with *Hin*dIII showed one band with SG24, PG265, PG269, PG316, and PG351; AM258 and PG337 had three bands of 14, 9.2, and 7.0 kb (Table 3). As *Hin*dIII has no site in the CTX prophage (23), four of the five 1998 O139 strains (PG265, PG269, PG316, and PG351), like the 1992 O139 strain (SG24), have the CTX prophage at only one site in the chromosome. RFLP with *Pst*I and *Bgl*II showed only one band each of 5.6 kb and 8 kb, respectively, in four of the 1998 O139 strains (PG265, PG269, PG316, and PG351), while the 1992 O139 strain (SG24) exhibited two bands of sizes 7.0 and 5.6 and 8.0 and 7.0 kb, respectively (Table 3). As *Pst*I and *Bgl*II have only a single site in the CTX prophage but not within the *ctxA* gene, these results indicate that most of the 1998 O139 strains have only one CTX prophage, unlike the 1992 O139 strain, which has two CTX prophages arranged in tandem (Figure 5). Further, as both the 8- and 5.6-kb bands are shared by the 1992 and 1998 *V. cholerae* O139 strains, the downstream regions of the CTX prophage in the 1992 and 1998 *V. cholerae* O139 strains are 97% similar,

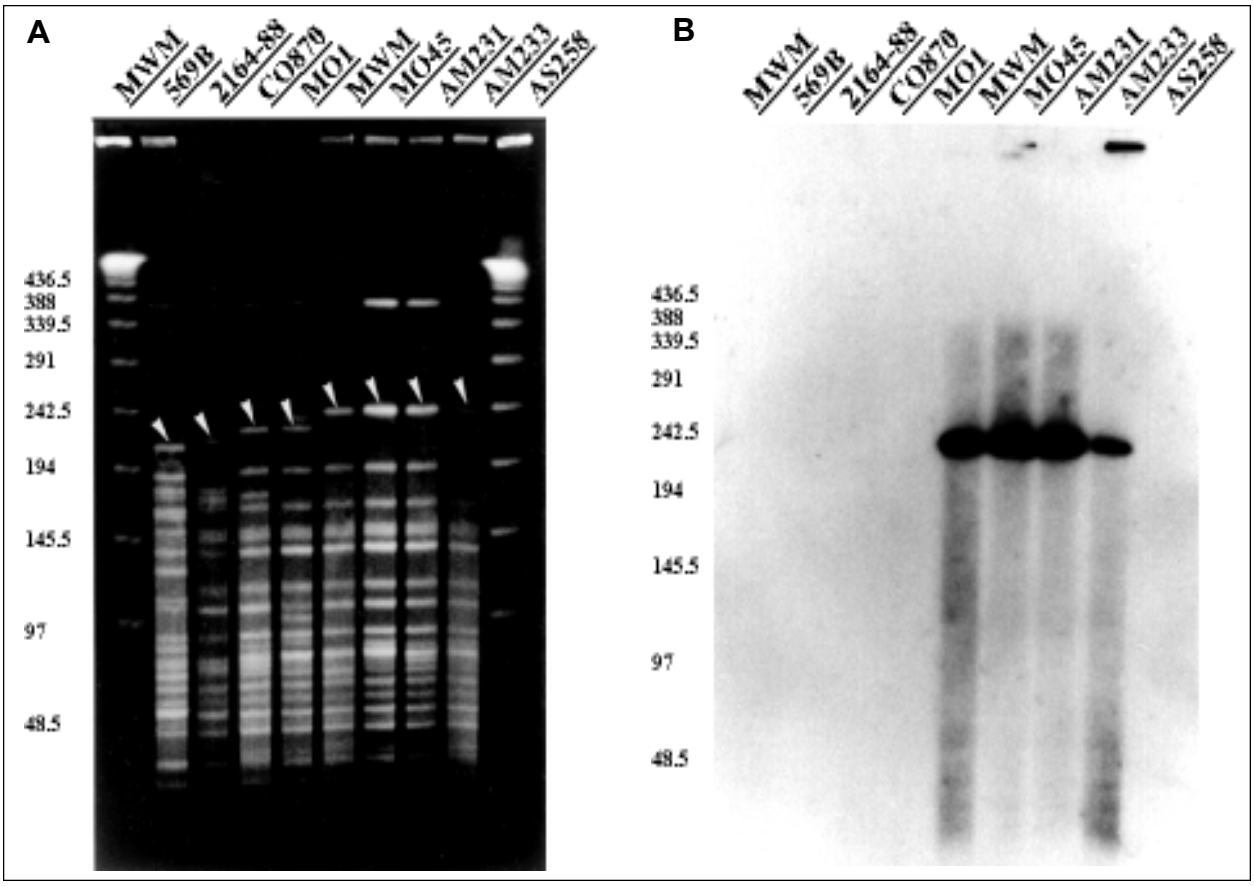

Figure 4. Pulsed-field gel electrophoresis profiles of the *V. cholerae* O139 strains (A) and Southern blot of the same gel with O139-specific probe. (B). The numbers at the top indicate the strain numbers. Lanes 1, MWM (bacteriophage λ ladder); 2, 569B (O1 classical Inaba); 3, 2164-88 (O1 El Tor Ogawa); 4, CO870 (O1 El Tor Ogawa); 5, MO1 (progenitor of the O139 strain); 6, MO45 (O139 strain isolated during 1992-93); 7, AM231 (O139 strain isolated during 1996-97); 8, AM233 (O139 strain isolated during 1996-97); 9, AS258 (O139 strain isolated during 1996-97); 10, MWM (bacteriophage λ ladder).

Table 3. Fragment sizes of restriction enzyme-digested genomic DNA of *Vibrio cholerae* O139 strains, isolated by *ctxA* probe, Calcutta, 1992-1998

Strain	Yr. isolated	Antibiogram	Fragment (kb) hybridized with the probe		
			*Hin*dIII	*Bgl*II	*Pst*I
SG24	1992	CoFzS	23	8.0, 7.0	9.3, 5.6
AM258	1996	ACCfFzNS	14.0, 9.2, 7.0	23.0	7.0, 5.6
PG265	1998	AFz	18.0	8.0	5.6
PG269	1998	AFz	18.0	8.0	5.6
PG316	1998	AFz	18.0	8.0	5.6
PG337	1998	AFzNS	14.0, 9.2, 7.0	23	7.0, 5.6
PG351	1998	AFz	16.0	8.0	5.6

suggesting that they may share the identical location in the *V. cholerae* genome. Southern blot of *Hin*dIII-digested genomic DNA, using a *ctxA* probe of the 1996 O139 strain (AM258), showed three bands, and the Southern blots of the *Pst*I- and *Bgl*II-digested genomic DNA, also using the *ctxA* probe, showed only two bands. As discussed (8,16), the strain has the same organization of the CTX prophage as the 1996-97 O139 strains isolated from Calcutta. It has three tandemly duplicated CTX prophages, with the second and third CTX prophages being new and differing from the first in having an altered restriction endonuclease site (8,9) (Figure 5). The Southern blots of the 1998 O139 strain PG337 exactly match those of the 1996-97 representative strain AM258 (Table 3), indicating that its structure resembles that of the 1996-O139 strains.

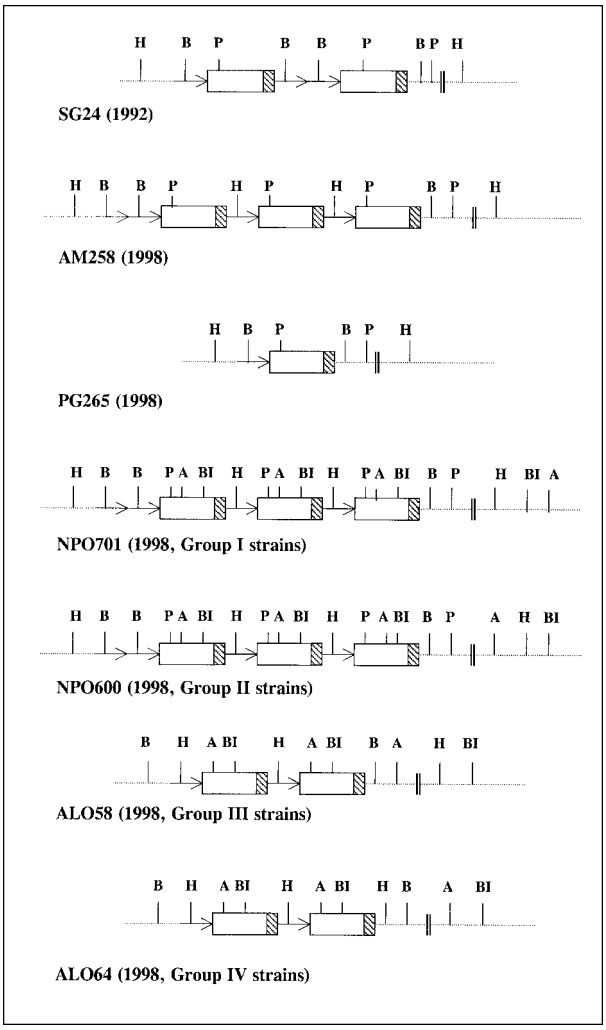

Figure 5. Schematic diagram of CTX prophage (not to scale) as deduced from Southern blot hybridization data. Arrows and boxes correspond to the restriction sites and core regions of CTX prophage, respectively, with hatched portions in the box representing *ctxAB* genes. Restriction site abbreviations: A, *Ava*I; BI, *Bgl*I; B, *Bgl*II; H, *Hin*dIII; P, *Pst*I.

Southern blot analysis of *Hin*dIII-digested genomic DNA of the 15 O139 strains isolated from different parts of India in 1998 showed four variations when probed with *ctxA*. Of the 15 strains examined, eight (designated group I strains) showed three bands of size 14, 9.2, and 7.0 kb (the pattern displayed by the 1996-97 O139 strains); two strains had three fragments of sizes 14, 12, and 7.0 kb (designated group II strains); and four had two fragments of sizes 23 and 7.0 kb (designated group III strains); only one had two bands of 14 and 7.0 kb (designated group IV strains) (Table 4). All group I and group II strains showed a single band of about 23 kb in the blot when digested with *Bgl*II and probed with *ctx*A (Table 4), indicating that in these strains the *Bgl*II site resides outside the CTX prophage, as described (8). Thus, group I and group II strains have the same unique organization of the CTX prophage as that of the 1996-97 O139 strains (8). The group III and group IV strains also showed a single band when digested with *Bgl*II and probed with *ctxA*, indicating that in these strains the *Bgl*II site may also be outside the CTX prophage (Table 4). Representative strains of the four types showed a common band of approximately 7 kb when digested with *Bgl*I, *Ava*I, and *Pst*I and probed with the *ctxA* probe (Table 4). Since all these enzymes have a single restriction site in the CTX prophage but not within the *ctxA* gene (23) and the size of an intact CTX prophage is approximately 7 kb, the 7-kb band in all these enzymes indicates a tandem duplication in all strains. All the group I strains have three CTX prophages in tandem (8,16), with the second and third prophages having a *Hin*dIII site instead of the *Bgl*II site in the RS region of the CTX prophage, like the 1996-97 O139 strains (Figure 5). The group II strains differ from the group I strains by the position of the second band in the blots (Table 4). Thus, the group II strains have exactly the same arrangement of the CTX prophage in their genome as the group I strains; the difference in the size of the second band could be due to the integration of the prophage at a different site in the genome (Figure 5).

The group III and group IV strains showed two bands in *Hin*dIII, *Bgl*I, and *Ava*I blots when probed with *ctxA*. There is no *Hin*dIII restriction site in the CTX prophage of El Tor and classical strains (23), while *Ava*I, *Bgl*II and *Bgl*I have only one site within the CTX prophage (23). In the $CTX^{ImmCalcutta}$ prophage, present in the 1996-97 O139 strains, the positions of the *Hin*dIII and the *Bgl*II enzymes have been interchanged (8,9). The presence of two bands in the blots with a common 7.0 kb in *Hin*dIII, *Ava*I, and *Bgl*I blots with *ctxA* and one band in the *Bgl*II blot (Table 4) with same probe indicates that group III and group IV have two copies of the CTX prophage in tandem, with both the CTX prophages in these strains being the new type of the O139 CTX prophage, $CTX^{ImmCalcutta}$ prophage (Figure 5). Thus, these O139 strains (all isolated in southern India) may derive from the 1996-97 O139 strains but lack the

Table 4. Restriction enzyme-digested fragments of genomic DNA of *Vibrio cholerae* O139 strains isolated with the ctxA probe, India, 1998

Strain no.	Place (Region) of isolation	Anti-biogram	Fragment size (kb) using *ctxA* probe				
			*Hind*III	*Bgl*II	*Bgl*I	*Ava*I	*Pst*I
NPO600	Nagpur (Central)	ACFzNaS	14, 12,7	23	23, 7	14, 7	9.5, 7
NPO701	Nagpur (Central)	ACNaS	14, 9.2, 7	23	12, 7	23, 7	7, 5
NPO702	Nagpur (Central)	AFzNaS	14, 12, 7	23	23, 7	14, 7	9.5, 7
NPO706	Nagpur (Central)	ACFzNaS	14, 9.2, 7	23	12, 7	23, 7	7, 5
NPO708	Nagpur (Central)	AFzNaS	14, 9.2, 7	23	12, 7	23, 7	7, 5
NPO709	Nagpur (Central)	AFzNaS	14, 9.2, 7	23	12, 7	23, 7	7,5
MUO7	Murshidabad (Eastern)	CFzS	14, 9.2, 7	23	12, 7	N.D.	N.D.
LHO359	Ludhiana (Northern)	AFzNaS	14, 9.2, 7	23	12, 7	N.D.	N.D.
LHO469	Ludhiana (Northern)	AFzNS	14, 9.2, 7	23	12, 7	N.D.	N.D.
LHO477	Ludhiana (Northern)	FzNaS	14, 9.2, 7	23	12, 7	N.D.	N.D.
ALO53	Alleppey (Southern)	FzS	23, 7	23	23, 7	23, 7	N.D.
ALO58	Alleppey (Southern)	ACFz	23, 7	23	23, 7	23, 7	N.D.
ALO59	Alleppey (Southern)	AFzNaS	23, 7	23	23, 7	23, 7	N.D.
ALO62	Alleppey (Southern)	FzS	23, 7	23	23, 7	23, 7	N.D.
ALO64	Alleppey (Southern)	ACFzS	14, 7	23	23, 7	23, 7	N.D.

El Tor CTX prophage of the three CTX prophages that they have in tandem. Alternatively, these group III and group IV strains may be a new type of O139 that lacks the El Tor CTX prophage. The only difference between the group III and group IV strains is in the *Hin*dIII blot, which may be due to polymorphism in the *Hin*dIII site (Table 4; Figure 5).

Discussion

The emergence of *V. cholerae* O139 is a puzzling event in the history of cholera. The sudden appearance of the O139 serogroup in late 1992, its rapid spread through Southeast Asia in 1993 followed by a quiescent period during 1994-95 and its subsequent emergence in 1996 and 1997 are inadequately understood, but characterize the unpredictable nature of the epidemiology of cholera. However, the reemergence of O139 in 1996 indicates that its appearance was not a one-time event and the serogroup has the potential to persist and spread to other continents.

Comparison of the O139 strains isolated during the 7-year study period revealed interesting patterns of antibiotic resistance to various common antibiotics. While the strains remained largely susceptible to ciprofloxacin, tetracycline, and gentamycin, resistance to ampicillin and susceptibility to cotrimoxazole (sulphamethoxazole, trimethoprim), chloramphenicol, and streptomycin increased during the period of the study. Resistance to cotrimoxazole (sulphamethoxazole and trimethoprim) and streptomycin is encoded by a 62-kb self-transmissible chromosomally integrated transposon termed the SXT element, which is not apparently linked to genes encoding O139 antigen (24). The SXT element was present in 1992-93 O139 strains and is probably silent or absent in 1998 O139 strains, which were sensitive to cotrimoxazole and streptomycin. The presence of this novel region in 1996 O139 strains is difficult to predict because these strains are susceptible to cotrimoxazole (sulphamethoxazole and trimethoprim) but resistant to streptomycin. However, this pattern of rapid shift is consistent with recent reports indicating an enhanced mobility in genetic elements, which confers resistance to antibiotics (25) and has also been observed in *V. cholerae* O1 strains (7). A multiple-antibiotic resistance plasmid belonging to incompatibility group C has been associated with drug-resistance plasmid of *V. cholerae* O139 (17). Since the multidrug-resistance plasmid is self-transmissible by conjugation, the incidence of plasmid-carrying strains and hence drug resistance may change, depending on the presence of these plasmids in O139 strains.

Molecular studies show continuous change in the structure and organization of CTX prophage during the study period, along with evolution of a new type of CTX prophage. The 1992-93 strains have two CTX prophages connected by an RS1 element, while the 1996 O139 strains have three CTX prophages arranged in tandem (8,22). Most of the 1998 O139 strains from Calcutta have only one CTX prophage, while those isolated from other parts of India have either the arrangement

of the 1996-97 O139 strains (group I and II strains) or have two CTX prophages arranged in tandem (group III and IV strains). However, as reported elsewhere (9), the 1996 O139 strains have two types of CTX prophages, with the first of the three an El Tor type CTX prophage and the second and third CTX prophages being a new type of CTX prophage that differs primarily in the *rstR* gene, the gene that codes for the repressor protein of CTX . In 1998, we observed two new clones of O139 at two epicenters, Calcutta and Alleppey. From the restriction profile data of the 1998 O139 strains, it can be predicted that most of the O139 strains from Calcutta have only the El Tor type CTX prophage and not the unique O139 CTX prophage of the 1996 O139 strains, while the reverse is the case with the south Indian (Alleppey) strains. Therefore, two different clones of O139 are circulating at two locations with different types of CTX prophages, indicating that reassortment in the genome is taking place in the O139 strains. Our study indicates a continuous emergence of new clones of toxigenic *V. cholerae*, possibly through natural selection involving unidentified factors and immunity of the host population (25-28). Another possibility is that the genetic reassortments observed here are random changes in the organism. The strains that gained advantage as a result of this rearrangement may have infected humans and become enriched inside the gastrointestinal tract so that they became detectable as new toxigenic strains. Molecular analysis of *V. cholerae* strains isolated during epidemics from 1961 to 1996 in Bangladesh revealed similar clonal diversity among strains isolated during different epidemics (25-29). These studies demonstrated three different ribotypes among the *V. cholerae* O139 isolated from Bangladesh, with different ribotypes often showing different CTX prophage genotypes (26,28).

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Mr. Basu holds a Masters in Biochemistry and is completing his doctoral degree in Microbiology under the supervision of G. Balakrish Nair, Deputy Director of the National Institute of Cholera and Enteric Diseases, the National Reference Centre for Cholera in India. Mr. Basu's doctoral thesis is an in-depth molecular analysis of the CTX genetic element in strains of *Vibrio cholerae* isolated in the last 3 decades, including recent isolates of *V. cholerae* O139.

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Multivariate Markovian Modeling of Tuberculosis: Forecast for the United States

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We have developed a computer-implemented, multivariate Markov chain model to project tuberculosis (TB) incidence in the United States from 1980 to 2010 in disaggregated demographic groups. Uncertainty in model parameters and in the projections is represented by fuzzy numbers. Projections are made under the assumption that current TB control measures will remain unchanged for the projection period. The projections of the model demonstrate an intermediate increase in national TB incidence (similar to that which actually occurred) followed by continuing decline. The rate of decline depends strongly on geographic, racial, and ethnic characteristics. The model predicts that the rate of decline in the number of cases among Hispanics will be slower than among white non-Hispanics and black non-Hispanics—a prediction supported by the most recent data.

After many years of decline, tuberculosis (TB) cases began to resurge in the United States in 1984, and continued to climb for a decade before declining again (1-4). Reemergent TB did not affect all segments of the population equally. While incidence continued to decline in some geographic areas, it increased in others, notably in the greater New York City area, southern Florida, the border areas of Texas, and urban California. Disadvantaged populations were disproportionately affected, with peak incidences in blacks and Hispanics now seen in young adults. TB in foreign-born persons accounts for nearly 40% of the total annual cases in the United States (5). Finally, HIV infection has played a large part in the recent increase in TB incidence. Effective TB control requires reliable estimates of future secular trends. We have constructed a population dynamics, mechanistic Markov chain model for the epidemiology of TB in the United States by separate modeling for disaggregated groups defined by race, ethnicity, age, and geography. With this model, we have projected TB incidence for the U.S. population at large, as well as for racial and ethnic and geographically defined groups within the population.

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The Study

In support of this work, we obtained datasets from the U.S. Bureau of the Census and the Centers for Disease Control and Prevention (6-19).

The epidemiology of TB in the United States has an underlying Markovian nature (20-22). Except in unknown or difficult-to-predict influxes of immigrants, future infections and cases of TB may be probabilistically estimated by knowing sufficiently well the present situation of susceptible, infected, and ill persons; rates of transition from healthy to infected and infected to ill; patterns of contact; and rates of fertility and death. Indeed, a Markov chain is completely specified by an initial state and transition probabilities. To model the future course of TB in the United States, we used the structure depicted in Figure 1.

The U.S. population was disaggregated into states multivariately defined by sociodemographic, health, and disease descriptors: age (single-year

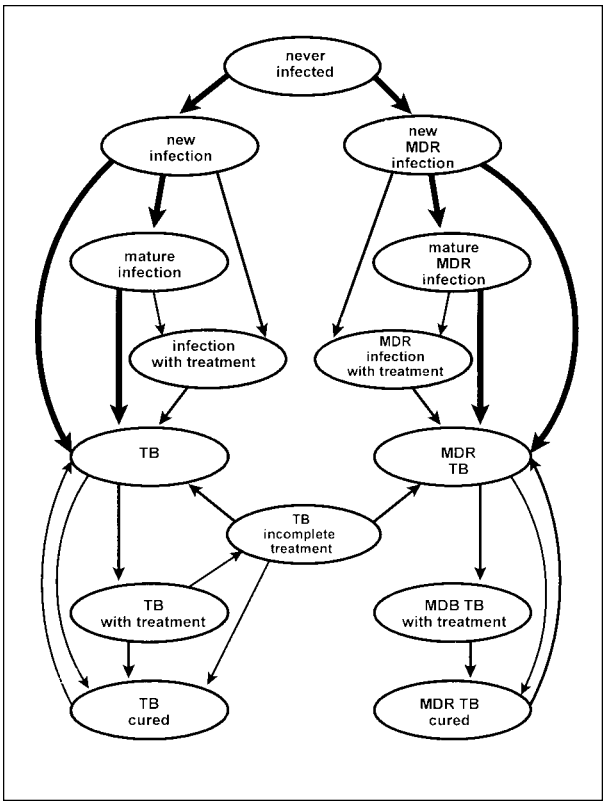

Figure 1. Tuberculosis model states and possible transitions. Thicker lines depict transitions considered in sensitivity analysis.

age groups); sex; race (white, black, Asian or Pacific islander, Native American or Alaskan native, or other race); ethnicity (Hispanic or non-Hispanic); TB infection or disease status; and location. One hundred sixty locations were defined: 60 corresponded to the largest standard metropolitan statistical areas (SMSAs) in the United States, 50 to groupings of smaller SMSAs in the 50 states, and 50 to groupings of non-SMSA counties in the 50 states. The impact of the HIV epidemic on the TB epidemic was addressed by dealing with certain location-specific populations in a way that recognizes that a fraction of them are dually affected. A state in the Markov chain was completely defined by the joint specification of all these descriptors.

In the absence of longitudinal data, the population database for the model was constructed by assembling population profiles for each of the individual years from historical surveys and population projections (5-17,23). These profiles consisted of the sizes of population groups defined multivariately by age, race, sex, ethnicity, and geographic location. The population projections of the Bureau of the Census incorporate expected immigration patterns.

Transition probabilities for the model were based on rates cited in the literature to describe how these transitions occur for individuals. The probability of transition from healthy to infected has been termed the "annual risk for infection." To estimate this probability from tuberculin skin test surveys, we used the catalytic method of Muench (19,24,25). This method relates incidence to prevalence, given several data points of the latter, under the assumption of an exponential model, the exponential curve being called "catalytic" at the time of Muench, who used graphic methods for estimation. The average number of susceptible persons infected by each infectious (i.e., sputum-positive) case in a geographic location in 1 year was termed the "contagious parameter" by Styblo, who noted the correspondence between the prevalence rate of the disease and the annual risk for infection (25,26). Since we assume that new active cases are treated during their year of occurrence, our use of the contagious parameter is consistent with that of Styblo.

An important assumption made in the model about the spread of infection is that each ill person infects susceptible persons entirely within race ethnicity location-specific subgroups, with the number of infections determined by the contagious parameter. The age-sex distribution of newly infected persons in the model was based on national TB contact investigation data (27). Time trends for contagious parameters for the various race ethnicity location-specific subgroups were based on quadratic regression fits of the total cases of TB for those subgroups over time from 1980 through 1993. For illustration, we present in Table 1 baseline values for the contagious parameter for 17- to 25-year-old white men, by state, derived from the U.S. naval recruits data (28-35).

The probability of transition from infected to ill is a product of host-parasite interactions and is determined by the biologic features of these interactions. A person with a new *Mycobacterium tuberculosis* infection (i.e., an infection of <1 year) is at much higher risk for clinical disease than someone with a mature infection, who has been at an ongoing risk in later years (36). These risks vary with age. Although not explicitly modeled, reinfection of cured persons is allowed at the same rate as infections with the same

Table 1. Baseline values of contagious parameters, for 17- to 25-year-old white men, by state

	New ACR[a]	ARI[b]	ARI/TB[c]
Alabama	0.13	38.2	293.6
Arizona	0.30	52.7	178.3
Arkansas	0.15	39.7	261.6
California	0.16	36.4	228.1
Colorado	0.19	18.9	97.4
Connecticut	0.12	15.7	134.2
Delaware	0.12	30.1	243.2
District of Columbia	0.15	55.6	376.1
Florida	0.07	27.4	410.5
Georgia	0.08	31.8	395.7
Idaho	0.10	13.2	135.3
Illinois	0.15	39.6	269.3
Indiana	0.16	30.7	198.0
Iowa	0.07	9.2	130.4
Kansas	0.07	12.6	176.9
Kentucky	0.28	50.6	177.9
Louisiana	0.10	35.4	339.6
Maine	0.14	25.2	179.9
Maryland	0.22	36.4	164.4
Massachusetts	0.14	28.9	203.9
Michigan	0.14	29.7	205.3
Minnesota	0.10	17.3	181.8
Mississippi	0.09	30.3	334.3
Missouri	0.14	32.9	231.8
Montana	0.11	13.6	123.2
Nebraska	0.09	16.4	174.5
Nevada	0.07	53.8	747.9
New Hampshire	0.11	16.8	153.6
New Jersey	0.15	23.7	161.6
New Mexico	0.22	29.1	130.9
New York	0.17	36.6	218.1
North Carolina	0.08	26.1	342.8
North Dakota	0.07	8.6	122.5
Ohio	0.14	27.1	198.2
Oklahoma	0.13	24.0	185.8
Oregon	0.12	32.0	257.0
Pennsylvania	0.17	32.1	183.8
Rhode Island	0.12	27.9	235.7
South Carolina	0.04	26.7	615.2
South Dakota	0.06	9.2	163.8
Tennessee	0.19	45.8	244.7
Texas	0.21	29.2	138.7
Utah	0.04	7.6	197.1
Vermont	0.18	18.3	99.3
Virginia	0.13	32.1	237.9
Washington	0.11	25.7	231.9
West Virginia	0.21	39.1	189.4
Wisconsin	0.08	22.1	260.1
Wyoming	0.15	10.3	66.7
Total	0.14	30.1	210.3

[a]ACR, Active case rate per 100,000 per year tuberculosis
[b]ARI, Annual rate of infection per 100,000 per year
[c]Contagious parameter

characteristics in the population. The case rates among persons with new and mature infections in the next year are based on the upper and lower bounds of rates reported in earlier studies, and are adjusted to calibrate the model to the historical time series (27,37-42) (Table 2).

The effects of the HIV epidemic and the emergence of multidrug-resistant TB are modeled through time-dependent exogenous inputs for appropriate geographic locations and, within these locations, for specific demographic subgroups, as dictated by trends in prevalence and dual-infection rates (43-45). These calculations result in time-dependent changes to the probabilities of transition from new or mature infection to clinical TB.

We established 1980 as the baseline year for all variables used in the model. The initial values of these variables were based on the Reports of Verified Cases of Tuberculosis (RVCT) files and published reports of TB incidence from Centers for Disease Control and Prevention. Because the RVCT program was not implemented nationwide until 1985, reporting for the years 1980 to 1984 was incomplete. To estimate the 1980 baseline values, we inferred values of missing data according to the multivariate structure observed in 1985. Baseline information also included counts of mature infections prevalent in 1980 and counts of new infections in 1980. These values were estimated by applying difference equations relating the numbers of cases, new infections, and mature infections in a given year to the numbers new infections, and mature infections in the previous year, where the relationships between the figures for the 2 years were dependent on model parameters such as survival rates, rates of transition from mature infection to disease, rates of transition from new infection to disease, and contagious parameters, and where each difference equation pertained to a specific multivariately defined subpopulation. Through repeated application of the difference equations, it was possible to express the number of mature infections in 1980 in terms of TB case counts for an arbitrary number of subsequent years for which actual data were available. Estimates thus obtained were examined for consistency and the most stable was selected. Because of the lack of reliable information, we did not attempt to address the underreporting of TB cases or underestimates of some populations.

Table 2. Literature-based lower, best, and upper estimates of the risk of developing tuberculosis for infected persons, by age and sex

	Acute risk		Ongoing risk	
Age	Male	Female	Male	Female
<1	0.013, 0.07, 0.07	0.013, 0.07, 0.07	0.0016, 0.004, 0.009	0.0016, 0.004, 0.009
1-4	0.013, 0.026, 0.026	0.013, 0.026, 0.026	0.0016, 0.004, 0.009	0.0016, 0.004, 0.009
5-14	0.002, 0.002, 0.006	0.002, 0.002, 0.006	0.0008, 0. 0008, 0.0013	0.0008, 0.0008, 0.0013
15-34	0.002, 0.01, 0.01	0.002, 0.02, 0.02	0.0003, 0.0013, 0.0042	0.0003, 0.0016, 0.005
35-54	0.0014, 0.009, 0.009	0.0014, 0.009, 0.009	0.0002, 0.0009, 0.0015	0.0002, 0.0009, 0.0015
55+	0.0019, 0.005, 0.005	0.0019, 0.005, 0.005	0.001, 0.001, 0.002	0.001, 0.001, 0.002

Computer implementation of the model was carried out on a Sun SPARCstation 5 with 80 megabytes of main memory and network access to a SPARCserver 1000E with approximately 100 gigabytes of online disk storage. All software was written in C++, an object-oriented programming language. A library of C++ objects and functions (46,47) was used as the basis for the representation of fuzzy numbers in the software.

Because of the extremely large sizes of the multidimensional arrays containing cross-sectional population data and year-to-year transition data, specialized data structures and algorithms were developed to take advantage of characteristics of these data that allowed them to be stored in a more compact form (48). For cross-sectional population data, a height-balanced binary search tree structure (49,50) was used. Within each tree node, both cell information and array subscript information were stored. Because the subscripts would occupy at least 9 bytes within each node of the tree if stored conventionally, a 4-byte compressed representation of the subscripts was stored within each node. For transition data, a simple linked-list structure was used in which each node contained both cell information and compressed subscript information. This approach resulted in a 96% reduction of storage space required for population data and a 99% reduction of storage space required for transition data (48).

Results

Figure 2 presents model projections of new cases of TB for the United States for 1980 through 2010 as well as actual data for white non-Hispanics, blacks, and Hispanics, the groupings used in TB case reporting in the United States. For these three groups, differences between actual counts and model predictions were generally <1,000 for blacks and Hispanics and <1,500 for white non-Hispanics.

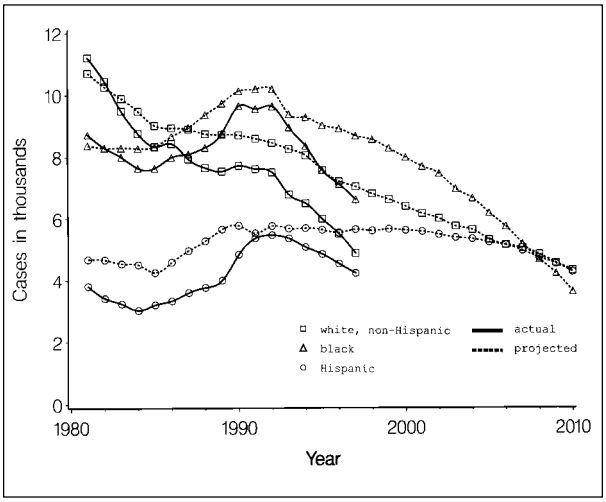

Figure 2. Total number of new cases of tuberculosis in white non-Hispanics, blacks, and Hispanics in the United States, 1980–2010.

Table 3 presents actual and projected TB case rates per 100,000 for each 5 years from 1980 to 2010 with results presented for racial, ethnic, and geographic groups. Generally, the model performs best, within the 1980 to 1995 validation period, for large subgroups with relatively high case rates. For example, it projects TB case rates to within 12% of actual rates for the U.S. population at large. Its performance is not as good in subgroups having lower case rates (e.g., there is a 23% projection error for non-Hispanic whites in 1995). In addition, projection errors are relatively high for smaller subgroups, especially those in which case reporting and population reporting are subject to inaccuracy (e.g., projection errors are consistently above 20% for Hispanics and nearly as high for American Indians and Asian/Pacific Islanders). The model's best performance tends to be among the older population: the model predicts case rates to

Table 3. Actual and projected tuberculosis rates per 100,000 for selected population groups, 1980 to 2010

	Year									
	1980	1985		1990		1995		2000	2005	2010
	Actual	Proj.	Actual	Proj.	Actual	Proj.	Actual	Proj.	Proj.	Proj.
All	(12.5)	10.6	(9.5)	11.3	(10.4)	9.7	(8.7)	8.3	6.6	4.6
By race/ethnicity										
White, not Hispanic	(6.6)	5.1	(4.7)	4.8	(4.2)	3.8	(3.1)	3.2	2.6	2.1
Black, not Hispanic	(34.3)	30.5	(28.0)	34.7	(33.0)	27.9	(23.9)	23.1	16.9	9.3
Hispanic	(25.6)	24.1	(18.2)	26.5	(22.1)	33.2	(18.0)	28.5	23.2	16.7
American Indian	(32.7)	31.5	(24.5)	26.3	(19.5)	19.2	(16.5)	16.6	11.4	7.0
Asian/Pacific Islander	(86.4)	39.9	(50.9)	34.9	(42.8)	27.1	(45.9)	20.1	13.1	6.2
By age										
Under 5	(1.1)	3.6	(4.4)	4.3	(5.1)	4.1	(4.7)	3.5	3.2	2.4
5-14	(0.8)	2.5	(1.4)	2.4	(1.9)	2.2	(1.7)	1.9	1.7	1.3
15-24	(2.8)	6.0	(4.3)	8.1	(5.2)	6.9	(4.7)	5.8	4.3	3.0
25-44	(8.4)	10.6	(9.4)	12.5	(12.0)	10.7	(9.9)	9.3	7.3	4.4
45-64	(19.6)	14.0	(14.0)	15.3	(13.7)	13.6	(11.5)	11.7	8.9	5.9
65+	(49.9)	27.1	(24.8)	20.1	(20.3)	16.1	(16.0)	13.4	11.1	8.5
Selected Metropolitan Areas										
New York City	(18.6)	24.9	(24.7)	41.7	(43.9)	35.2	(30.1)	30.3	25.3	17.9
San Francisco	(19.8)	13.8	(16.7)	17.9	(21.9)	18.3	(23.0)	17.4	13.2	5.1
Los Angeles	(24.8)	26.7	(19.2)	30.5	(23.6)	31.5	(19.1)	27.7	22.1	15.6
Miami	(28.0)	17.5	(23.7)	21.1	(23.6)	13.3	(19.1)	9.9	8.8	5.7

within 1% accuracy for the U.S. population aged 65 and over for 1990 and 1995. This is of significance in that the highest TB case rates are among the elderly.

Figure 3 presents model projections of the TB case rate quartiles, by state, for the year 2010. Note that the projected case rate for Wyoming spuriously falls into the highest quartile, as a result of its small population size. TB is projected to decrease in all parts of the United States and remain largely a problem of states receiving large numbers of immigrants.

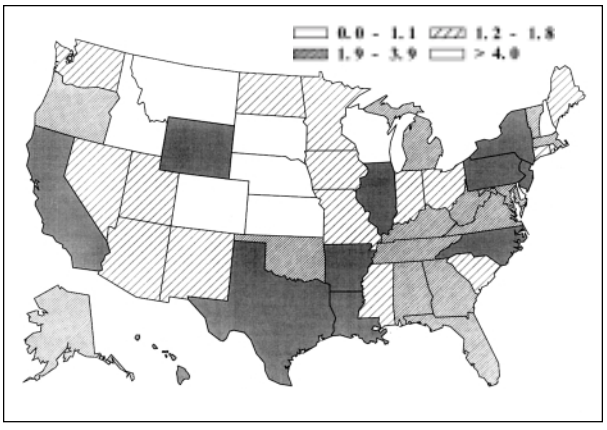

Figure 3. Projected quartiles of tuberculosis case rates per 100,000 by state, 2010.

In Figure 4, the model projection for the United States for 1980 through 2010 is presented together with actual numbers of newly reported TB cases for 1980 through 1997. Also depicted is the sensitivity of the model to changes in key parameters, seen in thicker lines in Figure 1. Specifically, changes were allowed for the contagious parameters and for rates of transition from healthy to infected and infected to ill.

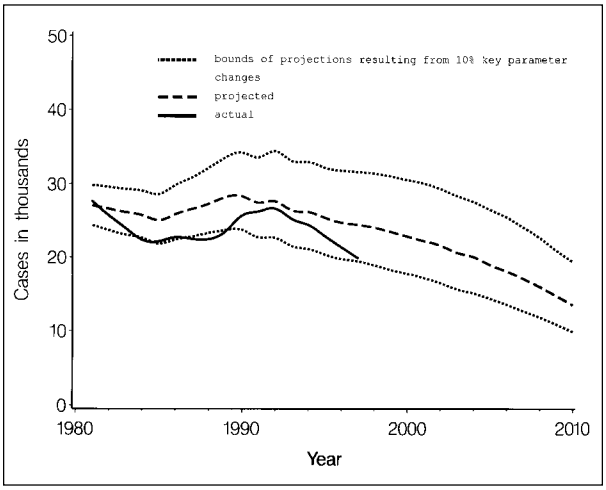

Figure 4. Total number of new cases of tuberculosis in the United States, 1980–2010 and sensitivity of model projections.

Specifically, above and below the central projection are lines representing projections from simultaneous 10% increases and decreases in these key parameters for each year in the projection period. A simultaneous decrease of 10% in the parameters for each year of the projection period results in a decrease of 27% (from 13,458 to 9,861) in projected TB cases in 2010. A simultaneous increase of 10% yields an increase of 44% (from 13,458 to 19,381) in projected cases in 2010. Not shown in the figure, a simultaneous decrease of 5% in the same parameters yields a decrease of 15% (from 13,458 to 11,459) for projected cases in 2010, and a simultaneous increase of 5% yields an increase of 19% (from 13,458 to 16,063) in 2010.

Conclusions

The studies of Waaler and colleagues, who carefully noted and credited other early workers in this field, mark the beginning of a modern approach to modeling and the thoughtful consideration of transition probabilities in modeling (51,52). ReVelle, Lynn, and Feldman, using many of the assumptions of Waaler and his colleagues, projected declining TB case rates in developing countries as a result of BCG vaccination (53). Azuma modeled TB incidence in Japan (54). Estimates of AIDS prevalence were used by Styblo (55), Schulzer and colleagues (56), and Heymann (57) to project TB incidence in sub-Saharan Africa. Modeling was used by Murray, Styblo, and Rouillon to examine the economic impact of TB in developing countries (58). Blower and colleagues used a differential equation-based model to examine the decline of TB epidemics and emphasize the importance of effective case treatment in TB control (59,60). Murray and colleagues used differential equation-based models to compare the global impact of different control strategies (61,62). Brewer and colleagues used simulation techniques to compare TB control policies (63).

Mathematical modeling of TB in the United States probably began in 1939 with the early work of Frost, who used age cohort analyses of secular trends to predict the future decline of TB (64). Ferebee developed a model for TB in the United States based on data from 1963 and used it to emphasize the potentially important contribution of chemoprophylaxis to TB control (65). The model we present is substantially more complex than Ferebee's and differs from her early work by disaggregating the American population. Given the marked heterogeneity of the population of the United States and the corresponding diversity of TB case rates in various American population groups, our approach offers major advantages over aggregate modeling both in improved accuracy of estimation of input data and in output that can be disaggregated to relate to segments of the population with very different TB control problems.

A highly disaggregated model incorporating population dynamics is appropriate. When applied to small groups, population dynamics are aptly described by a linear model, particularly when natural constraints are stated as totals. The model we constructed is Markovian in that it is independent of its history prior to its last known state. Our computer implementation of the theoretical model is clearly Markovian because its operation parallels the nature of a Markov chain, in the sense that the initial state was the state of our system in 1980, and the state in each subsequent year was generated from the previous year's state and 1-year transition rates. Since no information other than the previous year's state and 1-year transition rates are available to the computer software to generate the state for a year, the Markovian nature is established.

Our model for the intermediate years predicted (on the basis of 1980 baseline data) that TB would increase late in the ensuing decade and decline again. This prediction reasonably approximated the subsequent events. While the model does include changes in TB incidence related to the HIV epidemic and to immigration, it does not specifically incorporate changes in TB control measures, whose impacts are only addressed indirectly through net changes of transition probabilities in the model. The latter could be addressed by a submodel for appropriate population subgroups, and could affect projections.

The projections presented here suggest that the number of new cases and incidence rates of TB will now continue to decrease to a level approximately half the present level nationally. Measuring the extent to which increased control efforts contributed to the return of the TB epidemic to its formerly declining phase will require more detailed analysis.

The handling of geographic location necessarily relies on politically defined entities which determine how data are collected. Since disease patterns do not necessarily follow these

boundaries, the model is not uniformly accurate by geographic location. This may be seen in Table 3 where the TB case rate in 1995 is overestimated for Los Angeles and underestimated for Miami. However, the very fine age structure imposed has resulted in projections by age group within 20% accuracy for age 25 and older (Table 3). Refinement of the geographic characterization could result in corresponding increases in accuracy.

The model projections are smoother over time than the actual counts, as should be expected because random fluctuations are not built into the model (Figures 2,3). In Figure 4 the time series of actual cases is moving toward the lower 10% bound. This may be a temporary fluctuation (as in the mid-1980s) but could also indicate changes in transitions in the real-world disease process.

Errors of a projection model may arise from shortcomings of model structure, from inaccuracies in initial conditions, or from errors in the values of key model parameters. The structure of the Markov chain model is mechanistic, driven by highly detailed population dynamics. Each part of the mechanistic process may prompt suggestions for model refinements and discussions of variability; notably, occurrence of infection (66,67), time from infection to disease (68,69), and involvement of HIV (70) and immigrant status (71). The greatest potential for model inaccuracy due to initial conditions arises from estimates of the 1980 pool of prevalent infections. Errors thus introduced become less consequential with passing time, as persons infected before 1980 die. So, model projections in the latter part of the projection period are least affected by errors in the numbers of those infected before 1980. Model parameters not only have the obvious role of governing transitions for various population subgroups, but also, as reflected by their time dependency, are used to depict exogenous phenomena such as the HIV epidemic, implementation of TB control efforts, and changes in immigration patterns. Geographically focused changes of TB control efforts implemented after 1993 related to directly observed therapy could result in overestimation in projections in those areas.

Although the model does not allow mixing of groups from one location to another, this limitation is in large measure adjusted in the calculations of transition probabilities since these are based on actual data that implicitly reflect the movements of healthy, infected, and ill groups. The other type of mixing not accounted for in the model occurs in a location among different race-ethnicity subgroups. The most serious projection errors would then occur when a subgroup of uninfected persons lives in the same location as a subgroup of persons with active TB, for then any cases arising in the former subgroup would not be predicted by our model. This type of situation is likely to arise only in locations with very few active TB cases. Otherwise, the projection errors arise only from the extent to which the cross-spread of infection fails to cancel out (i.e., the net extent to which a subgroup infects others, over and above the number of the latter being infected by the former).

Although HIV status is addressed in this model, adding structure that more precisely reflects changes in transition rates of HIV-infected persons might also improve model projections. The model predicts that an increasing portion of cases will occur in Hispanics, and this prediction is supported by the most recent data, which show that the rate of decline in the number of cases in Hispanics is slower than for white non-Hispanics and for black non-Hispanics.

The foreign born account for a large fraction of the new cases seen in the United States (71). The model assumptions regarding the future contribution of the foreign born to the U.S. population are from the U.S. Bureau of the Census projections; therefore, this component of the population may be thought of as forming part of the backdrop against which the model projections are made. Departure from the patterns of immigration projected by the Census Bureau could cause two kinds of errors in model projections. A surge of immigration from countries with high rates of TB would soon be followed by increases in TB cases and case rates in the United States that would not be projected by the model. A different effect would be seen as a consequence of unexpected large increases in immigrants. Regardless of the TB status of the countries of origin, an increase in the number of immigrants would be reflected first in an artificial lowering of case rates because of the increase in denominators, followed by a longer-term slow increase in numbers of cases. The model could not recognize these changes within its current structure. These potential errors may be avoided as additional structure better reflecting immigrant status becomes incorporated into the

model so that unexpected influxes may be modeled as exogenous inputs.

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Serologic Response to Culture Filtrate Antigens of *Mycobacterium ulcerans* during Buruli Ulcer Disease

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Buruli ulcer (BU) is an emerging necrotic skin disease caused by *Mycobacterium ulcerans.* To assess the potential for a serodiagnostic test, we measured the humoral immune response of BU patients to *M. ulcerans* antigens and compared this response with delayed-type hypersensitivity responses to both Burulin and PPD. The delayed-type hypersensitivity response generally supported the diagnosis of BU, with overall reactivity to Burulin in 28 (71.8%) of 39 patients tested, compared with 3 (14%) of 21 healthy controls. However, this positive skin test response was observed primarily in patients with healed or active disease, and rarely in patients with early disease (p=0.009). When tested for a serologic response to *M. ulcerans* culture filtrate, 43 (70.5%) of 61 BU patients had antibodies to these antigens, compared with 10 (37.0%) of 27 controls and 4 (30.8%) of 13 tuberculosis patients. There was no correlation between disease stage and the onset of this serum antibody response. Our findings suggest that serologic testing may be useful in the diagnosis and surveillance of BU.

Buruli ulcer (BU) disease, caused by *Mycobacterium ulcerans*, is characterized by severe necrotizing ulcers. The disease, which is found worldwide but primarily in tropical climates (1,2), occurs predominantly in children ages 5 to 14 (3) and is associated with severe illness and permanent disabilities in >26% of patients (1,3). Over the past decade, the incidence of BU disease has dramatically increased in several West African countries, notably Ghana, Côte d'Ivoire, Benin, and Togo (1,2). In some West African communities, BU has replaced tuberculosis (TB) and leprosy as the most prevalent mycobacterial disease, affecting up to 22% of the population (4). However, the true global impact of the disease is unknown because reliable tools for its rapid diagnosis and surveillance are lacking. In addition, neither the mode of transmission nor the environmental reservoir for *M. ulcerans* is known, although observational studies suggest that *M. ulcerans* infection is transmitted through the skin after contact with contaminated water, vegetation (5), or insects (6).

Current treatment for cutaneous disease is primarily surgical. Ulcerative lesions require wide debridement and skin grafting in specialized health-care facilities, long hospital stays (7), and increased cost to the health-care system and the community (7). Antibiotics are ineffective once ulcers are present, possibly because *M. ulcerans* is absent from lesions or subcutaneous tissues have already been destroyed, preventing penetration of antimycobacterial drugs (3). Antimicrobial therapy has not been studied in preulcerative lesions because apparently patients rarely visit primary health-care providers with painless nodular lesions and diagnostic tests are not available to clearly identify *M. ulcerans* infection before it progresses to clinical disease. Early identification and surgical excision of these preulcerative nodular lesions can be curative and does not require inpatient care (8). We investigated the serologic response to the *M. ulcerans* culture filtrate (MUCF) of BU patients at different stages of disease to determine whether serodiagnosis is feasible as a tool for early diagnosis and intervention of BU disease.

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The Study

BU patients were identified through a case-control study conducted in 1991 in the Daloa region of Côte d'Ivoire (3). Patients with early ulcerative BU were identified by small, painless, or minimally painful nodules or edema, or ulcerative lesions for <2 weeks. Patients with ulcerative BU were identified by lesions that were chronic (>2 weeks duration), were painless or minimally painful, or had undermined edges. Patients with healed BU were identified by stellate scars with retraction in areas of prior ulceration. Descriptive and clinical data were obtained from all BU patients and controls (3). Blood specimens were collected from 82 BU patients and 164 healthy controls in the Daloa region.

Because TB is endemic in many of the areas where BU occurs, 13 TB patients from the United States were used as controls for cross-reactivity to MUCF. Use of sera from these patients ensured the lowest probability for exposure to *M. ulcerans*, so that only cross-reactivity to antigens common to both *M. tuberculosis* and *M. ulcerans* would be measured. TB patients were identified through an ongoing study in Atlanta by clinical presentation, a positive PPD response, and isolation of *M. tuberculosis* from sputum. All specimens tested negative for HIV and were strongly reactive to many of the culture filtrate antigens from *M. tuberculosis* (data not shown). Informed consent was obtained for all participants, and protocols were approved by the Institutional Review Boards of the Centers for Disease Control and Prevention and the Ministry of Health of Côte d'Ivoire for the BU study and the Institutional Review Board at Emory University for the TB study.

Skin Testing

During sera collection, Burulin (9) and PPD (purified protein derivative of *M. tuberculosis*, Connaught Laboratories, U.K.) were administered intradermally into the flexor surface of the forearms of 39 BU patients and 21 controls, and the diameter of induration was recorded at 48 and 72 hours. The criterion for using Burulin and PPD dual testing to confirm clinical cases of BU has been reported (3,9), and the cumulative delayed-type hypersensitivity (DTH) findings for this study have been described (Table 1) (3). Sera were also tested for reactivity to MUCF antigen by Western blot (Table 2).

Table 1. Burulin skin test responses of Buruli ulcer patients and healthy controls from the same villages

Clinical presentation	Burulin (+) only	Dual (+)	PPD (+) only	Dual (-)
Total BU patients (n=39) (%)	17 (43.6)	11 (28.2)	6 (15.4)	5 (12.8)
Healed (n = 16) (%)	9 (56.3)	6 (37.5)	1 (6.2)	0 (0.0)
Ulcerative (n = 17) (%)	7 (41.2)	4 (23.5)	4 (23.5)	2 (11.8)
Early ulcerative (n = 6) (%)	1 (16.7)	1 (16.7)	1 (16.7)	3 (50)
No scar or ulcer (n = 21) (%)	3 (14.3)	0 (0.0)	1 (4.8)	17 (81)

Each column provides the number (n) and percentage (%) of persons meeting the criteria for specific skin-test positivity. Dual (+) persons, being both PPD and Burulin (+), are not exclusive for either Burulin or PPD positivity and thus are not included in either of these two columns.

Persons were scored Burulin-only skin-test positive if the diameter of induration at the site of Burulin injection was 10 mm at 48 or 72 hours and the diameter of induration at the site of PPD injection was at least 3 mm smaller than in the Burulin response. Persons were scored as only PPD positive if the diameter of induration at the site of PPD injection was 10 mm at 48 or 72 hours and the diameter of induration at the site of Burulin injection was at least 3 mm smaller than that in the PPD response. Persons were scored as both Burulin and PPD skin-test positive if the diameters of induration of both the Burulin and PPD injection sites were 10 mm at 48 or 72 hours and the differences in the diameters of the zone of induration between the two was <3 mm. Persons were scored as skin test negative for both if the induration at both injection sites was <10 mm at 48 or 72 hours.

Table 2. Serologic and skin test responses of Buruli ulcer patients and healthy controls from the same villages

Clinical presentation	Total Burulin (+)[a]	AB to MUCF[b] (+)[c]
Total BU patients (n = 39) (%)	28 (71.8)	26 (66.7)
Healed (n = 16) (%)	15 (93.8)	11 (68.8)
Ulcerative (n = 17) (%)	11 (64.7)	11 (64.7)
Early ulcerative (n = 6) (%)	2 (33.4)	4 (66.7)
No scar or ulcer (n = 21) (%)	3 (14.3)	7 (33.3)

[a]Total Burulin (+) numbers and (%) are derived from columns one and two of Table 1 added together without regard to PPD status, and thus represent the highest possible number (percentage) of Burulin (+) BU patients.

[b]MUCF = *Mycobacterium ulcerans* culture filtrate

[c]Denotes all BU patients and controls with any antibodies detected by Western blot analysis against MUCF.

Preparation of MUCF Antigen for Serologic Analysis

M. ulcerans strain S-WT (from the Centers for Disease Control and Prevention culture collection; Atlanta) was recovered from a 1-ml lyophilisate in 10% glycerol by incubation in 5 ml Middlebrook 7H9 with OADC supplement (Remel, Lenexa, KS) at 32.5°C for 7 days. This 5-ml inoculum was passaged into 25 ml Middlebrook 7H9 broth supplemented with tryptose and glucose (7H9TG; 0.1% wt:vol and 2% wt:vol, respectively) and incubated at 32.5°C for 10 days, followed by passage into 100 ml of 7H9TG and growth at 32.5°C for 10 days. Cultivation in this protein- and serum-free media allowed isolation and analysis of specific secreted mycobacterial proteins. The 100-ml culture was passaged into 1L of 7H9TG, incubated at 32.5°C in 850-cm^2 roller bottles, and rotated at 90 rotations per hour. Bacilli were harvested during mid-log phase growth by centrifugation. The 1L of MUCF, containing mycobacterial proteins of interest, was concentrated at 4°C under N_2 by using a stirred-cell apparatus (Amicon Inc., Beverly, MA) with a 10-kDa molecular weight membrane. MUCF was exchanged into 0.1 M NH_4HCO_3 by dialysis, lyophilized, and purified by separation of residual small molecular weight contaminants from antigens of interest by size exclusion chromatography. MUCF was loaded onto a fast-performance liquid chromatography system (FPLC) (Pharmacia, Piscataway, NJ) equipped with a G-25 superfine Sephadex desalting column (Pharmacia, Piscataway, NJ), UV monitor, and conductivity monitor for separation and detection of proteins and salts, respectively. MUCF was eluted with an isocratic gradient of 0.1 M NH_4HCO_3. MUCF protein was quantitated by bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) and lyophilized until needed. Approximately 5 mg of MUCF protein was obtained per liter of culture.

Aliquots of 8 g of MUCF protein were resolved by discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10) by using 10% to 20% gradient 10-well gels (Novex, San Diego, CA). MUCF proteins were visualized by staining with silver (11), and this profile was reexamined throughout these experiments to ensure that the MUCF proteins did not degrade upon storage; no antigen variability was seen during our studies. 7H9TG media alone processed in the same way contained no proteins by silver stain analysis after SDS-PAGE and bicinchoninic acid assay.

Amino Acid Sequence Analyses

The N-terminal amino acid sequences of MUCF proteins were determined and compared with known mycobacterial sequences. One hundred micrograms of MUCF protein was resolved by two-dimensional gel electrophoresis (2D-GE) by the method of O'Farrell (12) with the modifications of Sonnenberg and Belisle for resolution of mycobacterial proteins (13). 2D-GE resolved proteins were transferred to 0.1 mm polyvinyl difluoride (PVDF) membrane (14) and visualized by staining with 0.5% Coomassie blue R250 in 40% methanol/10% acetic acid for 2 minutes. The N-terminal amino acid sequence was obtained by loading membranes containing the excised spot of interest onto a Procise-cLC automated Edman sequencer (Applied Biosystems, Foster City, CA) and reading the generated chromatographs against amino acid standards.

Statistical Analyses

Statistical analyses were done by the Mantel-Haenszel chi-square method. Statistical significance for the chi-square p values and Fisher's exact p values was evaluated at $\alpha = 0.05$. Fisher's exact p value was used when the expected cell count was <5. Odds ratios (OR) were determined by calculating the ratio of the odds of exposure among cases to that among controls. The test for specificity was determined by dividing the true negatives (n = 24; disease-negative and 70 kDa-negative) by the total number of controls (n = 27). All analyses were done on BU patients and healthy controls from the Daloa region of Côte d'Ivoire, where the disease is endemic, unless otherwise specified. All data were analyzed by using Exact 2.0b software (D. Martin and H. Austin, Atlanta, GA).

Results

Seventeen (43.6%) of the 39 skin-tested BU patients had induration of $\geq$ 10 mm within 72 hours of Burulin administration, with PPD induration at least 3 mm smaller than that with Burulin (Table 1). Only 3 (14.3%) of the 21 skin-tested healthy controls from the area had a similar DTH pattern (Table 1). In addition, 11 (28.2%) of the 39 BU patients had an equally strong response to Burulin and PPD, which

suggests that Burulin skin testing may not be reliable in regions where BU and TB are endemic and BCG vaccination is widely used (Table 1). Dual-positive responses included, 28 (71.8%) of the 39 BU patients were Burulin positive. However, when this total Burulin-positive population was delineated by disease stage, positive responses were observed in most of the patients with healed (93.8%) and ulcerative disease (64.7%), but in few (33.4%) patients with early ulcerative disease (Table 2). When healed and active Burulin responses were compared with the DTH response seen in early ulcerative BU patients, a significant difference between the disease stages was found (Fisher's exact p = 0.009).

These skin-tested BU patient and control populations from the Daloa region were then tested for reactivity of their sera to MUCF by Western blot, and Burulin induration was compared with serum antibody response (Table 2). Twenty-six (66.7%) of the 39 skin-tested BU patients had an antibody response to the MUCF, versus 7 (33.3%) of the 21 skin-tested controls (chi-square p = 0.014). BU patient populations had approximately the same positive antibody responses to the MUCF, regardless of disease stage. Specifically, 68.8% of the healed patients were antibody positive, with active ulcerative disease at 64.7% and early disease at 66.7% (Table 2). When the antibody responses of healed and active patients were compared with those seen in early BU patients, the serologic response did not differ significantly by disease stage (Fisher's exact p = 0.999). Therefore, serologic testing may be useful in the early diagnosis and surveillance of BU.

The antibody response to the MUCF was then determined for all 61 clinically diagnosed BU patients and 27 healthy controls, and antibody reactivity to individual antigens was recorded. Three *M. ulcerans* antigens of 70, 38/36, and 5 kDa were commonly recognized by BU patient sera antibodies (Figure). The 36-kDa antigen was found to be a degradation product of the 38-kDa antigen (unpub. results). Forty-three (70.5%) of 61 BU patient sera had antibodies to at least 1 of 3 antigens, compared with 10 (37.0%) of 27 sera from healthy controls from the area and 4 (30.8%) of 13 sera from TB patients (Table 3). The antibody response to the 70-kDa *M. ulcerans* antigen was associated with BU disease (OR = 12.33; chi-square p = 0.00002), and the response to this protein was consistent throughout early to late disease stage (OR = 12.4; chi-square p = 0.0000001; Table 3). Results were similar for the 38/36-kDa antigen (OR = 5.94; chi-square p = 0.0016), regardless of disease stage (OR = 3.00; chi-square p = 0.0026). The

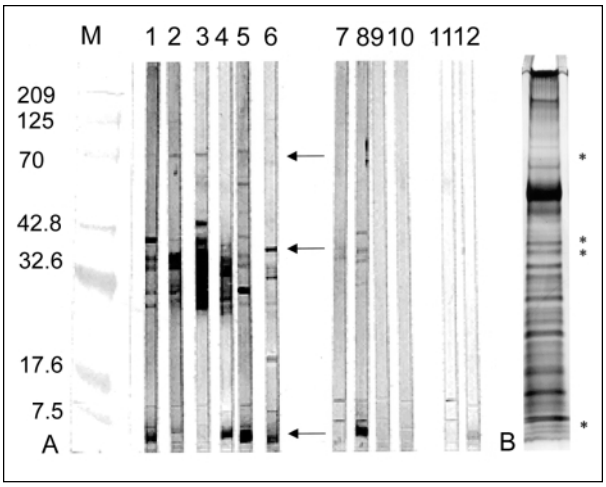

Figure. Western blot reactivity to and Silver stain analysis of *Mycobacterium ulcerans* culture filtrates (MUCF). A) Representative antibody responses to MUCF. M; molecular weight markers with annotations corresponding to molecular weight on the left; lanes 1-6, representative BU patient sera with reactivity to MUCF; lanes 7-10, representative antibody reactivity in healthy persons from the disease-endemic area; lanes 11-12, serologic reactivities to MUCF of two representative tuberculosis (TB) patients. TB patient sera that were serologically reactive to MUCF were included as a control for cross-reactivity. Regions of antibody reactivity corresponding to *M. ulcerans* antigens of 70, 38/36, and 5 kDa are noted (arrows). B) Silver-stained SDS-PAGE gel of MUCF. The identification of putative proteins corresponding to the serologically reactive 70, 38/36, and 5 kDa MUCF antigens are noted (stars). For Western blot analyses, aliquots (50 □g protein) of MUCF were resolved by discontinuous SDS-PAGE (10) with preparative 10% to 20% gradient 1-well gels (Novex, San Diego, CA) and then transferred to nitrocellulose (14). Nitrocellulose sheets were cut into 2-mm strips and stored in 5% skim milk in Tris-buffered saline, pH 7.6, until used. Antibody to MUCF was detected by probing nitrocellulose strips with sera at a 1:50 (vol:vol dilution). Bound serum antibodies were detected with alkaline phosphatase-conjugated anti-human antibody (Sigma Chemical Co., St. Louis, MO), and the substrate 4-Bromo-3-Chloro-2-Indoyl-1-Phosphate/Nitro blue tetrazoleum (Sigma Chemical Co., St. Louis, MO). All sera were tested in duplicate for confirmation of the antibody responses.

Table 3. Antibody responses to specific *Mycobacterium ulcerans* culture filtrate proteins in all Buruli ulcer patients and controls

Clinical presentation	5 kDa	36/38 kDa	70 kDa	All three	Any[a]
Total BU patients (n= 61) (%)	14 (22.9)	31 (50.8)	37 (60.7)	8 (13.1)	43 (70.5)
Healed (n = 29) (%)	8 (27.6)	15 (51.7)	17 (58.6)	7 (24.1)	20 (69.0)
Ulcerative (n = 22) (%)	6 (27.3)	10 (45.5)	14 (63.6)	2 (9.1)	16 (72.7)
Early ulcerative (n=10) (%)	0 (0.0)	6 (60.0)	6 (60.0)	0 (0.0)	7 (70.0)
No scar or ulcer (n= 27) (%)	7 (25.9)	4 (14.8)	3 (11.1)	1 (3.7)	10 (37.0)
U.S. TB patients (n= 13) (%)	3 (23.1)	3 (23.1)	0 (0.0)	0 (0.0)	4 (30.8)

[a]Denotes total number (percentage) of persons within the population with an antibody response to any one, or a mixture of, the three specified MUCF proteins. MUCF = *Mycobacterium ulcerans* culture filtrate

N-terminal amino acid sequence of the 38 kDa protein was DPAPAPAPRD, and had mycobacterial sequences that precede glycosylation sites (15). The serologic response to the 5-kDa antigen, although common in the seroreactive specimens tested, did not differ significantly between BU patients and healthy controls from disease-endemic areas or TB patients (chi-square p = 0.764 and Fisher's exact p = 0.999, respectively); nor were antibodies to this antigen observed in BU patients at the early ulcerative stage (Table 3). A cross-reactive serologic response to MUCF was observed in some TB patients who had not been exposed to *M. ulcerans* and in some healthy controls from disease-endemic areas (Figure; representative sample and Table 3). These positive responses may be due to exposure to other mycobacterial pathogens with similar antigens as *M. ulcerans* or BU disease undetectable by clinical diagnosis, respectively.

Conclusions

Diagnosis of BU currently relies on the clinical presentation of the ulcerative disease stage, by which time the infection has already caused damage, therapeutic options are limited, and outcome is associated with severe disease (3,7,8). Preulcerative nodules and edemous lesions, when identified, frequently contain numerous extracellular mycobacteria (1,2). Thus, diagnosis of BU disease at this early stage may lead to more effective treatment. Indeed, diagnosis of preulcerative BU disease is associated with a better therapeutic outcome and reduced illness (8), and accurate surveillance at the preulcerative stage would enable assessment of the prevalence and global impact of BU disease. Surveillance may also identify risk factors as well as prevention strategies for BU.

Consistent with previous reports (3,9), DTH to Burulin, a crude preparation of *M. ulcerans* lysate (9), was observed in many (71.8%) of the clinically diagnosed BU patients. This overall response was similar to the well-defined cell-mediated DTH responses reported for other mycobacterial infections (16-18), including *M. tuberculosis* (19,20). In contrast, in BU patients, the Burulin response was associated with ulcerative and healed disease, but not with early ulcerative disease. Thus, unlike other DTH-based monitoring programs that indicate mycobacterial infection before and during disease in immunocompetent persons, Burulin testing may be more useful for confirming ulcerative disease or monitoring past disease with *M. ulcerans* in conjunction with PPD results.

The marked shared reactivity to PPD and Burulin antigens by BU patients, as measured by similar induration upon intradermal injection, was expected to some degree, considering the commonality of antigenic lipids on the mycobacterial surface, genetic relatedness of mycobacterial pathogens, opportunity of exposure to both mycobacterial pathogens and environmental mycobacteria, and rate of BCG vaccination in the region. When Burulin-positive results were analyzed in conjunction with either the presence of a BCG vaccination scar or recollection of BCG vaccination, 12 of 14 BCG-vaccinated BU patients were Burulin positive, and 6 of these 12 patients were also PPD positive. Additionally, all the Burulin- and PPD-positive controls either recalled BCG vaccination or had a BCG scar (data not shown).

We found that a specific response to the MUCF was observed in BU patient sera, but unlike the Burulin DTH response, the antibody response did not correlate with disease stage

(Table 2). The antibody reactivity in the sera from some of the controls from disease-endemic areas may reflect preclinical infection with *M. ulcerans* or exposure to other mycobacteria and cross-reactivity to antigens common to mycobacterial species. Interestingly, 10 (55.6%) of the 18 antibody-negative BU patients, versus 15 (33%) of the 43 antibody-positive BU patients, recalled or had a BCG vaccination scar, which suggests that BCG vaccination did not contribute to the antibody response of BU patients to MUCF (data not shown).

Thirty percent of the TB patient sera were reactive to MUCF, with reactivity principally towards lower molecular weight proteins and not the 70- or 38/36-kDa proteins (Figure; Table 3). N-terminal amino acid sequence of one of these lower molecular weight proteins, a 31 kDa protein, was found to be FSRPGLPVEY and demonstrated homology with the mycolyl transferases found in *M. tuberculosis* and other mycobacteria (21). Thus, reactivity of the TB patient sera based on antibodies generated against common mycobacterial antigens is the likely explanation for the few TB patient sera reactive to MUCF.

Our preliminary results indicate the usefulness of the MUCF in detecting BU; specifically, individual antigens in the MUCF may be used for the development of a sensitive and specific test for BU (Table 3). Both the 70- and 38/36-kDa proteins were indicators of BU disease, and antibody responses to both of these proteins were similarly present in BU-reactive patient serum samples, regardless of disease stage. In addition, reactivity to the 70-kDa protein was specific for *M. ulcerans* infection (88.8%).

The 70- and 38/36-kDa proteins may be useful for the development of a serologic test for BU in areas where TB is endemic. Based on these results, we are isolating and characterizing these two *M. ulcerans* antigens for additional testing. The N-terminal sequence for the 38/36-kDa protein has already been determined as part of the purification process for this protein, and identification and purification of the 70-kDa protein are under way. If a serologic test proves to be diagnostic, early excision of nodules and antimycobacterial chemotherapy trials of patients could reduce the public health and socioeconomic impact of this emerging disease.

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Dr. Dobos is a senior postdoctoral fellow in the laboratory of Dr. C. Harold King at the Emory University School of Medicine. Her current research interests focus on bacterial proteomic analysis. She will continue studying the proteins of *Mycobacterium ulcerans* for development of future vaccine and diagnostic candidates.

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Haemophilus influenzae Type b and *Streptococcus pneumoniae* as Causes of Pneumonia among Children in Beijing, China

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To determine if *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* could be identified more often from the nasopharynx of patients with pneumonia than from control patients, we obtained nasopharyngeal swab specimens from 96 patients with chest x-ray-confirmed pneumonia and 214 age-matched control patients with diarrhea or dermatitis from the outpatient department at Beijing Children's Hospital. Pneumonia patients were more likely to be colonized with Hib and *S. pneumoniae* than control patients, even after the data were adjusted for possible confounding factors such as day-care attendance, the presence of other children in the household, and recent antibiotic use. In China, where blood cultures from pneumonia patients are rarely positive, the results of these nasopharyngeal cultures provide supporting evidence for the role of Hib and *S. pneumoniae* as causes of childhood pneumonia.

New vaccines for *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* effectively prevent disease caused by these pathogens (1,2). However, the relatively high cost of these vaccines inhibits their widespread use in developing countries. Therefore, for most developing countries, surveillance for disease caused by Hib and *S. pneumoniae* is needed to demonstrate that the investment in these highly effective vaccines is warranted (3).

Data on the incidence of Hib and pneumococcal disease in China are limited to a few meningitis surveillance studies, which suggest a surprisingly low incidence of confirmed Hib meningitis. In most cases the rate is 5 to 25 times lower than those observed in areas of North America, Europe, Africa, South America, and Oceania where careful surveillance studies have been carried out (4-7). However, the apparent low incidence in these studies is difficult to interpret because of concerns that physicians may not routinely perform lumbar punctures on all patients with suspected meningitis, that the laboratory methods may have been inadequate, and that widespread use of oral antibiotics for outpatients may have artificially reduced the yield of cultures (8). In part because of the uncertainty surrounding the local rate of Hib disease, China has not yet made Hib vaccination a routine infant vaccination. Many authors and organizations have called for further studies of the epidemiology in China, but few data are currently available (9-11).

Pneumonia is a leading cause of illness and death among children worldwide (12). Demonstrating the role of bacterial agents such as *H. influenzae* and *S. pneumoniae* in pneumonia, however, is difficult. The most widely accepted

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method for demonstrating that a bacterial agent causes pneumonia is isolation of the bacterium from cultures of blood or, in some cases, lung aspirates. However, blood cultures are rarely positive in pediatric pneumonia even when microbiologic techniques are optimal, and fastidious organisms such as Hib will not grow unless the appropriate culture medium is used. In China, parents and physicians often resist the collection of blood for cultures, and given the high rate of prior antibiotic use, the yield from these specimens is likely to be low.

Because alternatives are needed to traditional methods of documenting Hib and *S. pneumoniae* as causes of pneumonia in China, we designed a study to investigate the use of a readily available source of specimens that is likely to be positive more often than a blood culture. We compared the rates of isolation of Hib and *S. pneumoniae* from nasopharyngeal swabs and blood cultures of patients with radiographically confirmed pneumonia and a group of control patients without pneumonia.

The Study

The People's Republic of China is the most populous nation in the world, with an estimated population of >1.2 billion and >20 million births each year (13). Beijing, the capital, has a population of approximately 11 million people. Beijing Children's Hospital is one of the largest children's hospitals in Asia, with >700 beds in 24 wards. The outpatient department typically has 2,500 to 4,000 patient visits per day.

Before patients were enrolled, a study nurse or physician explained the study to the parent or guardian, answered questions, and obtained written informed consent. The protocol for this study was approved by the human subjects review committees at Beijing Children's Hospital and the Centers for Disease Control and Prevention.

Pneumonia cases were defined as illness in patients 2 to 60 months of age with radiographic evidence of pulmonary infiltrates and at least three of the following: fever (temperature 38.0°C), tachypnea (>50 breaths per min for infants <12 months old, and >40 breaths per min for children 12 to 60 months old), cough, auscultation findings indicative of lower respiratory disease (including rhonchi, crackles, or bronchial breath sounds), or chest indrawing.

Specimens for blood culture were collected from all 96 pneumonia patients. Urine specimens were obtained from 89 (93%) pneumonia patients and 199 (93%) controls.

Chest radiographs from all pneumonia patients were interpreted initially by a radiologist at Beijing Children's Hospital, who characterized the pattern of infiltrates as evidence of alveolar consolidation only, interstitial infiltrates only, or a mixed pattern with evidence of interstitial infiltrates and consolidation. The radiographs were then recorded by a digital camera and read later by one of the investigators who is a pediatrician (SFD). The pediatrician characterized the radiographs as having evidence of obvious or not obvious pneumonia. Both the radiologists and the pediatricians were blinded to the colonization status of the patients.

Control patients were children with a diagnosis of diarrhea or dermatitis who had no indication of respiratory tract disease. All subjects were recruited from patients attending the Outpatient Department. None of the participating children had received Hib vaccine. Of 121 eligible pneumonia patients identified in the Radiology department, 96 (79%) were enrolled; 214 controls were also enrolled, 169 with diarrhea and 45 with dermatitis. Cases and controls were frequency-matched by age in a 1-to-2 ratio in the following age categories: 2 to 5 months, 6 to 11 months, 12 to 35 months, and 36 to 60 months.

Laboratory Methods

After measuring the distance from the nares to the earlobe, a researcher inserted a fine flexible Dacron swab past the point of mild resistance to the posterior pharynx. The swab was rotated twice, withdrawn, and immediately used to make a uniform suspension in a skim milk/glucose/glycerol solution. The suspension was then plated onto a blood agar plate with gentamicin (for isolation of pneumococci) and a chocolate agar plate supplemented with X and V factors and bacitracin (for isolation of *H. influenzae*). Plates were refrigerated before inoculation and warmed to room temperature before use.

Within 3 hours of inoculation, blood agar plates were placed in an incubator at 35°C to 37°C and 5% to 10% CO_2, and individual colonies were isolated in the Microbiology and Immunology Laboratory, Beijing Children's Hospital. After 18 to 36 hours of incubation, the plates were assessed for the appearance of α-hemolytic colonies resembling streptococci. A second plate

was then streaked for confluent growth and an optochin disk placed on it. Strains inhibited by optochin were confirmed as pneumococci by bile solubility testing. For isolation of *H. influenzae*, X and V factor-supplemented chocolate agar plates were placed in an incubator at 35ºC to 37ºC and 5% to 10% CO_2, and single colonies were isolated. After overnight incubation, the plates were assessed each morning for 2 days for the appearance of large, flat, colorless-to-gray opaque colonies, without hemolysis or discoloration of the medium. *H. influenzae* were determined by dependency for X and V factors and absence of hemolysis on blood agar plates. All isolates were serotyped by using type-specific antiserum.

For blood cultures, which were done in the Clinical Microbiology Laboratory at Beijing Children's Hospital, 2 to 5 ml of blood was inoculated into blood culture bottles that contained agents to inhibit antibiotic activity. The bottles were incubated by the BacTec 9120 system (Organon Teknika, Durham, NC). All positive blood cultures were subcultured onto blood agar and chocolate agar plates. These plates were prepared by the same techniques and quality control used in the research laboratory at Beijing Children's Hospital. For colonies with appropriate morphologic features on subcultures, the same protocol for identification of pneumococcus and *H. influenzae* was used as described earlier. Reagents and media were checked weekly for their ability to support the growth of control strains of *H. influenzae* and *S. pneumoniae*.

Urine specimens were tested for antigens to *Legionella pneumophila* serogroup 1 by an optical immunoassay and for evidence of prior antimicrobial activity by a *Micrococcus luteus* inhibition assay. To determine if *Chlamydia pneumoniae* were present in the nasopharynx, nasopharyngeal swabs were cultured on HEp-2 cells by using a modification of standard techniques involving repeat centrifugation steps (14). *C. pneumoniae* cultures were performed only on a subset of 30 patients >2 years old.

Statistical Analysis

Recent day-care attendance was defined as attendance in the last month before enrollment. Exposure to other children was defined as residing in the same household with at least one other child <18 years old. All analyses were conducted by SAS (SAS for Windows v.6.12; SAS Institute, Cary, NC). Logistic regression was used to assess the independent association between colonization and pneumonia and to adjust for potential confounders. Odds ratios (OR) were calculated by comparing pneumonia patients with control patients without pneumonia. Separate ORs were calculated for both the clinical and radiologic definitions of pneumonia. ORs with 95% confidence intervals that do not include 1.00 and p values <0.05 were considered statistically significant.

Results

Four patients who were initially enrolled as pneumonia patients were later determined to have myocarditis and were thus excluded. One pneumonia patient had a blood culture positive for *S. pneumoniae*; no patients had a blood culture positive for *H. influenzae*. All urine specimens were negative for *L. pneumophila* serogroup 1. *C. pneumoniae* was not cultured from any of the 30 specimens tested. Eleven pneumonia patients were hospitalized. Among the pneumonia patients, 45 had a pattern of alveolar consolidation, 23 had an interstitial pattern, and 28 had a mixed pattern (both consolidation and interstitial).

Comparison of the patients with radiographic pneumonia and control patients showed that the distribution of ages was not consistently one pneumonia to two nonpneumonia patients in all age groups (Table 1). Although the differences in the distributions are not statistically significant, we adjusted all subsequent analyses by age to account for any residual confounding by this factor. Pneumonia patients were significantly more likely to have received antibiotics before attending the outpatient clinic, to attend day care, and to have other children in the household (Table 1; $p \leq 0.01$, for each comparison).

Of the 96 patients who had radiographic evidence of pneumonia, 32 (33%) were considered to have obvious pneumonia by the pediatrician's reading. The prevalence of Hib colonization was significantly greater among pneumonia patients than among patients without pneumonia (Table 2). The association was stronger, however, when the patients meeting the clinical definition of pneumonia were compared with the controls (OR = 5.8) than when the radiologist's definition of pneumonia was used (OR = 4.2). Multivariate analysis by logistic regression showed that the association remained after the data were

Table 1. Characteristics of pneumonia cases and diarrhea and dermatitis control patients, Beijing, China

Risk factor	Cases (n=96) No.	%	Controls (n=214) No.	%	p-value
Age group					0.265
2 to 5 months	20	20.8	40	18.7	
6 to 11 months	9	9.4	39	18.2	
12 to 35 months	41	42.7	83	38.9	
36 to 60 months	26	27.1	52	24.3	
Daycare attendance in the past 30 days	32	33.3	36	17.1	0.002
Recent antibiotic use	53	60.2	63	31.7	0.001
At least one other child in household	53	55.2	85	39.7	0.011
Three or more persons in household	75	78.1	154	72.0	0.254
At least one current cigarette smoker in household	67	69.8	139	65.0	0.404
Currently breastfeeding	31	32.3	89	41.6	0.120
Male sex	50	52.1	130	60.8	0.153
Caregiver Education Level					0.126
No schooling	11	11.5	10	4.7	
Primary (1-6 years)	17	17.7	53	24.8	
Middle (7-9 years)	33	34.4	82	38.3	
Senior (10-12 years)	22	22.9	38	17.7	
College or higher	13	13.5	31	14.5	

Table 2. Association of nasopharyngeal colonization with *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* and radiographically confirmed pneumonia, Beijing, China

	Controls (N=214)	Pneumonia defined by: Radiologist's reading (N=96)					Pediatrician's reading (N=32)				
	No. pos. (%)	No. pos. (%)	O.R.	95% C.I.	Adj.[a] O.R.	95% C.I.	No. pos. (%)	O.R.	95% C.I.	Adj.[a] O.R.	95% C.I.
Hib	4 (1.9)	7 (7.3)	4.23	1.20, 14.86	4.12	1.01, 16.80	3 (9.4)	5.77	1.21, 27.59	9.62	1.73, 53.40
S. pneumoniae	83 (38.8)	42 (43.8)	1.22	0.75, 1.99	1.84	1.03, 3.29	16 (50.0)	1.52	0.72, 3.23	3.03	1.20, 7,64

[a]Odds ratio adjusted for the following potential confounding factors: antecedent antibiotic use, recent day-care attendance, and other children in the household.

adjusted for potential confounders (recent antibiotic use, recent day-care attendance, and other children in the household), and in the case of the clinical definition of pneumonia, increased the strength of the association.

In univariate analysis, colonization with *S. pneumoniae* was not significantly higher among pneumonia patients than among patients without pneumonia (Table 2). This association, however, was confounded by the higher proportion of pneumonia patients with prior antibiotic use, an exposure that decreases colonization substantially. After the data were adjusted for prior antibiotic use, recent day-care attendance, and exposure to other children in the household, colonization with *S. pneumoniae* was significantly associated with pneumonia by either the radiographic or clinical definition of pneumonia.

Conclusions

We found that Hib and *S. pneumoniae* can be isolated significantly more often from the nasopharynx of children with radiographically confirmed pneumonia in China than from comparable children without pneumonia, demonstrating the potential role of these bacteria as a cause of pneumonia in Chinese children. These results suggest that further studies should be undertaken to quantify the role of these agents in China, the prevalent serotypes, and the potential impact of vaccines for their prevention.

The prevalence rates of Hib and *S. pneumoniae* colonization observed among controls are similar to those we reported in a study of outpatients at Beijing Children's Hospital, as well as by other investigators (15,16). The prevalence of Hib colonization among nonpneumonia patients (~2%) is consistent with that observed in other

studies of young children in China, but lower than commonly seen in other developing countries (5%-15%) (17-19). Even in industrialized countries such as the United States, Finland, and the United Kingdom, pre-vaccination colonization rates of 3% to 6% were generally observed (20). Host and environmental factors may play a role in this apparently low prevalence of Hib colonization in nonpneumonia patients. For example, widespread overuse of antibiotics in children and the relatively small family size as the result of the "one family, one child" policy may contribute to this observation.

The rate of positive blood cultures among pneumonia patients was lower (1%) than that generally observed in studies from other countries, but consistent with reports of a low rate of bacterial meningitis and invasive disease from mainland China and Taiwan (5,6,10). In rural Papua New Guinea, *S. pneumoniae* and *H. influenzae* were isolated from the blood of 11% and 12%, respectively, of children >6 years old with moderate or severe acute lower respiratory tract illness (21). However, a recent study from Dallas found no positive blood cultures among 106 ambulatory outpatients <5 years old with radiographically confirmed pneumonia (22). Thus, the rate of positive blood cultures was lower than that seen in hospital-based studies in developing countries but consistent with recent data from studies in industrialized countries of children with pneumonia who attend outpatient clinics.

Several steps were taken to ensure the quality of the blood specimens collected and their processing. For example, all blood specimens were collected before intravenous antibiotics were administered. The protocol for the study was to inoculate bottles with 2 and 5 ml of blood, a volume considered optimal for the 15-ml blood culture bottles used in the BacTec system, and the blood culture bottles contained resin-coated beads designed to neutralize the antibacterial activity of antecedent antibiotic use.

The observation that colonization with Hib and *S. pneumoniae* is more likely among pneumonia patients, especially those with radiographically obvious pneumonia, strengthens the contention that pneumonia is associated with these agents. This finding is consistent with the observation from studies with Hib conjugate vaccine showing that the protection afforded by these vaccines is most apparent in children with obvious radiographic pneumonia or alveolar consolidation and not apparent in children with milder forms of pneumonia (23,24). However, an inherent limitation of case-control studies is that they cannot establish that the exposure, in this case colonization with Hib or *S. pneumoniae*, preceded the onset of illness.

The potential role of other agents of pneumonia besides Hib and *S. pneumoniae* remains unclear. In this study, *H. influenzae* colonization (regardless of serotype) was also associated with pneumonia (data not shown). The observation that no patients had antigens to *L. pneumophila* serogroup 1 in urine is consistent with findings from the United States and elsewhere indicating that this agent rarely causes pneumonia among young children (25). The finding that none of 30 patients >2 years of age were positive for *C. pneumoniae* by culture suggests that if this organism causes pneumonia in this age group, alternative methods for detection are needed to define its role. The potential role of other agents such as *Mycoplasma pneumoniae* and viruses such as respiratory syncytial virus warrants further investigation. Characterizing the epidemiology of these agents may have important implications for the choice of empiric therapy in pneumonia patients and for the development of vaccines for pneumonia prevention.

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Bacteroides fragilis Enterotoxin Gene Sequences in Patients with Inflammatory Bowel Disease

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We identified enterotoxigenic *Bacteroides fragilis* in stool specimens of patients with inflammatory bowel disease and other gastrointestinal disorders. The organism was detected in 11 (13.2%) of 83 patients with inflammatory bowel disease. Of 57 patients with active disease, 19.3% were toxin positive; none of those with inactive disease had specimens positive for enterotoxigenic *Bacteroides fragilis* gene sequences.

Bacteroides fragilis, a gram-negative rod, constitutes 1% to 2% of the normal colonic bacterial microflora in humans (1,2). It is frequently associated with extraintestinal infections such as abscesses and soft tissue infections, as well as diarrheal diseases in animals and humans (3-6). Enterotoxigenic *B. fragilis* (ETBF) is an emerging enteric pathogen associated with diarrheal diseases in children, adults, and animals (4,7-9). The pathogen is unusual in children during the first year of life, but diarrhea associated with it is common in children 1 to 5 years of age, which suggests early acquisition of the organism and maternal protection. The pathogenicity of *B. fragilis* is related to its production of a potent enterotoxin, a zinc-containing metalloprotease with a molecular weight of 20,000 (10). The enterotoxin is tight-junction specific; causes rounding, swelling, and pyknosis of cultured enterocytes; and induces a fluid response in ligated intestinal loops and a cytotoxic response in the HT-29 colon cell line (7).

Ulcerative colitis and Crohn disease are inflammatory diseases of the gastrointestinal tract characterized by spontaneous remissions and relapses. Many microbial pathogens, particularly *Mycobacterium paratuberculosis*, paramyxoviruses, and *Listeria monocytogenes* have been implicated in the etiology of inflammatory bowel disease (IBD) (11). In addition, enteric pathogens such as *Campylobacter jejuni*, *Salmonella*, *Shigella*, *Yersinia*, and *Escherichia coli* have also been associated with relapses of IBD (12). We investigated the prevalence of ETBF in 83 patients with idiopathic IBD, in 18 patients with routine culture-negative diarrhea, and in a control population of 69 outpatients (Table 1).

Table 1. Demographic characteristics of patients with inflammatory bowel disease (IBD)

Patient characteristics	Patient group		
	IBD (n = 83)	Diarrhea patients (n = 18)	Controls (n=69)
Sex			
M	38	4	40
F	45	14	29
Age (yrs)	10-80	20-75	40-72
Mean =	45.5	45.6	58.2
Duration of disease (yrs)			
UC	1-13 (5.6)	1-6 (2.6)	NA
CD	1-20 (10.5)		
Therapeutic treatment			
5-ASA	58	0	0
5-ASA+Steroids	35	0	0
6-MP/ Azathioprine	20	0	0
MTX	1	0	0
Antibiotics	1	1	0
Antidiarrheals	1	3	8

The Study

The study protocol was approved by the Human Subjects Research Review Committee of

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the University of California, Davis. All samples were collected after informed consent was obtained. Of the 83 patients in the IBD group, 60 had Crohn disease, and 23 had ulcerative colitis (Table 2). Active disease was present in 68.6% of these patients on endoscopy (20 ulcerative colitis and 37 Crohn disease patients) in the form of mucosal erythema and ulceration. In the miscellaneous diarrhea group, 10 patients had irritable bowel syndrome; however, no mucosal erythema, edema, or ulceration was observed. None of the control patients had a history of diarrhea, and no erythema or ulceration was observed.

Table 2. *Bacteroides fragilis* enterotoxin gene amplification products in patients with inflammatory bowel disease (IBD)

Patient group (no.)	ETBF[a] Gene (+)	p value[b]
IBD (83)	11 (13.25%)	0.023
Active (57)	11 (19.3%)	0.0026
Inactive (26)	0	
Crohn disease (60)	5	
Active (37)	5	
Inactive (23)	0	
Ulcerative colitis (23)	6	
Active (20)	6	
Inactive (3)	0	
Diarrhea (18)	5 (27.8%)	0.0005
Control (69)	2 (2.89%)	

[a]ETBF = enterotoxigenic *Bacteroides fragilis*
[b]p value in relation to control group

Fecal specimens from the IBD group were collected endoscopically from patients undergoing colonoscopy or flexible sigmoidoscopy for evaluation of symptoms of diarrhea or abdominal pain. The fecal specimens from the 18 diarrhea patients with negative routine stool cultures and the 69 controls were collected by endoscopy.

Fecal specimens were cultured for *B. fragilis* in the selective medium Bacteroides Bile Esculin agar. Positive cultures were identified by using the RapID ANA II Panel (REMEL, Inc, Lenexa, KS). Plates were incubated anaerobically at 37°C for 48 hours. The presence of *B. fragilis* enterotoxin in the isolates was detected in the HT-29 colon cell line (13,14). HT-29 cells were grown and maintained in RPMI medium with glutamine (Gibco, Life Technologies, Inc., Grand Island, NY) supplemented with penicillin (100 IU/ml), streptomycin (100 □g/ml) (Sigma, Saint Louis, MO), and heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) at 12% in 25-ml flasks at 37°C in 5% CO_2. For the cytotoxicity assay, HT-29 cells were suspended in 20 ml of medium for each plate, and 180 □l/well were placed into 96-well tissue culture plates (Corning Glass Works, Corning, NY). The cells were allowed to attach and grow for 2 to 3 days. Supernatants from *B. fragilis* isolates grown on Brain Heart Infusion Broth were filtered through a 0.45-□m Acrodisk syringe filter (Gelman Sciences, Ann Arbor, MI), and 20-□l serial dilutions were placed into the wells in duplicate. The plates were incubated at 37°C for 3 to 4 hours under 5% CO_2 and examined for typical cytopathic changes. Cultures were considered positive for *B. fragilis* enterotoxin if a visible cytopathic effect was neutralized by specific antiserum. The highest dilution of the culture supernatant producing cytopathic changes in at least 50% of the cells after 3 to 4 hours of incubation was considered the cytotoxic titer. For the neutralization assay, dilutions of 1:25 anti-enterotoxin rabbit antiserum in phosphate-buffered saline were mixed with culture supernatants positive for enteroxin. After incubation for 30 minutes at 37°C, 20 □l of each mixture was inoculated into HT-29 cells as in the cytotoxicity assay. Neutralization was indicated by the lack of cytotoxic effect.

DNA was extracted from the fecal specimens and amplified by using specific primers (14) to detect *B. fragilis* enterotoxin gene sequences.[1] Chi-square was used to determine statistical significance.

[1]A 100-mg sample of stool was suspended in 400 □l of TES buffer (50 mM Tris [pH 8], 5 mM EDTA, 50 mM NaCl) and centrifuged at 1,000 x g for 3 min to remove large particles. The supernatant was then centrifuged at 5,000 x g for 7 min. The pellet was washed once in 200 □l of TES, and centrifuged at 5,000 x g for 3 min, and the supernatant was discarded. The pellet was suspended in 100 □l of sterile H_2O and boiled for 10 min, then centrifuged at 1,000 x g for 2 min and the supernatant containing the DNA extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with ethanol. The sequences of the primers and probes used and PCR conditions were as described (14). Amplification with the outer primers RS-3 (5' TGAAGTTAGTGCCCAGATGCAGG 3') and RS-4 (5' GCTCAGCGCCCAGTATA TGACC 3') yielded a 367-bp product. Amplification of this product with the inner primers RS-1 (5' TGCGGCGAACTCGGTTAATGC 3') and RS-2 (5'AGCTGGGTTGTAGACATCCCACTGG' 3') amplified a 290-bp product. The reaction mixtures were prepared in 1X PCR buffer (50 mM KCl, 20 mM Tris HCl, 2.5 mM $MgCl_2$, 100 □g bovine serum albumin per ml [pH 8.4]) and contained per reaction 20 pmol of the respective primers, 0.1 mM concentrations each of 2'-deoxynucleoside 5'-triphosphate, 2 U of recombinant DNA polymerase (rTaq) (Perkin Elmer, Norwalk, CT), and 10 □l purified fecal DNA. The final reaction volume was adjusted to 100 □l with sterile deonized water. The PCR profile included a denaturing step at 95°C for 30 sec, followed by a 60°C annealing step for 30 sec, with extension at 72°C for 30 sec. The outer PCR was performed for 35 cycles in a thermal cycler (MJ Research). Amplification with the inner primers was done for 30 cycles. Negative controls included a blank containing all PCR reagents with no DNA. As control for amplifiable DNA in the stool specimens, primers targeting the 16S rRNA gene of enteric bacteria were used as described by Kato et al. (15).

ETBF was cultured from four patients with IBD and two patients with diarrhea. However, *B. fragilis* enterotoxin gene sequences were detected in the stools of 11 (13.25%) of 83 patients with idiopathic IBD (five with Crohn disease and six with ulcerative colitis). All 11 patients positive for ETBF had active disease by endoscopic and histologic tests (Table 2). No ETBF was found in patients with inactive disease. The Crohn disease patients who were positive for enterotoxin gene sequences had superficial mucosal disease. In the control group, 2 (2.9%) of 69 patients were positive for *B. fragilis* enterotoxin gene sequences. Enterotoxin amplification products were also detected in the specimens of 5 (27.7%) of 18 patients with diarrhea due to miscellaneous causes (Table 2). All the specimens were positive when amplified with primers specific for the 16S rRNA gene of enteric bacteria.

Conclusions

The normal colonic microflora of humans is a complex ecosystem of approximately 500 species of aerobic and anaerobic microorganisms. Although the gut of the newborn infant is sterile, *Bacteroides* species—the predominant anaerobic constituent of the colonic flora—appear at approximately 10 days, are established by 2 weeks, and usually remain constant lifelong (1,2). In breast-fed infants, *Bifidobacterium* are the predominant population, and *Bacteroides* group organisms remain undetectable. However, after weaning, the *Bacteroides* group organisms increase and *Bifidobacterium* organisms decrease substantially (16).

The exact etiology of idiopathic IBD is still unknown, although a potential role for infectious agents or toxins that may stimulate an inflammatory response has been suggested (16,17). For example, *Peptostreptococcus*, *Coprococcus*, and *Bacteroides* sp. have been reported in patients with Crohn disease (17). A role for these microorganisms in the disease process is also suggested by the clinical responses of some patients to antibiotics (18). Observations of *B. thetaiotaomicron* in patients with ulcerative colitis (16) and *B. vulgatus* in guinea pigs with experimentally induced ulcerative colitis (19) suggest that microorganisms may influence the development or maintenance of intestinal inflammation in IBD.

In this study, *B. fragilis* enterotoxin gene sequences were detected by nested polymerase chain reaction (PCR) in the stools of 13.2% of patients with inflammatory bowel disease and 2.9% controls. The low recovery of ETBF in culture may be due to the length of time from specimen collection to processing in the laboratory (most specimens were kept frozen for at least 2 weeks before culturing). Similar results have been reported by Sack et al. (9), who found a marked reduction in the recovery of *B. fragilis* with time. Amplification of enterotoxin gene directly in the stools of these patients appears to be a more sensitive detection method. In a previous study (14), we found 100% correlation between PCR and enterotoxin production in isolates; we therefore did not routinely perform the HT-29 cell assay in specimens of these patients.

In the IBD group, all the patients positive for ETBF had active disease, which suggests an association with disease activation or flare-up. ETBF was also found in patients with ulcerative proctitis, collagenous colitis, and microscopic colitis. In patients with Crohn disease, ETBF was usually seen in the colonic superficial inflammatory disease type. The presence of ETBF in IBD patients may represent alterations of endogenous bacterial flora, which may be related to either the etiology or flare-up of the disease or both. Colonization with ETBF may be acquired early in life or may be a de novo infection related to flare-ups of the disease.

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Outbreak among Drug Users Caused by a Clonal Strain of Group A Streptococcus

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We describe an outbreak among drug users of severe soft-tissue infections caused by a clonal strain of group A streptococcus of M-type 25. Cases (n = 19) in drug users were defined as infections (mainly needle abscesses) due to the outbreak strain. Comparison with controls showed that infected drug users bought drugs more often at a specific place. Drug purchase and use habits may have contributed to this outbreak.

In the 1980s, reemergence of severe group A streptococcus (GAS) infections, especially toxic shock syndrome, necrotizing fasciitis, and bacteremia associated with high death rates, was observed (1,2). Temporal and geographic clustering of cases with severe GAS infection has been described (3,4). Outbreaks caused by clonal strains have been reported in households, schools, and hospitals (5).

In September 1997, a sudden increase was observed in needle abscesses due to GAS among drug users hospitalized in Berne, Switzerland. Analysis of GAS isolates suggested that the outbreak was caused by clonal strains. An outbreak investigation included a case-control study of potential sources and risk factors for acquisition of GAS infection among drug users in Berne. To our knowledge, this is the first report of an outbreak among drug users of invasive GAS infections caused by clonal strains.

The Study

Cases of GAS infection were identified through culture records of the Institute of Medical Microbiology, University of Berne, Switzerland. During September to December 1997, all GAS isolates from any site were prospectively stored and included in the study. Cases were defined as GAS infection in drug users from September 22 to November 20, 1997, due to the outbreak strain, as determined by pulsed-field gel electrophoresis (PFGE).

During December 15-20, 1997, controls were randomly chosen from drug users visiting a government shelter for drug use and needle exchange in Berne. An exclusion criterion was a skin infection at an injection site since September.

Drug users with cases, as well as controls, were interviewed by a standardized questionnaire including age, employment, recent infections, and drug use habits (Table). Crude odds ratios and 95% confidence intervals were calculated with EpiInfo software, version 6.02 (CDC, Atlanta, GA). Categorical data were compared with Fisher's exact test or the chi-square test, and continuous data with Student's t-test. To identify independent risk factors, logistic regression analysis was performed in EGRET (Seattle, WA).

GAS was isolated on sheep blood agar and identified by gram staining and the bacitracin test (6). Throat swabs were obtained at the time of the interview from the 55 controls. Culture specimens were taken with moistened (sterile saline) swabs from spoons (n = 10) and filters (n = 2) used for drug preparation.

Samples of cocaine confiscated by police during September 15-28 and October 12-20 were cultured for GAS. The drug specimens had been stored for 1 to 6 weeks in a dry place before culture. For each cocaine specimen, three 0.2-g samples were dissolved in sterile saline. One sample was inoculated into tryptic soy broth (TSB); the other two were filtered through sterile 0.45-□m membranes. One filter was placed on a sheep blood agar plate, and the other was

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Table. Sociodemographic and drug use habits of 19 drug users with group A streptococcal infection and 55 healthy controls, Berne, Switzerland, 1997

Variable	Cases n=19 N	%	Controls n=55 N	%	Odds Ratio	95% CI[a]
Age						
Mean (yrs)	30.3		30.7			
Range (yrs)	20-43		21-48			
Sex						
Male	9	47.4	38	69.1	Ref.	0.76-8.26
Female	10	52.6	17	30.9	2.48	
Employed						
No	12	63.2	33	60.0	Ref.	
Yes	6	31.6	15	27.3	1.10	0.30-4.00
Unknown	1	5.3	7	12.7		
Daytime shelter						
Home or work	7	36.9	20	36.4	Ref.	
Street	8	42.1	20	36.4	1.14	0.30-4.40
Unknown	4	21.1	15	27.2		
Nighttime shelter						
Home	14	73.7	29	52.7	Ref.	
Street	3	15.8	10	18.2	0.62	0.10-3.06
Unknown	2	10.5	16	29.1		
Sharing of paraphernalia for drug use						
Filter						
No	9	47.4	34	61.8	Ref.	
Yes	8	42.1	21	38.2	1.44	0.42-4.92
Unknown	2	10.5	0	0.0		
Spoon						
No	8	42.1	28	50.9	Ref.	
Yes	9	47.4	27	49.1	1.08	0.32-3.68
Unknown	2	10.5	0	0.0		
Needle						
No	15	78.9	49	89.1	Ref.	
Yes	1	5.3	5	9.1	0.65	0.03-6.68
Unknown	3	15.8	1	1.8		
Cocaine heating						
No	16	84.2	35	63.6		
Yes	0	0.0	6	10.9	p = 0.17	
Unknown	3	15.8	14	25.5		
Mode of drug use						
Intravenous[b]	17	89.3	55	100.0		
Mucosal only	1	5.3	0	0.0		
Unknown	1	5.3	0	0.0		
Illegal drug used						
Cocaine	2	10.5	7	127		
Heroin	0	0.0	2	3.6		
Both	16	84.2	46	83.6		
Unknown	1	5.3	0	0.0		
Methadone program[c]						
No	18	94.7	40	72.7	Ref.	
Yes	1	5.3	15	27.3	0.15	0.01-1.23
No. of drug use(s)						
≤1 per day	4	21.1	21	38.2	Ref.	
>1 per day	13	68.4	29	52.7	2.35	0.59-10.04
Unknown	2	10.5	5	9.1		
Cocaine used						
≤1 g per day	7	36.8	30	54.5	Ref.	
>1 g per day	8	42.1	10	18.2	3.43	0.84-14.34
Unknown	4	21.1	15	27.3		
Nationality of dealer(s)						
Not A	4	21.1	15	27.3	Ref.	
Only A	6	31.6	13	23.6	1.73[d]	0.32-9.59
Several including A	4	21.1	20	36.4		
Unknown	5	26.3	71	2.7		
Dealer location						
Place X	8	42.1	1	1.8	27.43	2.57-696.75
Other places	7	36.8	24	43.6	Ref.	p <0.001
Not Berne	0	0.0	1	1.8		
Unknown	4	21.1	29	52.7		

[a]95% confidence interval
[b]Includes combined intravenous and mucosal drug use for one case and seven controls.
[c]Government-approved methadone program.
[d]Comparing "only nationality A" and "not A."

inoculated into 500 ml TSB. Cultures were incubated at 37°C. Subcultures from TSB were placed on sheep blood agar at 12-hour intervals until the broth became turbid.

PFGE was performed on GAS isolates as described (7). Briefly, whole genomic DNA was restricted with SmaI, and fragments were separated in a CHEF DRIII unit (BioRad, Glattbrugg, Switzerland) under the following conditions: 0.5xTRIS-borate-EDTA running buffer, 6 volts/cm, 14°C, 120°C angle, and a 1.2- to 54-s ramped switch time for 18 hours. Gels were stained with bromide, and banding patterns were compared. Only isolates with identical banding patterns were considered to belong to the same clone.

The M-type of the outbreak strain was determined by PCR amplification and sequencing of a region of the emm gene (3,8). The presence of the pyrogenic exotoxin A gene was evaluated as described (3).

PFGE analysis of isolates showed that 19 of the 21 infections were caused by the same clone (Figure). Most of these infections (16 of 19) were needle abscesses at the injection site; two were complicated by erysipelas and one by osteomyelitis distant from the needle abscess. None of the patients had streptococcal toxic shock syndrome (1). Seventeen cases required inpatient treatment, including surgery; all patients recovered. All patients lived (n = 16) or purchased drugs in Berne (n = 3).

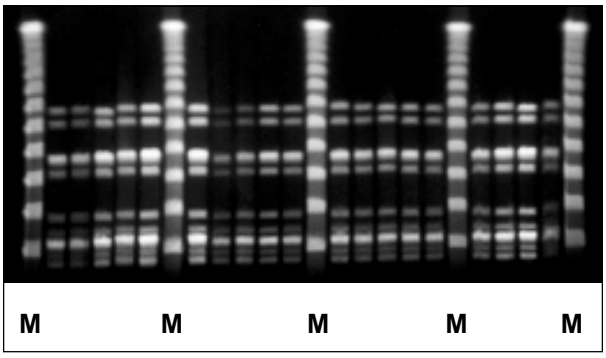

Figure. Pulsed-field gel electrophoresis of group A streptococci causing clinical infection in 19 drug users, Berne, Switzerland.
M = molecular weight standards. All other lanes represent PFGE pattern of group A streptococcus isolates from 19 drug users.

Cases and controls did not differ by mean age, current employment status, sharing of paraphernalia (e.g., needles, spoons, and filters) for drug use, and place of shelter (Table).

No GAS could be isolated from the eight confiscated cocaine samples. Five (9%) of 55 controls were colonized by GAS at the time of the interview; three of them carried the outbreak strain. Unfortunately, four of the five colonized controls refused to disclose the location and nationality of their drug dealer(s). One admitted buying drugs in various places, including place X, and from dealers of several nationalities, including nationality A. He was colonized by the outbreak strain.

The outbreak strain was M-type 25, which did not carry the gene encoding for the pyrogenic exotoxin A. The strain was susceptible to penicillin, clindamycin, and erythromycin, as determined by E-testing.

Conclusions

During the past decade, GAS infections have attracted increased attention because of their worldwide reemergence. Molecular typing studies suggested that this might have been due to the intercontinental spread of a virulent clone of M-type 1 (3). Isolates of other M-types have since been associated with clusters of severe infections (9). Person-to-person spread by respiratory droplets from colonized patients or asymptomatic carriers has been thought to be the main mode of transmission. Vaginal carriage of GAS in health-care workers has been associated with nosocomial spread of GAS (10). Acquisition of GAS by contaminated food and foodborne GAS outbreaks has also been described (11).

The epidemic of GAS infections among drug users described here is to our knowledge the first report of a clonal epidemic with GAS in this patient population. GAS is commonly isolated from soft-tissue infections in intravenous drug users (12,13). High rates of pharyngeal carriage with epidemic clones may play a role in this high frequency of GAS infections, although no clonality of such GAS isolates was found in two studies (14,15).

The case-control study showed a strong association between infection and purchase of drugs at X, a place commonly used for drug dealing in Berne. The association with place X

was strong enough that bias due to the high refusal rate among controls is unlikely.

Outbreaks of infections with other pathogens were previously described in drug users (e.g., *C. tetani, C. botulinum, Candida albicans*, and hepatitis A virus) (16-20). These outbreaks could be due to contamination of the drug or drug paraphernalia. In our population, sharing paraphernalia such as needles, spoons, and filters was reported infrequently and did not differ between cases and controls. We believe that this strain of GAS was spread through cocaine or its containers. Drug dealers and users often hide cocaine in their mouths during police raids. Therefore, GAS may be spread to drug users by contamination of the plastic bags containing the cocaine or the cocaine itself from persons with GAS colonization of the mouth and throat. The strong association of the outbreak with a common place of drug purchase suggests that one or several drug dealers there were colonized by the outbreak strain and spread it by contaminated drug containers or respiratory droplets. Infected drug users may also have become colonized by hiding the drug containers in their mouths. Alternatively, GAS may have entered the subcutaneous tissue directly by contaminated drug or handling paraphernalia with contaminated hands. Although we were not able to culture GAS from cocaine samples, the drug cannot be ruled out as a potential vehicle of GAS, since these samples had been stored for some weeks and GAS may not survive in sufficient numbers in dry (cocaine) powder.

We obtained a throat culture from only one of the infected drug users, because they had already received antibiotics for at least 1-2 days when they were identified by the microbiologic studies. The single throat culture showed carriage of the outbreak strain in the throat as well as at the site of infection (erysipelas of the leg). Oropharyngeal carriage of the outbreak strain was also found in 5.4% of our controls; the strain was not found in randomly selected isolates from nondrug users, indicating that the outbreak strain was probably circulating mainly in the drug user population.

Needle abscesses in drug users have been associated with cocaine use. The local vasoconstriction induced by cocaine may predispose to abscess formation (13,21). Cocaine, unlike heroin, is usually not heated before injection, since it is thought to lose its activity when heated. Failure to heat the drug likely increases the risk of inoculating pathogens. In our study, none of the 19 cases heated the dissolved cocaine, while six of the controls did. Some reported that they started to heat the drug after hearing about the outbreak.

From the end of the outbreak in November 1997 to May 1998, we observed three sporadic cases of infection due to the outbreak strain among drug users, but not the general population. A retrospective analysis by PFGE of clinical GAS isolates cultured in our institution demonstrated that the outbreak strain has been circulating among drug users in Berne since at least February 1997. This study also revealed two previous clonal GAS outbreaks among drug users in 1993 (unpubl. obs.). These findings suggest that GAS outbreaks may be observed among drug users more frequently than previously appreciated and that their propagation may involve transmission of the outbreak clones by mechanisms related to drug purchase and use.

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Erythromycin Resistance in *Streptococcus pyogenes* in Italy

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In a prospective study of acute pharyngitis in Italian children, 69 (38.3%) of 180 isolates of *Streptococcus pyogenes* were resistant to macrolides. *S. pyogenes* was eradicated in 12 (63.1%) of 19 patients with erythromycin-resistant *S. pyogenes* treated with clarithromycin and in 22 (88%) of 25 patients with erythromycin-susceptible strains. The constitutive-resistant phenotype was correlated with failure of macrolide treatment.

Over the last decade, severe infections due to *Streptococcus pyogenes* and its complications have reemerged in several parts of the world (1-3). *S. pyogenes* is uniformly susceptible to penicillin, which remains the drug of choice for treating infections by this organism. Erythromycin and other macrolides have been recommended as alternative treatment for patients allergic to penicillin (2,4); however, resistance to erythromycin and related drugs in *S. pyogenes* has become widespread (5). Resistance to erythromycin was first described in 1955 in the United Kingdom (6) and, more recently, has been reported in Japan (7), Finland (8), Taiwan (9), Australia (10), the United States (11), Spain (12,13), and Italy (14-16).

From 1991 to 1996 in Genoa, the percentage of *S. pyogenes* resistant or with intermediate resistance to erythromycin increased from 0% to 50% (17). This abrupt increase in the rate of erythromycin-resistant strains is of concern, since erythromycin has been effective against most *S. pyogenes* isolates.

We investigated the prevalence and distribution of macrolide resistance phenotypes among *S. pyogenes* and carried out a clinical study in patients with *S. pyogenes* pharyngitis to correlate clinical and microbiologic outcomes with in vitro susceptibility patterns.

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The Study

Ten pediatricians in Genoa (population 700,000) participated in this study. Children included in the study had to have two or more of the following signs and symptoms: oropharyngeal erythema, fever and sore throat, tonsillar exudate or cervical lymphadenitis, and strawberry tongue.

S. pyogenes was confirmed by culture of throat swabs in agar blood; ß-hemolytic colonies were identified as *S. pyogenes* by the bacitracin disk (Difco Laboratories, Detroit, MI) and latex-agglutination test (Streptex, Wellcome, U.K.). Minimum inhibitory concentrations (MICs) for penicillin, cefixime, ceftriaxone, chloramphenicol, rifampin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin were determined by using the PASCO MIC gram-positive panel (Difco Laboratories, Detroit, MI), supplemented with horse blood. MICs for clindamycin, erythromycin, azithromycin, and clarithromycin were determined by using E-test strips (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar supplemented with 5% horse blood incubated in an atmosphere containing 5% carbon dioxide. Phenotypes of macrolide resistance were differentiated according to the description of Seppala et al. (18) and Suttcliffe et al. (19). Resistance to both erythromycin and clindamycin indicated a constitutive type of resistance (CR), blunting of the clindamycin zone of inhibition proximal to erythromycin indicated an inducible type of resistance (IR), and susceptibility to clindamycin without blunting indicated the M-phenotype of

resistance. For all antibiotics tested, the break-points suggested by the National Committee for Clinical Laboratory Standards were used (20,21).

At their physicians' discretion, eligible patients received a 10-day course of one of the following drugs: amoxicillin 75 mg/kg three times a day; amoxicillin/clavulanic acid 50 mg/kg twice a day; cefaclor 50 mg/kg twice a day; or clarithromycin 15 mg/kg twice a day. The attending physician was blinded to the results of microbiologic tests. Fisher's exact test and the chi-square test were performed by using Epi Info, version 6. For all tests, a p value of ≤ 0.05 was considered statistically significant.

Six hundred children ages 1-13 years (median age 7.0) with acute pharyngitis were observed, and 180 (30%) whose throat cultures were positive for *S. pyogenes* were included in the study. Amoxicillin was prescribed to 42 patients, amoxicillin/clavulanic acid to 56, cefaclor to 35, and clarithromycin to 44. The clinical cure rates were 79.5% (35 of 44) in the clarithromycin group, 92% (39 of 42) in the amoxicillin group (p = 0.14 for comparison with clarithromycin), 100% (56 of 56) in the amoxicillin/clavulanic acid group (p = 0.0003 for comparison with clarithromycin), and 97.1% (34 of 35) in the cefaclor group (p = 0.03 for comparison with clarithromycin).

Results of post-treatment throat swabs were available from 159 patients. Bacterial eradication response rates were 77.2% (34 of 44) with clarithromycin, 88.8% (32 of 36) with amoxicillin group (p = 0.28 for comparison with clarithromycin), 95.8% (46 of 48) with amoxicillin/clavulanic acid (p = 0.03 for comparison with clarithromycin), and 90.3% (28 of 31) with cefaclor (p = 0.24 for comparison with clarithromycin). All 180 strains were susceptible to penicillin (MIC_{90} <0.06 □g/l) and other ß-lactams tested (Table 1). Overall, 69 (38.3%) of the 180 isolates were resistant to one or more macrolides, 7 (3.9%) to clindamycin, and 21 (11.6%) to the 16-member macrolide rokitamycin. Sixty-two percent (43 of 69 strains) of the erythromycin-resistant strains showed the M phenotype of resistance, 11.5% (8 strains) the CR phenotype, and 26.0% (18 strains) the IR phenotype.

Among the 159 patients, 19 (43.1%) of 44 treated with clarithromycin, 16 (44.4%) of 36 treated with amoxicillin, 13 (27.0%) of 48 treated with amoxicillin/clavulanic acid, and 8 (25.8%) of 31 treated with cefaclor had *S. pyogenes* resistant to erythromycin at the first swab collected before treatment.

S. pyogenes was eradicated in 12 (63.1%) of the 19 patients with erythromycin-resistant isolates and in 22 (88.0%) of 25 patients with erythromycin-susceptible isolates treated with clarithromycin (p = 0.07). As a control, the results of ß-lactam treatment were also studied. The rates of microbiologic eradication in patients with erythromycin-resistant isolates were 87.5% (14 of 16) for amoxicillin, 100% (13 of 13) for amoxicillin/clavulanic acid, and 100% (8 of 8) for cefaclor. Rates of microbiologic eradication for erythromycin-susceptible strains were 90% (18 of 20) for amoxicillin, 94.2% (33 of 35) for amoxicillin/clavulanic acid, and 86.9% (20 of 23) for cefaclor (p = 1.0; p = 1.0; p = 0.54, respectively, for comparison with erythromycin-resistant isolates).

In clarithromycin-treated patients, 6 of the 7 treatment failures were related to isolates with a CR phenotype (p = 0.0002 for comparison of percentages with other phenotypes of resistance, and p = 0.0001 for comparison with erythromycin-susceptible isolates) (Table 2).

Table 1. In vitro susceptibility[a] of *Streptococcus pyogenes* from 180 pharyngitis patients

		Erythromycin-resistant		
	Erythromycin susceptible	M-phenotype	IR[b]	CR[c]
Erythromycin	0.25	32	64	>128
Clarithromycin	0.5	16	64	>128
Azithromycin	0.25	16	64	>128
Clindamycin	0.5	1	1	>128
Rifampin	1	1	1	2
Chloramphenicol	≤4	≤4	≤4	≤4
Ciprofloxacin	0.25	1	2	2

[a]MIC_{90} in □g/ml; [b]IR = Inducible-type resistance; [c]CR = Constitutive-type resistance.

Table 2. Frequency of clarithromycin failure, by susceptibility profile

	No. patients treated	No. treatment failed (%)	p value
Erythromycin-susceptible	25	3 (12)	
Erythromycin-resistant	19	7 (36.8)	0.07[a]
CR[b]	6	6 (100)	0.0001[a]
other phenotypes	13	1 (7.6)	1.0[a]; 0.0002[c]
Total	44	10 (22.7)	

[a]For comparison of percentage of failure with erythromycin-susceptible; [b]CR = Constitutive-type resistance;[c]For comparison of percentage of failure between CR and other phenotypes of resistance.

Conclusions

Our results show that in Genoa, 38% of *S. pyogenes* isolated from pharyngitis patients are erythromycin resistant. Sixty-three percent of such isolates belonged to a recently reported, noninducible M phenotype, described as having low-level resistance to erythromycin and sensitivity to clindamycin and 16-member macrolides (18). Twenty-six percent of resistant strains were classified as IR phenotype, characterized by low-level resistance to erythromycin and inducible resistance to 16-member macrolides and clindamycin after exposure to subinhibitory concentrations of erythromycin (5). The remaining isolates (11.5%) showed the CR phenotype, characterized by high-level resistance to macrolides and clindamycin.

Our study also examined whether in-vitro resistance could be a good predictor of clinical outcome in children with pharyngitis. Although physicians were instructed to choose the antibiotic without regard to clinical signs and symptoms, a bias due to selective antibiotic choice based on clinical presentation cannot be excluded. Clarithromycin was prescribed to 44 patients, 19 with erythromycin-resistant isolates and 25 with erythromycin-susceptible isolates. Although the rate of microbiologic eradication did not differ between patients with erythromycin-resistant isolates and those with erythromycin-susceptible isolates (63.1% vs. 88.0%; $p = 0.07$), a clear trend was observed toward a higher rate of eradication among erythromycin-susceptible isolates.

When results in clarithromycin-treated patients were analyzed by phenotype of resistance, the rate of treatment failure was 100% (6 patients) for CR phenotype, compared with 7.6% (1 of 13 patients) for other phenotypes ($p = 0.0002$) and 12% (3 of 25 patients) for erythromycin-susceptible isolates ($p = 0.0001$).

Failure of erythromycin to eradicate group A streptococci with high levels of resistance to erythromycin and lyncosamide has been reported (22,23). Seppala et al. (8), in a retrospective analysis of medical records, found that erythromycin failed in 47% of pharyngitis patients with erythromycin-resistant isolates, a rate significantly higher than the 4% observed in patients with erythromycin-susceptible isolates. The susceptibility profile of these strains, however, was consistent with phenotypes other than CR.

The eradication rate in patients with isolates belonging to phenotypes other than CR, thus showing low levels of resistance to macrolides, was comparable with that observed for erythromycin-susceptible isolates. However, our findings suggest that CR phenotype will be an accurate predictor of in-vivo failure of macrolides in the treatment of streptococcal pharyngitis. Whether the discrepancy between our results and those of a previous Finnish study (8) should be attributed to differing macrolides remains to be proven by large, well-controlled studies. Despite in-vitro cross-resistance with other 14-member macrolides, clarithromycin is characterized by elevated concentrations attained in different tissues (including tonsil tissue) because of its improved pharmacokinetic profile (5,24).

Because only a few alternative antimicrobial agents can be used to treat pharyngitis in patients allergic to ß-lactams, adequate interventions include a controlled use of macrolides and surveillance for the susceptibility of group A streptococci. Determining erythromycin resistance phenotypes seems to be a useful tool, particularly in areas where macrolides are frequently prescribed. Should the CR phenotype, reported infrequently at present, become prevalent, its high-level resistance may threaten the efficacy of macrolides and clindamycin in the treatment of streptococcal pharyngitis.

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Sin Nombre Virus (SNV) Ig Isotype Antibody Response during Acute and Convalescent Phases of Hantavirus Pulmonary Syndrome

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Serum samples from 22 hantavirus pulmonary syndrome (HPS) patients were tested for Sin Nombre virus (SNV)-reactive antibodies. In the acute phase of HPS, 100% and 67% of the samples tested positive for SNV-specific immunoglobulin (Ig) M and IgA, respectively. Among the virus-specific IgG antibodies, the most prevalent were IgG3 (in 97% of samples), followed by IgG1 (70%), IgG2 (30%), and IgG4 (3%).

Hantaviruses are associated with hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans (1). Sin Nombre virus (SNV), a newly identified hantavirus, was identified as the causative agent in a 1993 outbreak of HPS with a >60% case-fatality rate in the southwestern United States (2). SNV and other American hantaviruses associated with HPS have a specific rodent reservoir; transmission was inferred to occur primarily by inhalation of infected aerosols from the rodent urine and excreta (3,4). However, an outbreak with human-to-human transmission of HPS caused by Andes virus has also been reported from South America (5). HPS is clinically characterized as an acute febrile illness associated with headache, malaise, and myalgia that proceeds to thrombocytopenia, pulmonary edema, and hypotension or shock (1,2). In fatal cases, death usually occurs 1 to 2 days after onset of respiratory symptoms. Although SNV-specific $CD4^+$ and $CD8^+$ T lymphocytes have been documented (6), the virus-specific humoral immune response has not yet been well defined.

The different patterns of increase in immunoglobulin (Ig) classes are associated with Th1- or Th2-type immune responses in mice (7). In some parasitic diseases, increased antigen-specific IgG4 subclass levels correspond to a variable extent with the induction of antigen-specific T-cell anergy, sometimes associated with serious or disseminated disease (8,9). Antibody responses to polysaccharide antigens have been reported to be predominantly IgG1/IgG2 (10). In viral diseases, IgG1 and IgG3 subclasses were detected predominantly in primary infections, while IgG2 and IgG4 subclasses were more characteristic of recurrent infections (11-14).

Ig class- and subclass-specific titers of antibodies to SNV, their kinetic appearance, and their role in preventing disease or death in HPS patients has not been reported. To address this issue, we developed SNV-specific, immunoglobulin class/subclass-specific enzyme-linked immunosorbent assays and evaluated the levels of SNV-specific IgA, IgM, and IgG subclass antibodies in the serum samples from HPS patients. Ig class- and subclass-specific titers were compared in the sera of patients who survived and those who died.

The Study

Thirty-three serum samples were obtained from 22 patients hospitalized with HPS in 1993. Patients were diagnosed as having HPS on the basis of results from immunohistochemistry

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analysis, alone or in combination with serologic studies or reverse transcription-polymerase chain reaction (16). The samples were divided into three groups with respect to the time of collection: acute phase (1-8 days posthospitalization, 12 samples), early convalescent phase (9-30 days posthospitalization, 9 samples), and late convalescent phase (31 days to 1.5 years posthospitalization, 12 samples).

Human Ig antibodies were captured by affinity-purified, polyclonal, monospecific sheep antihuman IgM, IgG1, IgG2, IgG3, IgG4, and IgA reactive antibodies (IgM: Biosource, Camarillo, CA; IgG1, IgG2, IgG3, IgG4, IgA: The Binding Site, San Diego, CA). The capture antibodies were coated onto 96-well high-binding microtiter plates (Corning Costar Corporation, Charlotte, NC) at 4°C for a minimum of 16 hours (0.5 mg/ml in 25mM sodium carbonate buffer pH 9.6; 0.1 ml/well). Sera and detection antibodies were diluted in PBS, pH 7.4 to 7.6, containing 0.05% Tween 20 supplemented with 4% milk (PBSM). Initially, all plates were washed and blocked with PBSM for 30 minutes at room temperature. Fourfold dilutions of the plasma samples in a volume of 0.1 ml were incubated for 1 hour at 37°C, then the plates were washed and SNV-infected Vero E6 cell slurries (CDC, Atlanta, GA; SPR 268) and uninfected control slurries (CDC, Atlanta, GA; SPR 391), diluted 1:10, were added to appropriate wells. After incubation for 1 hour at 37°C, the plates were washed and incubated with a polyclonal rabbit anti-SNV serum (CDC, Atlanta, GA; diluted 1:1000, 0.1 ml/well) as described. Then the plates were washed and an alkaline phosphatase-conjugated sheep Ig anti-rabbit Ig (Southern Biotechnology Associates, Birmingham, AL) diluted 1:4000 (0.1 ml/well) was added to each well. After incubation for 1 hour at 37°C, the plates were washed and the substrate p-nitrophenylphosphate (Bio-Rad, Hercules, CA) was added. The reaction was stopped by the addition of 0.1 ml 0.4 M NaOH, and the optical density was read at 405 nm.

Where applicable, antibody titers are presented as geometric mean titers (GMT), with the range of positive titers. Antibody titers for significant differences between survivors and patients who died in the acute-phase group were compared by unpaired Student's t-test.

SNV-specific IgM, which had the highest titers overall (GMT 15,097, range 1,600-102,400), was detected in all acute-phase samples and 89% of samples from the early convalescent phase (Table). SNV-specific IgM was also detected in one sample from the late convalescent phase (titer 25,600 at day 67), a phenomenon occasionally observed in other HPS convalescent-phase samples (data not shown). SNV-specific serum IgA was detected in 67% of acute-phase samples and 78% of samples from the early convalescent phase, but the titers were generally lower (GMT 9,406, range 100 to 409,600) than the IgM titers. Two of the 12 patients during the late convalescent phase had detectable virus-specific IgA, although the titers were relatively low (1,600 and 100). Patients who had very low or undetectable SNV-specific IgA generally also had lower titers of SNV-specific IgM during the acute phase of illness.

In the IgG class, the highest SNV-specific antibody titers were predominantly of the IgG3 subclass (GMT 4,935, range 100-409,600), detected in 97% of samples. SNV-specific IgG1 antibodies were also detected in 76% of samples, but in much lower titers (GMT 447, range 100-6,400). SNV-specific IgG2 antibodies were detected in only 30% of samples (GMT 348, range 100-6,400), and only one serum tested positive for SNV-specific IgG4, with a very low titer (1:100).

The Ig class- and subclass-specific antibody titers were further analyzed with regard to the outcome of the disease. The 12 patients who had samples drawn during the acute phase of infection included six who died and six who survived. The SNV-specific antibody titers of these two groups did not differ significantly in any Ig class or subclass tested ($p > 0.1$ in each Ig class or IgG subclass group, data not shown).

When total IgG, IgA, and IgM concentrations were measured in 18 serum samples from randomly chosen HPS patients, 12% of the IgM values (reference range 56-332 mg/dl), 39% of the IgA values (reference range 70-312 mg/dl) and 56% of the IgG values (reference range 639-1349 mg/dl) fell outside the normal reference range. Interestingly, increased titers of both SNV-specific IgA and IgM did not correlate with patients' levels of total serum IgA and IgM. This lack of correlation suggests that the increase of SNV-specific IgM and IgA antibodies is not due to nonspecific polyclonal activation of the immune system.

As expected, SNV-specific IgM antibodies are observed during the initial phase of disease; both SNV-specific serum IgA and IgM antibodies peaked early during the acute phase. These levels were

Table. Total Ig concentrations and specific Sin Nombre virus Ig titers in sera from patients with hantavirus pulmonary syndrome

Patient[a]	Days[b]	IgM	IgG1	IgG2	IgG3	IgG4	IgA	Total IgM [mg/dl]	Total IgG [mg/dl]	Total IgA [mg/dl]	Phase
7(d)[c]	1	25,600	400	0	1,600	0	25,600	264	1,000	336	Acute
8(d)	1	1,600	0	0	100	0	0	559	<333	<67	Acute
9(d)	1	25,600	400	0	6,400	0	102,400	295	802	<40	Acute
6.1	2	102,400	6,400	100	25,600	0	25,600	280	1,590	492	Acute
10(d)	2	25,600	100	0	400	0	0	nd[d]	4,840	234	Acute
5.1	2	25,600	100	0	1,600	0	409,600	1,080	176	342	Acute
21.1	4	102,400	100	6,400	409,600	0	0	409,600	nd	nd	Acute
23.1	7	25,600	100	100	400	0	25,600	nd	nd	nd	Acute
1.1	7	1,600	400	100	6,400	0	400	142	401	108	Acute
11(d)	7	102,400	100	0	400	0	6,400	nd	nd	nd	Acute
23.2	8	25,600	0	0	100	0	0	nd	nd	nd	Acute
12(d)	8	6,400	0	0	0	0	0	108	<381	<67	Acute
4.3	10	6,400	0	0	1,600	0	25,600	nd	nd	nd	Early conv.[e]
2.1	12	25,600	6,400	1,600	25,600	0	409,600	225	508	125	Early conv.
21.2	13	102,400	100	6,400	409,600	100	409,600	nd	nd	nd	Early conv.
23.3	13	6,400	0	0	100	0	100	nd	nd	nd	Early conv.
3.1	16	6,400	6,400	100	6,400	0	0	322	1,170	290	Early conv.
1.2	21	25,600	6,400	400	25,600	0	0	nd	nd	nd	Early conv.
4.1	24	1,600	0	0	6,400	0	6,400	nd	nd	nd	Early conv.
13	24	0	6,400	0	102,400	0	102,400	nd	nd	nd	Early conv.
14	30	1,600	0	0	1,600	0	100	nd	nd	nd	Early conv.
15	34	0	100	0	6,400	0	0	201	1,400	263	Late conv.
3.2	35	0	6,400	0	6,400	0	0	176	2,110	221	Late conv.
4.2	59	0	0	0	1,600	0	1,600	144	1,310	95	Late conv.
2.2	66	0	1,600	100	6,400	0	0	126	1,140	140	Late conv.
16	66	0	100	0	25,600	0	0	69	977	82	Late conv.
20.2	67	0	400	100	6,400	0	100	nd	nd	nd	Late conv.
6.2	67	25,600	6,400	0	102,400	0	100	219	1,700	403	Late conv.
17.1	92	0	100	0	102,400	0	0	107	1,000	162	Late conv.
5.2	145	0	100	0	1,600	0	0	78	1,260	288	Late conv.
18	330	0	100	0	25,600	0	0	nd	nd	nd	Late conv.
17.2	330	0	100	0	6,400	0	0	nd	nd	nd	Late conv.
19	547	0	100	0	400	0	0	nd	nd	nd	Late conv.

[a]Decimal values indicate sequential samples from one patient.
[b]Days after patient reported to hospital.
[c](d) = patient died.
[d]nd = not done.
[e]conv. = convalescent.

maintained during both the acute and the early convalescent phases. SNV-specific antibodies of IgG1 and IgG3 subclasses appeared during the first 10 days, coinciding with the appearance of symptoms. However, high titers were maintained throughout early (IgG1) or early and late convalescent phases (IgG3). In 5 (70%) of the 7 samples that tested positive for SNV-specific IgG2 antibodies, these titers appeared during the acute and early convalescent phases but were lost thereafter.

Conclusions

We tested serum and plasma samples from HPS patients for the presence of IgA and IgM class and the IgG subclass-specific antibody titers against one of the SNV antigens. Although the design of this study is cross sectional, we observed a characteristic pattern of appearance of specific Ig classes/subclasses during the course of infection. In addition, sequential samples from several patients were included in the study (represented by the decimal values in the Table)

and the data from these samples show trends similar to those observed in the composite with single samples. SNV-specific IgM and IgA antibodies appeared in high titers predominantly during the early phases of infection (in 95% and 71% of samples, respectively). In our IgG subclass-specific assays, most samples contained IgG3 and IgG1 subclass-specific SNV-reactive titers throughout illness. The detection of substantial IgG3 antibody titers early in hospitalization can be explained by the fact that since infection occurred several days before onset of illness, there was sufficient time to allow a switch to the IgG class to occur. In contrast with other primary viral infections, a substantial number of samples showed high titers of SNV antigen-reactive antibodies belonging to the IgG3 subclass rather than the IgG1 subclass. It is possible that in SNV infection, specific sets of cytokines may be produced that preferentially stimulate the production of IgG3 subclass antibody.

The similarity in antibody titers between deceased and surviving patients is an important observation. Regardless of the eventual outcome of infection, the sera from patients during hospitalization with either HPS or HFRS contained high levels of antibody. Abundant viral antigens were present within endothelial cells of the pulmonary microvasculature, as well as significant levels of $CD8^+$ T-cell infiltrates and evidence of circulating pro-inflammatory cytokines in serum. These findings suggest that the disease is most likely secondary to immunopathologic mechanisms (6,15,16). Thus, antibodies may well play an important role in containing the initial viremic phase of the infection, but T-cell activation may have an important role in inducing disease. In addition, there may be epitopes of the virus antigens that are not detected by the assays used in the studies reported here and antibodies against these epitopes may distinguish infected persons who do or do not become ill. Further studies to identify differences in the viral epitopes recognized by sera from patients who died compared with those who survived, as well as comparison of antibody functionality, are needed to address these issues. Finally, anti-SNV IgM antibodies were detected in 100% of the patients in the acute phase of the infection in relatively high titers (1,600 to 102,400). This finding confirms that the SNV-specific IgM-capture enzyme immunoassay we describe can be used as a valuable tool in the early diagnosis of HPS.

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Bovine Tuberculosis and the Endangered Iberian Lynx

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We report the first case of bovine tuberculosis in a free-living Iberian lynx (*Lynx pardina*), an extremely endangered feline, from Doñana National Park in Spain. The isolate (*Mycobacterium bovis*) correlates by molecular characterization with other isolates from wild ungulates in the park, strongly suggesting an epidemiologic link.

Mycobacterium bovis infects many animal species, with wild and free-ranging domestic ungulates being the main reservoirs in nature (1). Carnivores generally acquire the infection by eating infected food (2,3), but reports of tuberculosis (TB) in wild carnivores are rare (2-6). However, TB monitoring in free-living carnivores has provided increased reports of the disease (7). TB does not pose a serious threat to most wild carnivore populations but could have a devastating effect in a small and divided population such as the Iberian lynx. This species is the most endangered feline in the world (8), with a declining number of animals living in reduced and isolated areas in Spain and Portugal.

An adult male Iberian lynx, seen limping in August 1998, died in October 1998. We could perform only direct and radiologic examinations of the empty carcass. The right elbow joint was enlarged, with fistulization. On X-ray, the lesion was diagnosed as septic arthritis on the basis of radiolucent areas and sclerosis, along with bony excrescences. A fragment from the right elbow joint, a small fecal sample, and a nasal swab were collected and routinely processed for detection of mycobacteria by auramine acid-fast staining, culture, and polymerase chain reaction (PCR) (9-11).

Auramine staining of a smear from the elbow lesion was performed by the method of Smithwick (9), and transmission fluorescence microscopy at x400 showed acid-fast bacilli. Samples were simultaneously processed for culture following standard procedures: a suspension was obtained from a nasal swab, and the other samples were homogenized in sterile distilled water in a tissue homogenizer. The homogenates and suspension were then decontaminated with hexadecylpyridinium chloride and centrifuged, and sediments were inoculated onto Coletsos and Löwenstein-Jensen media for mycobacteria; they were incubated at 37°C and inspected weekly for growth (10). Colonies were examined for acid-fast bacilli by the Ziehl-Neelsen technique (9). Identification procedures were performed as described elsewhere, by means of direct PCR either on the processed samples or on isolated colonies (10), and specific mycobacterial molecular characterization was done by spoligotyping (11). The only positive sample was the elbow joint: direct PCR was positive for *M. tuberculosis* complex. An isolate consisting of acid-fast bacilli on selective media was identified as *M. bovis*. Additionally, molecular characterization by spoligotyping showed that the lynx isolate was identical to other isolates from samples of wild ungulates living in the park, including nine wild boars (*Sus scrofa*) and four fallow deer (*Dama dama*) studied during the past 3 years.

Once TB has been confirmed in a free-living lynx, it is important to determine whether a single animal has been affected or the infection has spread within the population. From 1993, postmortem examinations have been performed

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on 21 lynxes in conditions that permitted reliable interpretation of pathologic findings. Any granulomatous lesions found during necropsy, as well as fecal samples taken regularly from temporarily or permanently captive animals, were tested by acid-fast staining, direct PCR, and culture. Radiologic screening has also been performed on captive and live-captured animals.

TB had never been diagnosed in lynxes before this case. However, because of the insidious nature of the infection, this case will not be the last; other lynxes could already be infected. The prevalence of the infection in the species is extremely difficult to determine because the number of lynxes is low and very few animals can be monitored, since captures are limited to a minimum. In addition, rapid, specific, and accurate in vivo diagnosis of TB in lynxes is difficult. A reliable, rapid technique for diagnosis is urgently needed to ensure that any captured animal will be correctly diagnosed and treated.

Another important consideration is the role of wild ungulates and domestic livestock as reservoirs. Populations of red deer (*Cervus elaphus*), fallow deer, and wild boar live in the National Park. Traditional farming in the area means that many domestic animals (mainly free-ranging cattle) coexist with wild species. All these species are susceptible to TB and could act as reservoirs (1). Carnivore populations are generally considered spill-over hosts that become incidentally infected and therefore are unlikely to maintain the disease without an external source of infection (3,6,7). Because eradication campaigns are conducted in domestic cattle, the prevalence of bovine TB is low, but cattle are not TB free. Wild ungulates have been monitored over the last 11 years; a few isolated cases were detected before 1993, although the number of cases has increased dramatically in recent years. This observation may represent a real increase in prevalence or a higher intensity of surveillance. Therefore, surveys of the prevalence of TB among wild ungulates in the Park need to be enhanced to allow a more accurate assessment of the problem.

The isolate of *M. bovis* from the lynx correlates exactly by spoligotyping with isolates obtained recently from wild boars and fallow deer living in the same area, suggesting a common source of infection. As fallow deer are known to be part of the lynx's diet (12), these results are not surprising and strongly suggest interspecies infection. However, since no cultures from cattle are available, the molecular comparison of strains isolated from wild and domestic animals is not possible and a potential epidemiologic link among them cannot be demonstrated. Nevertheless, TB transmission between domestic cattle and wild ungulates sharing the same pastures has been described (13). The different patterns of lesions in red and fallow deer (mainly respiratory) and wild boars (digestive) suggest the probable routes of infection, but the modes of transmission need to be elucidated.

Infectious diseases affecting both domestic and wild animals become a problem for both conservationists and farmers. In this case, the concern is not only transmission between cattle and wild ungulates, but transmission to the Iberian lynx, an endangered species protected by strict conservation policies. Thus, health requirements for domestic animals entering the park should be strengthened to prevent TB, and livestock populations must be managed and controlled, as a total ban seems unfeasible.

The role of lagomorphs in this case is speculative, as TB has not been diagnosed in these species at the park. Although naturally acquired TB seems to be extremely rare in rabbits, it is not uncommon in hares (14). Since these animals are a basic item of the lynx's diet (12), monitoring TB in lagomorphs must be included in a prevention program so that a potential source of infection should not be overlooked.

The detection of bovine TB in an Iberian lynx leads to another question: what should be done with a free-living lynx with a positive TB diagnosis? Intraspecies transmission seems quite unlikely because of the rare contact between lynxes except during mating season. However, basic rules of animal health dictate that infected animals should be isolated to interrupt the transmission cycle of many infectious diseases, including TB.

Anti-TB therapy in animals is controversial, and euthanasia is the most commonly accepted option. However, the critical situation of the Iberian lynx, with a declining population of under 1,000, makes every animal essential. Multidrug, long-term, daily therapy has been used in domestic cats (2,3), but extrapolating this treatment to lynxes maintained in captivity would be difficult. Although therapy must be the choice, adequate facilities and containment and preventive measures must be in place to ensure

that disease will not spread during treatment. In addition, the likelihood of success of treatment, which depends on the severity of the disease and the general condition of the animal, must be evaluated in light of the well-known collateral effects of antimycobacterial drugs (2,3). Finally, even if treatment is successful, the animals will probably not be able to live in the wild again because of possible physical or behavioral changes.

In conclusion, survival of many species already depends on human management of populations (15); in this case, every measure must be adopted to ensure that bovine TB will not threaten the endangered Iberian lynx.

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Haff Disease: From the Baltic Sea to the U.S. Shore

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Haff disease, identified in Europe in 1924, is unexplained rhabdomyolysis in a person who ate fish in the 24 hours before onset of illness. We describe a series of six U.S. patients from 1997 and report new epidemiologic and etiologic aspects. Although Haff disease is traditionally an epidemic foodborne illness, these six cases occurred in two clusters and as one sporadic case.

In the summer and fall of 1924, physicians near the Königsberger Haff shores along the Baltic coast recognized an outbreak of an illness characterized by sudden, severe muscular rigidity (1-3). No neurologic abnormalities, fever, splenomegaly, or hepatomegaly were observed (1). Patients often had coffee-colored urine. The clinical spectrum of disease varied, few patients died, and most survivors recovered quickly. In the following 9 years, similar outbreaks, affecting an estimated 1,000 persons, occurred seasonally in the summer and fall along the coast of the "haff" (a shallow lagoon). Recent ingestion of fish, usually cooked, was common among those who became ill; species of fish included burbot, eel, and pike. Seabirds and cats reportedly died after eating fish in the wild. Because of the absence of fever and the fact that fish had been cooked, known infectious causes were eliminated. Several toxic etiologies were proposed but could not be confirmed. Among these was arsenic poisoning (4), which is still cited in modern medical dictionaries as the cause of Haff disease (5). From 1934 until 1984, other outbreaks resembling Haff disease were described in Sweden and the Soviet Union (6-9). The first two cases reported in the United States occurred in Texas in June 1984; through 1996, only four more cases were reported: two from Los Angeles, California, in 1985, and two from San Francisco, California, in April 1986 (M. Tormey, pers. comm.). All patients had eaten buffalo fish before onset of illness. Tests of the remains of one of the fish meals suggested a neutral lipid as a causative agent. Reports of six cases of Haff disease from California and Missouri during a 5-month period (March-August) in 1997 prompted an investigation with the objectives of describing the epidemiology and clinical characteristics of the 1997 U.S. cases of Haff disease, tracing back implicated fish, and elucidating the cause of Haff disease.

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The Study

Based on the clinical description of the original cases from 1924 to 1933, we defined a case of Haff disease as illness in a person with unexplained rhabdomyolysis who had eaten fish in the 24 hours before onset of symptoms. The laboratory marker used to define rhabdomyolysis was a fivefold or greater elevation in creatine kinase (CK) levels, with a muscle/brain (MB) fraction <5% (10). Cases were identified through county and state epidemiologists, as well as the Food and Drug Administration (FDA) laboratories in Bothell, Washington, and Dauphin Island, Alabama. We interviewed all persons with cases reported to the Los Angeles County Health Department or FDA. We reviewed medical records for demographic information, medical history, course of illness, and food exposures,

including method of preparation and source of food purchase. For the California cases (March 8-9 and August 8), we conducted active surveillance in city or community hospitals near the case-patients' residence during the 5 days surrounding the dates of onset. Surveillance included reviews of laboratory logs (for cases with highly elevated CK but a low MB fraction) and emergency room logs for all patients with a diagnosis of suspected fish poisoning.

State and local environmental management staff visited stores and markets where fish was bought to trace the implicated fish lots. We interviewed fish wholesalers regarding the number and location of fishermen catching buffalo fish. Because fish eaten by the patients originated from Louisiana and Missouri, we discussed with health departments and environmental experts possible sources of fish intoxication. Recovered leftovers and uncooked fish from the same lot were tested for sodium channel-active biotoxins (e.g., ciguatoxin [the toxin of ciguatera] or saxitoxin [the toxin of paralytic shellfish poisoning]), and cyanobacterial toxins (e.g., microcystin or nodularin, which are toxins of blue-green algae) (11,12). Samples from the Bakersfield case were tested for organophosphates, and other samples were tested for arsenic.

To characterize the physicochemical properties of the toxin, extract from both cooked and uncooked fish was partitioned into water-soluble, nonpolar lipid (hexane) and polar lipid (chloroform) fractions. These fractions were then administered intraperitoneally and orally to laboratory mice. Fractions causing toxicity to mice were further analyzed for identification of the toxin.

From March through August 1997, two clusters of Haff disease cases occurred in Los Angeles, California, and St. Louis, Missouri (13); one isolated case occurred in Bakersfield, California. The Los Angeles cluster consisted of two sisters who lived together and a third patient who was admitted to the same hospital during the same weekend. The St. Louis cluster was a married couple. The four meals, all eaten at home, contained buffalo fish. Six (75%) of the eight persons who ate buffalo fish became ill. All patients were >30 years of age (33 to 87 years); five were Ukrainian immigrants, and one was African American. Three of the six patients were taking medications, including aspirin, codeine, and simvastatin, that could have exacerbated rhabdomyolysis. Two persons who vomited shortly after the meal had either milder symptoms or lower laboratory values. The median incubation time to onset of symptoms after fish ingestion was 8 hours (6 to 21 hours). All patients were hospitalized, none died, and the median hospital stay was 3 days. Clinically, five of six patients had rapid onset of generalized muscular pain and rigidity, so severe that in one case ventilation was required (Table). In one patient who had chest pain only, the diagnosis of Haff disease was made through the exclusion of other causes of chest pain and the epidemiologic link with two other case-patients who had eaten buffalo fish purchased from the same market.

Table. Symptoms of Haff disease cases, United States, 1997

Symptom (n = 6[a])	No. reports
Myalgia	5
Muscular stiffness	5
Pain to light touch	5
Dry mouth	3
Painful breathing	2
Chest pain	2
Nausea or vomiting (within 1 hour after the meal)	2
Numbness of thighs	1
Numbness of whole body	1

[a]One patient may have had multiple symptoms.

The predominant laboratory abnormalities were elevated CK and myoglobin. Other muscle enzymes, such as glutamate oxalate transaminase, glutamate pyruvate transaminase, and lactate dehydrogenase, were also elevated. The CK was elevated to a mean of 12,700 IU/L (normal is usually <300 IU/L) (Figure). At the peak of the CK, the MB fraction was <5% in all cases. The mean maximum myoglobin level, tested in three patients, was 6,997 IU/L (upper limit of normal 100 IU/L). Treatment consisted of intravenous fluids, in addition to mannitol or bicarbonate. Several of the patients had variable sequelae, including weakness and fatigue, for several months after the acute stage. Active surveillance has identified no further cases.

Buffalo fish (*Ictiobus cyprinellus*) is a bottom-feeding freshwater fish similar to carp. The fish was fried (four cases) or cooked for 3.5 hours as "gefilte fish" (two cases). Fish were purchased

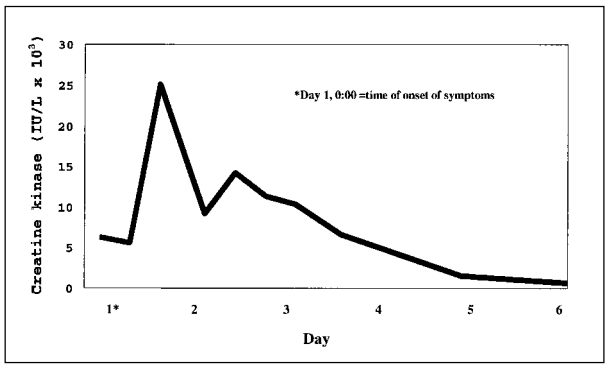

Figure. Creatine kinase elevation after the onset of symptoms in a patient with Haff disease, California, 1997.

locally either in a supermarket (California) or alive from a fish tank at a market (Missouri). Fish lots were caught by approximately 25 commercial fishermen from rivers in Louisiana (three incidents) or five fishermen in rivers and lakes near St. Louis, Missouri (one incident). No specific bodies of water could be identified as the origin of implicated fish. No fish or unusual animal die-off was noted in the areas where buffalo fish were caught.

Tests of the fish for toxins were either negative or below toxicity thresholds. The fact that the eaten fish was thoroughly cooked suggests that the presumed toxin is heat-stable. Mice fed hexane-soluble products extracted from cooked fish had behavioral changes consistent with muscle impairment; bladders contained red-brown urine.

Conclusions

Historically, Haff disease has been identified during seasonal outbreaks in Europe. This report documents that it may also occur sporadically or in small clusters. Ten of the 12 U.S. cases of Haff disease reported since 1984 occurred during March through August (M. Tormey, pers. comm.). All patients had eaten buffalo fish before becoming ill; 8 of the 12 patients were California residents, although the buffalo fish was caught in Louisiana or Missouri waters.

Most reported cases or outbreaks of Haff disease have been associated with freshwater fish, unlike most other seafood-related illnesses (e.g., ciguatera, scombroid fish poisoning, or paralytic shellfish poisoning), which are associated with saltwater fish (14). The clinical symptoms of Haff disease also differ from those of any other fish-related toxic or bacterial illness. Symptoms of rhabdomyolysis predominate and neurologic features are absent, in contrast to ciguatera or the various forms of shellfish poisoning (14). Because of the spectrum of symptoms, the potential for misdiagnosis is considerable. Furthermore, an increasing number of health-conscious persons prefer diets that include fish. In 1998, more than 4 billion pounds of fish, both domestic and imported, were eaten in the United States (website of National Oceanic and Atmospheric Administration: www.nmfs.org) Unusual smell or taste does not help identify toxic fish, and normal cooking methods cannot detoxify a fish capable of causing Haff disease. Because Haff disease may occur not only in epidemics but also in small clusters or sporadically, fish consumption should be included in the history of patients with unexplained rhabdomyolysis.

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Dr. Buchholz, who was with the Centers for Disease Control and Prevention, at the time of this study, is a medical epidemiologist with the Los Angeles County Department of Health Services. His interests include foodborne disease epidemiology, outbreak investigations, and surveillance for listeriosis and influenza.

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Chlamydia pneumoniae Infection in a Breeding Colony of African Clawed Frogs (*Xenopus tropicalis)*

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More than 90% of a breeding colony of clawed frogs (*Xenopus tropicalis)* imported to the United States from western Africa died in an epizootic of chlamydiosis. Chlamydial inclusions were observed by light and electron microscopy in the liver of an infected frog. *Chlamydia pneumoniae* was isolated in cell cultures from four frogs. A cutaneous infection by a chytridiomycete fungus observed in two frogs could have been a cofactor in the die-off.

Chlamydia infections cause disease in humans, birds, and mammals. Of the four currently recognized *Chlamydia* species, *C. psittaci* is the most important animal pathogen. Psittacosis, which can manifest as severe enteric and respiratory illness in many avian species, is highly contagious and can be transmitted to humans and many other mammals (1). *C. pecorum,* the newest species to be recognized, appears to have a highly restricted host range. Infections due to *C. pecorum* have been associated with sporadic encephalitis, polyarthritis, pneumonia, and conjunctivitis in pigs, sheep, cattle, and koalas (2,3). *C. trachomatis,* an agent responsible for millions of cases of ocular and urogenital infections worldwide, causes most chlamydial infections in humans. *C. pneumoniae*, an acute respiratory tract pathogen of cosmopolitan distribution, may be linked with chronic diseases such as coronary atherosclerosis and multiple sclerosis (1,4,5).

Initial reports of infections due to *C. pneumoniae* suggested that the organism's host range was limited to humans. Subsequently, infections due to *C. pneumoniae* have been documented in koalas, a horse, and most recently, a giant barred frog (*Mixophyes iteratus*) from Australia (6-8). We report an epizootic of chlamydiosis due to *C. pneumoniae* in a commercial colony of African clawed frogs (*Xenopus tropicalis*).

In July 1998, a biologic supply company located in the midwestern United States imported 241 *X. tropicalis* from western Africa to start a breeding colony. Upon arrival, the frogs were placed in quarantine. Within 4 months, 220 (91%) of the frogs had died. Before death, most of the frogs had sloughing of the skin and lethargy and appeared bloated. Two frogs were submitted to our veterinary diagnostic laboratory for necropsy. On external examination, the frogs were 5.5 cm and 5.2 cm from snout to vent and weighed 6.5 gm and 29.5 gm, respectively. Both frogs had roughening and early sloughing of the posterior skin. The smaller frog had scattered subserosal intestinal and hepatic nodules containing tetrathrydian cestodes. The larger frog was extremely edematous, with clear gelatinous fluid in the subcutaneous tissues and body cavities. In hematoxylin- and eosin-stained 4-□m sections of liver from the larger frog, there was moderate to severe lymphohistiocytic hepatitis with individual hepatocyte necrosis, fibrosis, and numerous granular basophilic bodies resembling bacteria within hepatocytes and histiocytes. Transmission electron microscopy of liver tissue from the larger frog confirmed the presence of bacteria replicating within vacuoles of infected cells. The organisms had a biphasic structure consistent with a *Chlamydia spp*. (Figure). Other

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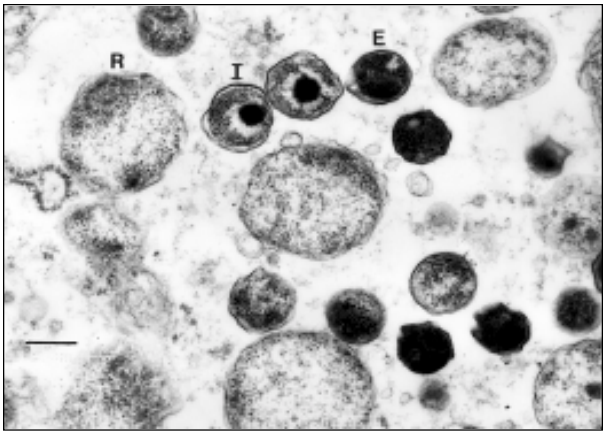

Figure. Transmission electron micrograph of chlamydial particles in liver from a captive African clawed frog (*Xenopus tropicalis)*. Note the reticulate bodies (R), intermediate bodies (I), and highly condensed elementary bodies (E). Bar, 270 nm.

lesions present in both frogs included mild to moderate lymphohistiocytic interstitial pneumonia and mild lymphocytic interstitial nephritis. No chlamydial inclusions were observed in other organs by light microscopy. Both frogs had multifocal epidermal hyperplasia with numerous intracorneal fungal organisms characterized by thick-walled sporangia containing multiple 2-□m to 3-□m round spores. Many of the sporangia had single tubular extensions (discharge papillae) directed toward the skin surface, and transmission electron microscopy showed the spores had flagella. These features were considered diagnostic of a chytridiomycete fungus (9).

To further characterize the *Chlamydia* species, liver samples were submitted for culture from the larger frog and three additional frogs obtained subsequently from the same breeding colony. Tissue homogenates were injected into shell-vials having monolayers of either buffalo green monkey kidney (BGMK), Hep-2, McCoy, or HeLa - 229 cells. Cultures were incubated at 35°C for 72 hours and then the cell monolayers were stained with a fluorescein-conjugated monoclonal antibody specific for the genus *Chlamydia* (Pathfinder, Kallestad, California). *Chlamydia* grew from all four liver specimens and in all the cell lines used. The organism grew better, with more and larger inclusions, in the McCoy and BGMK cell lines.

Chlamydia was identified by sequence analysis of the 16S rDNA and major outer membrane protein A (*ompA*) genes. Bacterial DNA was extracted by lysis (Generations, Gentra, Maryland) of liver and cell culture specimens from each of the four frogs. The broad-range eubacterial primers FD1(5'-AGAGTTTG ATCCTGGCTCAG-3') and RD1(5'-AAGGAGGTG ATCCAGCC-3') were used in a polymerase chain reaction (PCR) to amplify a 1,520-bp segment of the 16S rDNA gene (10); primers 20CHOMP (5'-TTAGAGGTRAGWATGAARAA-3') and CHOMP371 (5'-TAGAAICKGAATTGIGCRTTI AYGTGIGCIGC-3') were used to amplify a 1,200-bp segment of the *omp*A gene (11). Agarose gel electrophoresis of the PCR products yielded single bands for each specimen. Amplified products were column purified to remove unincorporated primers and nucleotides, and sense and antisense strands were sequenced by using nested primers in an automated laser fluorescent sequencer (ALF Express, Amersham Pharmacia Biotech, New Jersey). Sequence fragments were aligned with the assembly software program DNAsis 2.5 (Hitachi, California), and ambiguities resolved. A contiguous sequence was established from the overlapping regions, which were used as a query sequence in the GenBank database.

The 1,520-bp 16S rDNA sequences from the *Chlamydia* infecting the four *X. tropicalis* (GenBank-AF139200) were identical to one another and had 99.1%, 99.3%, and 99.4% homology with published sequences for *C. pneumoniae* strains N16 (U68426.2), CWL026 (AE001668), and TW-183 (L06108), respectively. In addition, there was 100% homology with the 235-bp segment of 16S rDNA reported for the *C. pneumoniae* isolated recently from the giant barred frog from Australia (AF102832). In contrast, there was 96.0% sequence homology with *C. psittaci* strain 6BC (U68447.2), 95.7% homology with *C. pecorum* strain E58 (D88317.1), and 93.8% homology with *C. trachomatis* strain D/UW-3/CX (AE001345).

The sequence of a 279-bp segment of the variable domain IV region of the *ompA* gene was determined for one of the frog *Chlamydia* isolates (AF184214). There were six nucleotide differences (97.8% homology) from the giant barred frog biovar of *C. pneumoniae* (AF102830) and three differences (98.9% homology) from *C. pneumoniae* strain CWL029 (AE001652). Less than 75% homology was observed with the *omp*A variable domain IV sequences reported for the three other *Chlamydia* species (results not shown).

Previous reports of confirmed *Chlamydia* infections among captive amphibians in North America involved *X. laevis*, another member of the African clawed frog family (12-14). These reports occurred before the discovery and molecular characterization of *C. pneumoniae*, and the *Chlamydia* species involved were either not known or presumed to be *C. psittaci*. Our study is the second documented report of *C. pneumoniae* involving an amphibian and the first describing an epizootic in a captive population of *X. tropicalis*.

The spectrum of clinical disease and histopathologic lesions associated with chlamydial infections in amphibians is unknown. In our study, only two animals were evaluated histologically. The more severely affected animal had active hepatitis with hepatocyte necrosis, lymphohistiocytic infiltrates, and inclusion bodies within hepatocytes and sinusoidal histiocytes. These lesions are similar to those observed in a 1984 outbreak of chlamydiosis among laboratory-bred *X. laevis;* in that outbreak, hepatosplenomegaly and histologic evidence of active hepatitis were the major pathologic findings in affected animals (12). In the recent case of *C. pneumoniae* involving a free-ranging giant barred frog, the predominant necropsy findings were chronic mononuclear pneumonia, nonregenerative anemia, and pancytopenia (8). Additional studies will be necessary to determine the epidemiology and virulence of *C. pneumoniae* in captive and free-ranging populations of amphibians and reptiles, as well as the pathogenicity and transmissibility of this agent to humans and other mammals.

This breeding colony had >90% deaths during the 4-month epizootic, and the few surviving frogs appeared ill and were subsequently euthanized. We were unable to determine the prevalence of *C. pneumoniae* infection in the colony but believe that it was very high, since all four frogs submitted for culture were positive. The crowding and stress associated with the captive environment may have contributed to spread of infection within the colony. The potential for zoonotic transmission of *C. pneumoniae* from amphibians to humans has important public health implications, especially in situations involving frequent and prolonged human contact with amphibians in crowded conditions, such as commercial breeding colonies or zoos. Until more is known about the host range of this pathogen, chlamydiosis should at least be considered in the differential diagnosis of an unexplained respiratory or febrile illness in persons exposed to amphibians.

Whether *Chlamydia* was the primary pathogen responsible for the die-off or served as a cofactor with the chytrid fungus or other parasites was difficult to determine. Although the *Chlamydia* infection is the primary focus of this report, the presence of cutaneous chytridiomycosis in two of the frogs is also important. This highly pathogenic zoosporic fungus has only been found in the American and Australian continents, where it has been implicated as a major factor in amphibian deaths and population declines in the rain forests (9). The origin of the chytrid fungus found in our cases was not determined. However, the rapid onset of illness affecting the frogs after their arrival in the United States suggests that at least some of the frogs may have been infected before capture.

The pipid frog *X. tropicalis* has a diploid genome and short generation time, which make it an ideal model organism for multigenerational genetic analysis. Current demand for this species is higher than most biologic supply companies can meet. Continued importation of *X. tropicalis* and other amphibians from regions of the world experiencing poorly understood population declines raises concern about the inadvertent spread of virulent pathogens to naive populations of amphibians and reptiles, as well as transmission of these agents to mammals. Until more is known about the epidemiology and prevention of these infections, caution must be exercised in transportation, husbandry, and human contact with these animals.

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The Impact of Health Communication and Enhanced Laboratory-Based Surveillance on Detection of Cyclosporiasis Outbreaks in California

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We investigated the timing of diagnosis, influence of media information on testing for *Cyclospora*, and the method used to identify cases during eight cyclosporiasis outbreaks in California in spring of 1997. We found that Internet information, media reports, and enhanced laboratory surveillance improved detection of these outbreaks.

Cyclospora cayetanensis, a recently identified coccidian parasite of humans (1), causes explosive watery diarrhea that can persist for several weeks (2). The oocysts cannot be detected by standard ova and parasite testing; methods specific for *Cyclospora* include modified acid-fast or other stains, autofluorescence with ultraviolet epifluorescence microscopy, and wet mount under phase-contrast microscopy (3). The infection can be treated with trimethoprim-sulfamethoxazole (TMP-SMX) (4). Eight outbreaks in California were among the 41 event-associated clusters of cyclosporiasis associated with Guatemalan raspberries identified in the United States and Canada in 1997 (5).

We examined factors contributing to the identification of outbreaks of this emerging infectious disease. The detection of outbreaks was enhanced by Internet information that patients brought to their physicians, media reports, and enhanced laboratory-based (ELB) surveillance. ELB surveillance, part of the California Emerging Infections Program (EIP), is a cooperative agreement between the California State Health Department, the University of California Berkeley School of Public Health, selected local counties, and the Centers for Disease Control and Prevention.

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The Study

An outbreak of cyclosporiasis was defined as diarrhea (three or more loose stools per day for at least 3 days) in two or more persons who shared a meal and became ill within 2 weeks, and laboratory confirmation of *Cyclospora* infection in at least one person. The first person with laboratory-confirmed *Cyclospora* infection identified in an outbreak was designated the index patient.

We interviewed each index patient about the onset of symptoms and medical care, and we reviewed laboratory records to determine the date of testing for *Cyclospora*. Through information that we gathered from patients and their physicians, we determined the reason for testing for *Cyclospora* (e.g., symptoms alone, symptoms and media attention, or symptoms and Internet information). We interviewed all guests at a wedding reception (event 3, Table) to determine the proportion of ill persons who sought medical attention, submitted stool specimens tested specifically for *Cyclospora,* and received recommended treatment for *Cyclospora* infections, all with respect to timing of media coverage of *Cyclospora* outbreaks.

To assess the impact of ELB surveillance on outbreak detection, we determined the proportion of outbreaks identified by ELB surveillance. All 22 laboratories in eight participating counties

Table. Event-associated outbreaks of cyclosporiasis, California, 1997

Outbreak number, type month/day of event	Event to symptoms	Symptoms to medical care	Seeking of medical care[a]	Timing of stool collection	Stool testing[a]	Stool collection to *Cyclospora* verification	Method of outbreak detection[b]
1, Banquet, 4/1	7 days	None–patient was physician	No	39 days after onset	No[c]	2 days	Report of illness cluster by patient[d]
2, Conference, 4/17	7 days	None–patient was physician	No	19 days after onset	No[e]	6 days	Report of illness cluster by physician[d]
3, Wedding, 5/3	7 days	18 days	Yes (I) bride sent information	1 day after visiting physician	Yes (I) patient requested	1 day	Interview of index patient identified through ELB[f] surveillance, 4 days
4, Barbecue, 5/10	8 days	1 day and repeat phone calls for 2 weeks	No	15 days after visiting physician	Yes (M) patient requested	1 day	Interview of index patient identified through ELB[f] surveillance, 6 days
5, Picnic, 5/11	7 days	18 days	Yes (M and I) Internet searches after media reports	1 day after visiting physician	Yes (I) patient requested	4 days	Interview of index patient identified through ELB[f] surveillance, 41 days
6, Card Party, 5/14	7 days	14 days	No	24 days after visiting physician	Yes (M) patient notified health dept., which recommended testing	3 days	Report of illness cluster by patient[d]
7, Dinner, 5/21	5 days	3 days	No	1 day after visiting physician	Yes (M) physician saw TV show on outbreaks	3 days	Interview of index patient identified through ELB[f] surveillance, 3 days
8, Luncheon, 5/24	6 days	1 day	Yes (M, and I) Internet searches after media reports	2 days after visiting physician	Yes (I) patient requested	1 day	Interview of index patient identified through ELB[f] surveillance, 3 days
Median (range)	7 days (5-8 days)	8.5 days (1-18 days)	2 days (1-24 days)		2.5 days (1-6 days)		4 days (3-41 days) For ELB surveillance notifications for all outbreaks

[a]Prompted by Internet (I) or Media (M) information.
[b]By local health department, time from laboratory verification to notification of the health department (if applicable).
[c]Testing was requested by the patient based on her knowledge of tropical medicine.
[d]The health department was aware of pending test results and the index patient was known to the health department prior to laboratory verification.
[e]Specific testing was not requested by a physician but was conducted by a laboratorian who had just read a journal article about *Cyclospora*.
[f]ELB denotes enhanced laboratory-based surveillance.

in northern California submitted biweekly or monthly laboratory reports on *Cyclospora* to EIP. EIP staff then forwarded case information to the local health departments for follow-up.

The eight index patients were tested for *Cyclospora* infections a median of 18.5 days after symptom onset (range 3-39 days, Table); 6 (75%) were diagnosed >14 days after onset (events 1-6, Table). The two index cases diagnosed within 1 week of disease onset occurred after media announcements about clusters of cyclosporiasis in the United States were widespread in late May 1997 (events 7 and 8, Table). Testing for 6 (75%) index patients was apparently prompted by this media coverage (events 3-8, Table). After reading newspaper articles, three index patients (events 3, 5, and 8) obtained information on the diagnosis and treatment of *Cyclospora* from Internet searches, brought this information to their physicians, and requested testing. One index patient (event 4) read about other clusters in the United States in the newspaper and requested testing from her physician. Another index patient (event 6) suspected that she and a group of friends had *Cyclospora* infections after reading a list of symptoms in a newspaper article and reported this cluster to the local health department, which then recommended testing for *Cyclospora.* One index patient (event 7) was tested by a clinician who watched a television report about cyclosporiasis the morning of the patient's visit to the clinic.

Within 2 weeks after a wedding party in May (event 3, Table), 30 (65%) of 46 guests had diarrhea; within 3 weeks, 13 sought medical attention. Although seven patients submitted stool specimens, none was tested for *Cyclospora*. Approximately 3 weeks after the wedding, several ill guests and the bride (who had not eaten the mixed berry dessert and was not ill) read media accounts of a *Cyclospora* outbreak that had occurred in Reno, Nevada. They recognized similar symptoms in themselves or their family members, obtained more information about *Cyclospora* from the CDC Web site, and distributed it to other guests. All 16 guests who brought that Internet information to their physicians received appropriate treatment with TMP-SMX; symptoms resolved promptly (typically within 24 hours). Three of these 16 guests were tested for *Cyclospora,* and stool specimens were positive.

In three outbreaks (events 1, 2, and 6, Table), the index patient was tested after a suspected foodborne outbreak had been reported to the local health department by symptomatic patients (events 1 and 6, Table) or an infectious disease specialist (event 2, Table). Index patients from these three outbreaks were identified by the health department within 1 day of their laboratory verification.

In six outbreaks, patients lived in counties with ELB surveillance; 5 (83%) of these outbreaks were detected by interviewing index patients with positive specimens identified through ELB surveillance (events 3, 4, 5, 7, and 8, Table). The health departments were notified of 4 of the 5 index cases detected through ELB surveillance 3 to 6 days after laboratory confirmation.

The detection of these outbreaks was delayed primarily because the diagnosis of cases was delayed, rather than because health departments were not notified of positive laboratory results (Table). The delays in diagnosis were due to delays in seeking medical care and receiving evaluation by a physician. For example, the index patients in outbreaks 3 and 5 sought medical attention after $\geq$ 14 days of symptoms, after learning about other outbreaks of cyclosporiasis, conducting Internet searches on diagnosis and treatment, and bringing this information to their physicians; both were tested promptly. In contrast, the index patient in outbreak 6 was tested 24 days after seeking medical attention, in response to a recommendation by the local health department; this cluster of illnesses was reported to the health department when some of the patients learned about the cyclosporiasis outbreaks in the news.

The wedding outbreak investigation (event 3) best illustrates delays in clinician evaluations and effects of Internet information provided by patients on proper diagnosis and treatment (6). Thirteen (43%) of ill persons consulted physicians, and 54% of these submitted stool specimens; however, disease diagnosis was delayed because none of the physicians ordered specific laboratory testing that could identify *Cyclospora* (3). Patients were not tested until media attention about other clusters of cyclosporiasis in the United States led the bride and ill guests to conduct Internet searches.

Conclusions

The role that the media and Internet information played in testing for *Cyclospora* in these outbreaks was striking. Most index patients prompted their physicians to test for *Cyclospora* by providing them with Internet or media information. At least seven of the ill wedding guests contacted their physicians several times over a 2- to 3-week period but were not tested or treated properly until they provided information on *Cyclospora*. The two index patients who did not have a delay in diagnosis were prompted by widespread media reports to seek both Internet information on *Cyclospora* and medical attention. In the only instance in which appropriate testing was ordered without prompting by the patient or the health department, the physician had watched a television report on cyclosporiasis that morning.

Several factors can contribute to delays in testing for *Cyclospora,* and all of these factors were noted in the wedding outbreak. Persons with diarrheal illnesses often do not seek medical attention; 57% of ill persons in the wedding outbreak did not. Since *Cyclospora* is a new infection, many physicians are not familiar with its symptoms and treatment. Physicians often do not request testing of stool; 46% of ill persons who sought medical care in the wedding outbreak did not have any stool testing.

Detection of cases not only influences detection of clusters but also prompts appropriate medical treatment with TMP-SMX. When a clinical decision is made to assess a patient for a parasitic gastrointestinal infection (e.g., patients with prolonged diarrhea), clinicians should order both routine ova and parasite examination and specific testing for *Cyclospora* and *Cryptosporidium*. Laboratory-confirmed cases should be promptly reported so that outbreaks can be detected and investigated, ongoing transmission can be interrupted, and other ill persons can be provided with effective treatment. Because symptoms can be prolonged, investigation even 1 month after a common event can lead to effective treatment.

Cyclospora is not yet reportable in California; however, clinicians are required to report all unusual diseases and outbreaks to the local health departments. Nevertheless, diseases that are not reportable are rarely reported. Therefore, *Cyclospora* outbreaks identified through ELB surveillance (83% of outbreaks that involved residents of the counties with this surveillance system) might not have been detected without enhanced surveillance.

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Norwalk-Like Viral Gastroenteritis Outbreak in U.S. Army Trainees

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An outbreak of acute gastroenteritis hospitalized 99 (12%) of 835 U.S. Army trainees at Fort Bliss, El Paso, Texas, from August 27 to September 1, 1998. Reverse transcriptase polymerase chain reaction tests for Norwalk-like virus were positive for genogroup 2. Gastroenteritis was associated with one post dining facility and with soft drinks.

Norwalk viral gastroenteritis has been identified as an important cause of illness among military troops (1-3). We report the epidemiologic, environmental, and laboratory investigations of a large foodborne outbreak of acute gastroenteritis in military trainees in which a Norwalk-like virus, genogroup 2 (NLV2), was identified by reverse transcriptase-polymerase chain reaction (RT-PCR).

The Outbreak

From August 27 to September 1, 1998, 99 soldiers with acute gastroenteritis, assigned to the Fort Bliss Air Defense Artillery Training site, El Paso, Texas, were admitted to the William Beaumont Army Medical Center for observation and hydration. The criteria for admission were lack of medical care in barracks and isolation of cases to contain the outbreak. All soldiers admitted to the medical center with gastroenteritis were isolated in a temporary medical facility. The most prominent symptoms were acute nausea, vomiting, abdominal pain, diarrhea, and fever (Table 1). The median hospital stay was 24 hours (12 to 72 hours). Initial clinical laboratory evaluations suggested acute viral gastroenteritis (Table 2). All soldiers were from the same unit and lived near each other in the training area compound, a rectangular block of 15 buildings. The hospitalization rate for gastroenteritis in this

Table 1. Frequency of symptoms in hospitalized soldiers

Symptom	No.[a]	%
Nausea	79/90	88
Vomiting	72/90	80
Abdominal pain	68/90	76
Diarrhea	60/90	67
Fever/chills	37/90	41
Headache	20/90	22
Photophobia/eye pain	3/90	3

[a]90 records were abstracted from 99 hospitalized soldiers

Table 2. Frequency of selected findings in hospitalized soldiers

Sign/finding	No.	%
Tmax > 99.5°F	28 / 90	38
Tmax > 100.4°F	17 / 90	19
WBC > 10,600	10 / 60[a]	17
Platelets < 150,000	22 / 60[a]	37

[a]Sixty of 90 abstracted records reported white blood cells and platelet counts.
Tmax, maximum temperature.
WBC, white blood cells.

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unit for the week of August 27 was 99 (12%) of 835. The compound had two dining facilities, DF1 and DF2, located across from each other in the center of the compound. Preliminary interviews with hospitalized patients implicated DF1, which was closed on August 28. Although some patients preferred one dining facility over the other, in general they dined as directed by their drill sergeants or where the wait was shortest. In addition to U.S. soldiers, the unit trained small numbers of US marines and Japanese defense force personnel, who also used these dining facilities.

Patients, food handlers, facility engineers, post public health officers, and training unit officers were interviewed, and the training compound and dining facilities were inspected. Sign-in rosters, menus, and meal preparation documents, as well as daily personnel status reports and training schedules, were reviewed. Cases were mapped by building of residence. Foodborne outbreak questionnaires were administered to 86 of the hospitalized patients available for interview; a randomly chosen (using the last digit of Social Security numbers: 3,5,7) group of 198 soldiers who had not sought medical care; and US marines and 15 Japanese defense force troops (total 323 questionnaires).

Because of the time elapsed since the start of the outbreak, the questionnaire was designed with a food preference format, based on the foods listed on the previous week's menu. Data were abstracted from 90 inpatient records and 323 questionnaires and meal sign-in rosters from August 25 to August 27, 1998, and were analyzed for trends by SPSS, a statistical software package. The case definition for acute gastroenteritis was three or more loose diarrheal stools (with or without vomiting) within a 24-hour period in the week before the outbreak, with or without admission to the hospital. To better characterize the point source of the outbreak, 98 (77.8%) of 126 cases with onset on August 27 to August 28 were termed "first wave" cases and were compared with propagated cases and controls for association with selected exposures (Table 3).

Limited food samples and cultures of the ice cream and soft drink dispensers were obtained and sent for analysis—the ice cream dispensers had been turned off and left at room temperature for 36 hours before sampling. Post water production facilities were reviewed, and water distribution maps were obtained from the Fort Bliss engineers. Construction in the area had necessitated closure of a nearby water main on August 27, but the main was reopened on the same day. Water samples were taken from multiple sites in the training compound and elsewhere on post. Stool samples were collected from all hospitalized patients and processed for *Salmonella*, *Shigella*, *Campylobacter*, and *Yersinia*. The first 15 samples were processed for *Aeromonas*, *Plesiomonas,* and *Escherichia coli* O157:H7, as well as for fecal leukocytes and ova or parasites, including staining for *Cyclospora* and *Cryptosporidia*. Twenty-four specimens were also sent to the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, for electron microscopy and PCR testing for NLVs (4).

Of the 222 U.S. soldiers and marines selected to complete a questionnaire as controls, 31 (14.0%) also met the case definition for acute gastroenteritis. Extrapolating to 736 nonhospitalized U.S. soldiers and 24 marines, 106 (14.0%) of

Table 3. Odds ratios (OR) for selected foods and dining facilities

Exposure	Univariate analysis OR	Univariate 95% CI	Multivariate analysis OR	Multivariate 95% CI
Ever ate at DF1 in week prior[a]	9.8	(2.8, 40.2)	7.3	(2.0, 26.4)
Ate in DF1 preferentially[b]	3.7	(2.0, 6.9)	2.4	(1.3, 4.5)
Ever ate at DF2 in week prior[a]	1.1	(0.5, 2.3)	0.6	(0.2, 1.4)
Soft drinks	3.8	(2.0, 7.2)	2.6	(1.3, 5.0)
Crumb cake	2.4	(1.2, 4.8)	1.8	(0.8, 3.8)
Ice cream	1.7	(1.1, 3.0)	1.1	(0.6, 2.0)
Cinnamon rolls	1.7	(0.8, 3.7)	1.3	(0.6, 3.0)
Pie	1.5	(0.9, 2.7)	1.1	(0.6, 2.0)
Ice	1.5	(0.8, 2.9)	1.1	(0.6, 2.0)

Analysis based on inclusion of 98 "first-wave" cases only, as described in text.
Odds ratios (OR) defined as consumed item *vs*. did not, in all cells except:
[a]OR = 'Yes' vs. 'No'; [b]OR = DF1 vs. any other dining facility.

760 would have been expected to have had unreported illness. Added to the 99 hospitalized troops, this yields a crude unit attack rate among all U.S. trainees of (106 + 99) (23.9%) of 859. Of the 15 Japanese soldiers who were interviewed, nine (60%) had been ill and met the case definition. In all, 40 (16.9%) of the 237 controls also met the case definition and were reclassified as case-patients. An epidemic curve, based on time of symptom onset in cases, was constructed from the 126 identified cases of acute gastroenteritis with completed questionnaires (Figure).

Mapping of the outbreak cases demonstrated a discrete geographic clustering in the training compound, with the exceptions of the Japanese soldiers and two U.S. Army officers. Interviews with the Japanese troops found that they all had used DF1 exclusively for morning and evening meals during the week before illness. The two army officers reported eating just one meal at DF1 at lunch on August 26, with soft drinks and pudding pie as the only food in common. Interviews with food handlers found that a confection baker had become acutely ill while baking in the DF1 facility between 2 a.m. and 4 a.m. on August 26. A DF1 housekeeper (not food handler) also reported self-limited gastrointestinal illness between August 27 and August 29. None of the workers in DF2 reported illness.

Univariate analysis of the abstracted questionnaire data showed that soldiers who ate in DF1 in the week before the outbreak were 9.8 times as likely to contract acute gastroenteritis as those who did not use DF1 (95% CI: 2.8, 40.7). Although univariate analysis indicated that illness was also associated with eating crumb cake and cinnamon rolls prepared by the baker, as well as ice cream and soda from DF1, multivariate analysis did not support these associations. Multivariate analysis indicated that the best predictors of illness were dining in DF1 and drinking soda from the dining facility (Table 3).

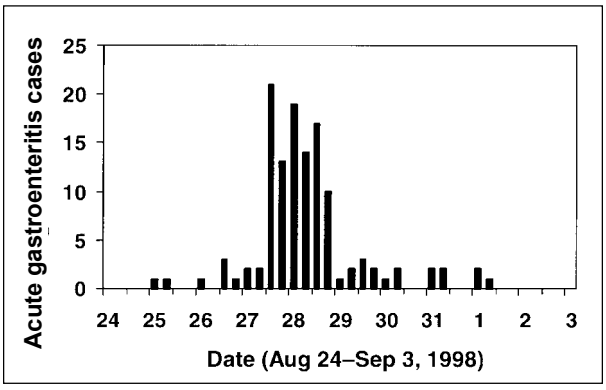

Figure. Date of onset of acute gastroenteritis symptoms (AGE) in 126 soldiers, week of August 24, 1998.

All post water samples tested negative for fecal coliforms. General sanitation in the dining facilities was satisfactory, and no back-siphoning hazards were found. The soft drink dispensers had antisiphoning valves. Cultures of food specimens were negative except for nonpathogenic coliform bacteria (*Citrobacter diversus* and *Serratia liquefaciens*) isolated from the ice cream dispenser in DF1 and *Enterobacter cloacae* coliform bacteria isolated from the soda fountain in DF2. All stool cultures from hospitalized trainees were negative for bacterial fecal pathogens. No ova or parasites, fecal leukocytes, or bacteriologic pathogens were found. RT-PCR was positive for NLV2 in 17 of 24 stool specimens submitted to CDC for analysis. Electron microscopy was performed on seven specimens, and all were positive for 30-nm particles consistent with NLV.

Conclusions

This gastroenteritis outbreak was notable for the explosive onset of an intense but brief illness with a short incubation period of 24 to 36 hours. The outbreak curve was characteristic of a point-source, propagated, foodborne illness, with clinical and epidemiologic features suggestive of NLV gastroenteritis, which was subsequently confirmed by RT-PCR (5-14).

NLVs have been identified as the predominant cause of viral gastroenteritis and have been implicated in 42% to 96% of nonbacterial gastroenteritis outbreaks since 1976 (5,10-12).

Although contaminated water is often the vehicle of outbreaks, the water distribution on Fort Bliss is within a closed loop system, and the tight geographic distribution of cases was inconsistent with proximal contamination of the general water supply. In addition, ice from the dining facilities was not associated with illness (Table 3). Interviews supported the hypothesis that the outbreak was caused by point-source contamination of food or drink in DF1.

Statistical analysis showed a strong association between illness and dining in DF1 and weaker associations with several food items, particularly crumb cake prepared by the ill confectioner on the morning of August 26 and

served at meals on August 26 and 27. The weakness of the association could be due in part to recall bias, since the onset of illness occurred fully a week before the investigation. Both pre- and postsymptomatic contamination of foods has been documented in outbreaks traced to food handlers, which could account for a few early, sporadic cases before the major outbreak. The strong statistical association of gastroenteritis with soda drinking suggests contamination of the soft drink dispenser in DF1—a distinct possibility, as NLVs are hardy and persistent in the environment, resisting both disinfection and chlorination (5,8,10). Bacterial contamination of the ice cream dispenser in DF1 and the soda fountain in DF2, while an incidental finding, implies that mechanical transmission of pathogenic organisms is possible in these facilities. A confounding effect, such as a tendency of ill soldiers to drink more soda, could also explain this association. Secondary person-to-person transmission promotes viral propagation in outbreaks and maintains these viruses in circulation. The prompt closure of DF1 and use of temporary medical facilities for quarantine likely decreased secondary propagation in this outbreak.

Limitations of this investigation include inability to identify the viral agent from the confection baker, failure to obtain acute- and convalescent-phase sera from ill DF1 staff and case-patients, design limitations in using food preferences in the survey, and inability to identify the agent in suspect foods, since NLVs cannot be cultured (5-10).

NLVs are distributed worldwide; serum antibody to Norwalk virus in some countries approaches 100% seroprevalence in adults. The ubiquitous nature and persistence of these viral agents make similar future outbreaks of NLV gastroenteritis a near certainty. RT-PCR is a valuable diagnostic tool in the identification of NLVs as the etiologic agent in this setting.

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Preventing Zoonotic Diseases in Immunocompromised Persons: The Role of Physicians and Veterinarians

To the Editor: We read with great interest the article by Grant and Olsen on the role of physicians and veterinarians in preventing zoonotic diseases in immunocompromised persons (1) and the letter by Barton et al. on the risk of pregnant women and young infants for pet-associated illnesses (2). Essentially all aspects and conclusions of the study by Grant and Olsen are also valid in Europe.

In Austria, veterinarians are well educated in zoonotic infections. However, it is impossible for practitioners to know all zoonotic agents in detail. In addition to immunocompromised persons, pregnant women, and young infants, persons in certain occupations are at higher risk for zoonotic infections. Veterinarians are one of these groups. We have completed seroepidemiologic studies involving veterinarians (3) and are testing other groups at high risk, such as slaughterhouse workers, farmers, and zoo employees.

We surveyed 52% of the veterinarians in an Austrian federal state who agreed to participate in the study. They completed a questionnaire and provided case histories so that risk factors could be assessed. We also obtained blood samples. The sera were tested for antibodies to viral, bacterial, and parasitic zoonotic agents. After correlating the serologic results with the statements in the questionnaire, a statistical analysis, and another questionnaire of selected participants, we found transmission of zoonotic agents from animals to veterinarians for influenza A virus H1N1 (prevalence of the infection was much higher among veterinarians than in the general population; a significant number of seropositive veterinarians were swine practitioners [chi-square = $p^{xx} \leq .01$]); *Coxiella burnetii* (veterinarians who removed bovine placenta without gloves had a higher risk [chi-square = $p^{xxx} \leq .001$] of acquiring *Coxiella burnetii* infections); *Brucella* sp.*; Chlamydia psittaci; Leptospira* sp.; *Toxoplasma gondii;* and *Toxocara canis/cati* (antibody prevalence was 20 times higher among veterinarians than in the general Austrian population). As a result of the survey, veterinarians know about their profession-specific risk factors and take adequate measures to prevent infections; also, they are more qualified to advise pet owners and persons of other professions at high risk.

In addition to the pathogens listed by Grant and Olsen and the agents listed in this letter, some other zoonotic agents present (at least in Central Europe) are cowpox (mainly acquired from cats; 4-6) and parapox viruses, lymphocytic choriomeningitis virus, Newcastle disease virus, *Erysipelothrix rhusiopathiae,* and *Capillaria hepatica* (7). On the other hand, animals are not the source of human disease such as leukemia; there is no evidence that feline leukemia virus and feline immunodeficiency virus, for example, are transmitted to humans (8). The risk of acquiring a zoonotic infection from a pet animal is definitely lower than the emotional and health benefits of pet ownership.

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Reply to Drs. Nowotny and Deutz

To the Editor: We thank Drs. Nowotny and Deutz for their Letter to the Editor regarding our letter in the January-February issue of Emerging Infectious Diseases. We agree that increased education and research efforts regarding zoonoses would benefit not only at-risk patients, but also veterinary and human health professionals. We also applaud their efforts to provide serologic evidence of exposure to zoonotic pathogens among veterinarians in Austria. However, readers should be aware of the zoonotic potential of two of the pathogens in their screening. Specifically, although both animals and humans suffer from respiratory syncytial virus infections, evidence is minimal for interspecies transmission of the domestic animal and human strains. Similarly, bovine viral diarrhea virus is an important bovine pathogen, but there is little evidence for its ability to infect humans.

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International Law and Infectious Diseases.
David P. Fidler. Oxford University Press, 1999.

This thoughtful book should find its readership among health professionals, lawyers, international relations scholars, and activists addressing issues of infectious diseases in the context of public health, international trade, environment protection, or war. Its chapters include the following topics: a historical overview, International Health Regulations, international legal framework, trade law, human rights law, war and weapons, environmental law, international relations, and a Draft Framework Convention on Global Infectious Disease Prevention and Control.

Early attempts at controlling the epidemic spread of diseases by quarantining international shipping vessels were costly and ineffective. Beginning in the mid-19th century, a series of international sanitary conferences sought legal agreements among states to reconcile the competing demands of commerce and public health. As microbes and their vectors do not respect international boundaries, David Fidler has chosen a rich subject for this study of international legal regulation.

In democracies at least, rational trade policies may be swayed by popular concerns; for example, bans on certain foodstuffs may be more driven by the public's fear of disease than by good science. The International Health Regulations, which are supposed to address these issues, are outdated, limited in scope, and not respected. Fidler therefore proposes a broader international convention on global infectious disease prevention and control, which would incorporate revised international health regulations; he provides a draft convention for consideration.

As AIDS now kills more people globally than any other infectious disease, Fidler's discussion of the right to health and the confluence of human rights and public health in the context of the AIDS pandemic is of particular interest. Yet his draft convention of nation-states does not address either the incapacity of the states that are worst affected or the importance of active participation by nongovernmental organizations and transnational corporations. Present-day international law seems incapable of addressing the broader issue of collective international responsibility to act in the face of the global AIDS pandemic.

The book is well paced and scholarly. Fidler's analysis is cautious, but he does not shrink from discussing the failure of the World Health Organization to apply the successes of international legal regulation in fields such as international trade, aviation, labor standards, and the environment to infectious diseases and public health. One of the many ironies delineated in the book is the resurgence of interest in international legal solutions to public health challenges when science and medicine fail to provide enduring national solutions.

Interest in international public health law waned in the 20th century, when vaccination and improvements in public services and hygiene reduced or eliminated the threats of smallpox, plague, cholera, and yellow fever. Yet today, the increasing speed and scale of global trade and population movements pose new risks from emerging and reemerging diseases. Fidler's proposed international convention on global infectious disease prevention and control would represent progress toward addressing these concerns and deserves serious consideration.

David Patterson
Geneva, Switzerland

Oxford Handbook of Tropical Medicine.
Michael Eddleston and Stephen Pierini. Oxford University Press, New York, 1999.

In judging a new textbook, one is initially influenced by the authors' reputation and qualifications. The authors of this Oxford Handbook, Michael Eddleston and Stephen Pierini, are listed on the front cover, but their names do not appear again. In the introduction, these mystery authors tell us that the Oxford Handbook of Tropical Medicine was written to answer a need for a soft-cover, pocket-sized (18 x 10 x 2.5 cm), inexpensive, lightweight (300 grams) handbook of clinical medicine in the tropics for "junior doctors" who work in the developing world, where few laboratory tests are available and technical and human resources may be lacking. This book was written in collaboration with World Health Organization (WHO) staff, as evidenced in the acknowledgments, in which no fewer than 22 WHO consultants appear, as well as the foreword by David Heymann, Executive Director, Communicable Diseases, WHO.

So what's in this handbook, and how is it organized? The first 16 pages make up the introduction, which briefly covers such topics as WHO's Essential Drugs Programme, outbreak investigations, universal and isolation precautions, and integrated management of childhood diseases. The next section (160 pages) covers five major infectious disease areas (malaria, HIV and sexually transmitted diseases, tuberculosis, diarrheal diseases, and acute respiratory infections). Except for the section on fevers and systemic signs, the rest of the book is systems based, covering most internal medicine topics, including cardiology, chest medicine, renal diseases, gastroenterology, neurology, dermatology, endocrinology, hematology, nutrition, injuries and poisoning, and immunization. Each section spans 30-50 pages of very small print, for which those of us over 40 will need the assistance of a magnifying glass.

Before we tell you what we thought of the book, let us point out two refreshing features. The authors ask that readers provide comments and criticisms for improving future editions. (This is a clue as to the identity of the authors.) The second is the authors' expectation that readers would and should adapt the book for local conditions. To this end, they have included blank pages throughout the book for the reader to add and modify treatments and diagnostic tests as necessary.

This book focuses on diseases, both infectious and non-infectious that are seen in tropical developing countries and on therapies that are available there. You will not find imipenem, moxifloxacin, insulin pumps, or even culture and sensitivity data. Drugs recommended may not always be ideal, but they are likely to be available locally. Another major feature of the infectious disease section is the liberal use of excellent WHO algorithms, which are particularly useful for the inexperienced physician.

Disease descriptions are concisely written and organized with a section on etiology/life cycle, clinical features, diagnosis, management, and control. Most diseases are summarized in one page or less, except for major diseases such as malaria, tuberculosis, and HIV. A good part of the book is problem-based, by symptoms, and this is by far its major strength. This problem-oriented approach is ideal for the rural developing world, where "medicine by intuition" is often practiced, and clinical skills, knowledge, and judgment are all that may be available for disease management.

The major infectious disease sections and the one on nutrition are excellent. The systems sections are very good but somewhat lacking in perspective. Differential diagnoses are always listed, but the inexperienced physician may have some difficulty sorting out the top five conditions to be considered. Clearly, these vary in different parts of the world (hence the blank pages), but there are common problems everywhere that tend to head most lists. We particularly loved the section on how to do a burr hole, complete with diagrams (a worthwhile technique to practice on your teenage children).

We were a bit disappointed that Bacille Calmette-Guérin vaccine was covered in only seven lines, that bed nets were not mentioned for malaria control, that typhoid was not included in the differential diagnosis of lymphocytosis or prolonged fever, that short-course therapy was not recommended for typhoid fever, and that tinidazole or single-dose metronidazole were not suggested for invasive amebiasis. In addition, the book is somewhat inconsistent about which diagnostic tests are recommended: on the one hand, bacterial cultures are almost never available in rural areas of the tropics, but little

emphasis is given to presumptive treatment. On the other hand, in the nephrology section, the authors surprisingly recommend an autoantibody screen and complements, urine pH, and calcium levels.

Enough of nitpicking. The bottom line? This is an excellent first edition of a handbook of tropical and internal medicine for the rural practitioner. It is a comprehensive, concise, well written, and (for the most part) practical handbook that provides a wealth of information on diagnosis, treatment, and decision making. We recommend it highly for medical students, residents, and even infectious and tropical disease consultants planning to work in the tropics or to care for patients from the tropics.

Given their level of training, the authors have done a remarkable job. We have decided, on the basis of the following clues, that the authors of this handbook are probably medical residents, or "registrars" in the British system. First, the book is dedicated to their parents rather than their spouses, so they are probably young and unmarried. They acknowledge their "long-suffering mentors, David Warrell and David Theakston," who send "fresh-faced medical students out to remote corners of the world …" This book was almost certainly written by "kids," recent medical students who had an international health experience during training. In this world of academic medicine, it is a shame that credibility is accorded only those who have more initials *after* their names than *in* their names. We should judge an excellent book such as this one by its contents and not by the prestige of its authors. As Butch Cassidy said to the Sundance Kid, "Who are those guys?" In this case, it doesn't matter.

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Conference Summary

Institute of Medicine's Forum on Emerging Infections: Workshop on Managed-Care Systems and Emerging Infections

The Institute of Medicine's Forum on Emerging Infections was established in 1996 in response to a request from the National Center for Infectious Diseases, Centers for Disease Control and Prevention, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The forum provides structured opportunities for representatives of academia, industry, professional and special-interest groups, and government agencies to examine and discuss scientific and policy issues related to research and prevention, detection, and management of emerging infectious diseases. The forum, which fosters the exchange of ideas, identifies areas for study, clarifies policy issues, and updates decision-makers, seeks to illuminate issues rather than resolve them; it does not provide advice or recommendations on any policy pending before any agency or organization. Its strengths are the diversity and dedication of its membership.

The forum sponsors a series of workshops. The first of these addressed public- and private-sector collaboration (1). The second explored many aspects of antimicrobial resistance, surveillance, and response (2). The third workshop (3), which we summarize here, examined opportunities posed for managed-care systems and challenges to their ability to address the threat of emerging infectious diseases.

Representatives of managed-care organizations, hospitals, government agencies, pharmaceutical companies, and academia convened to discuss issues, suggest solutions, and highlight impediments to be overcome in five key areas: basic and clinical infectious disease research, clinical practice guidelines, emerging infections surveillance and monitoring, education and outreach, and drug formularies and product development. A common theme of the discussions was the heterogeneity and rapid evolution of the managed-care industry. Although a few large health maintenance organizations (HMOs) were mentioned as effective research partners, others have different capabilities and corporate cultures. The participants recognized that, as the restructuring of the nation's health-care system evolves, forging stronger partnerships with managed-care systems will likely have a positive effect not only on health-care delivery but on many aspects of public health.

The workshop revealed that the structure of managed-care organizations provides few incentives for taking the broader and longer-term public health view. Some incentives, even those concerning quality assurance for individual patients and formulary restrictions, may actually intensify public health problems. For example, one presenter cited a study in which, for every condition except otitis media, increased formulary restrictions were associated with increased numbers of physician office visits, emergency department visits, hospitalizations, and prescriptions, along with an increased cost of prescriptions over a 12-month period. The evidence regarding managed care's actual performance and impact on emerging infections needs clarification. Some managed-care plans have integrated services and sophisticated research capabilities, whereas others provide little more than cost reimbursement for conventional health-care services.

The managed-care industry by itself cannot be expected to develop and implement solutions for those challenges. The industry could become a more productive partner in combatting emerging infections. Incentives might include support to cover the marginal costs of research and demonstration activities, for example, the gathering of drug-dispensing data and crude surveillance for multidrug resistance among the organisms that cause tuberculosis and sexually transmitted diseases. Some HMOs may garner a competitive advantage in being viewed as progressive, research-oriented organizations. Major purchasers of managed care will also have an important role alongside the managed-care industry in developing and implementing solutions to emerging infections problems. Likewise, academic health centers and government agencies can act as catalysts, as well as partners in research, to allow greater participation by managed care in addressing emerging infections.

The need for better information to support the provision of quality health care was also

addressed by the workshop. For example, an expert at the workshop reported on preliminary studies indicating that formulary practices may have an adverse impact on antibiotic resistance, prompting the need for additional comprehensive data about these practices and their impact. The use of outcomes information was identified as one way to develop and implement new clinical practice guidelines. Another promising outcome of the workshop was the identification of the potential for integrated, electronic information systems to assist physicians in diagnosing and treating infectious diseases; managed-care organizations in tracking antibiotic use, costs, and outcomes; and public health agencies in monitoring and even preventing emerging infections and antibiotic resistance.

Many of the issues raised during the workshop, including drug formularies and surveillance, have international as well as domestic implications. For example, there are three health systems in Latin America—private, public, and employee systems—but many of the providers in the region work with all three systems. As governments face increasing pressure to downsize, the impact of that change on the vital public health functions of surveillance, control, and prevention of infectious diseases concerns all systems. In the United States, the National Institutes of Health, the Centers for Disease Control and Prevention, and private groups that are working on these issues should be involved in international deliberations, and managed care should be part of foreign policy initiatives in the area of infectious diseases.

For information, contact the Forum on Emerging Infections, Division of Health Sciences Policy, Institute of Medicine, The National Academies, 2101 Constitution Avenue N.W., Washington, D.C. 20418, USA; telephone: 202-334-1888; fax: 202-334-1329; e-mail: bugs@nas.edu. For information on the Institute of Medicine, visit the Institute's home page at http://www.iom.edu.

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Upcoming Events

First United Kingdom Workshop on Borna Disease Virus: Borna Disease Virus: A Veterinary and Public Health Problem?
Rhondda, Wales, March 23-24, 2000

Borna Disease Virus (BDV) is endemic in parts of Europe, infects a broad range of species, and causes a rare meningoencephalitis in horses and sheep. The virus has not been clearly linked to any human disease, but an association between infection with the virus and certain neuropsychiatric disorders has been suggested. However, the methods used in previous studies and the significance of the findings are controversial.

This first United Kingdom Workshop on BDV is organized by the Public Health Laboratory Service, the Health and Safety Executive, the Ministry of Agriculture, and the Welsh Development Agency. The Workshop will bring together agencies and researchers in the United Kingdom interested in the diagnosis, pathology, and epidemiology of BDV in human and animal populations; provide opportunities for collaboration and sharing of expertise; disseminate the latest findings from research on BDV in Europe and elsewhere; and provide guidance for veterinary and public health policy-makers in developing surveillance and research programs.

The program will include sessions on the detection, pathology, and epidemiology of BDV in animal and human populations. Keynote speakers will be from Europe and the United States. The registration fee of £69 includes delegate package, meals, and proceedings. Delegates must make their own arrangements for accommodations. For more information, visit our web site: http://www.cdsc.wales.nhs.uk/bcon.htm.

10th International Symposium on Viral Hepatitis and Liver Disease
Atlanta, Georgia
April 9–13, 2000

The preliminary program of the 10th International Symposium on Viral Hepatitis and Liver Disease is now available for viewing on the conference web site, http://www.hep2000.com

For additional information, contact the Organizing Secretariat at info@hep2000.com or by telephone at 404-233-4490.

The Johns Hopkins University Graduate Summer Institute of Epidemiology and Biostatistics
Baltimore, Maryland

The Graduate Summer Institute, sponsored by the Johns Hopkins University School of Public Health, Baltimore, MD, provides an opportunity for the physician, nurse, or health professional to enroll in courses for academic or continuing education credit.

Cost is $675 for one course; $1,000 for two courses; $175 for each additional course (non-credit 3-week courses). Application deadline is June 1, 2000. For information contact Ayuesha Khan, Department of Epidemiology, School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205. Phone: 410/955-7158; fax: 410/955-0863; e-mail: akhan@jhsph.edu.

Negative Strand Viruses 2000 Eleventh International Conference on Negative Strand Viruses
Québec City, Québec, Canada
June 24-29, 2000

For information, contact the Conference Committee at Negative Strand Viruses 2000, P.O. Box 33799, Decatur, GA 30033-799, USA; telephone: 404-728-0564; fax: 404-728-0032. The e-mail address is nsv2000@aol.com and the web site address is http: www.nsv2000.com.

5th International Meeting on Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases
Hyderabad, India
November 12-16, 2000

The conference is sponsored by the Centers for Disease Control and Prevention, USA; Institut de Recherche pour le Développement and the Centre National de la Recherche Scientifique, France; and the Indian Council of Medical Research and the Department of Biotechnology, India. Objectives are to integrate epidemiologic, molecular biologic, and evolutionary genetics approaches in the areas of diagnosis, strain typing, species identification, pathogenesis, antigenic variation, drug and vaccine resistance, host susceptibility, and vector specificity; to foster interactions between epidemiologists and laboratory scientists working on the same pathogens; and to provide a forum for health-care providers, public health professionals, policy-makers, epidemiologists, laboratory scientists, and program managers to discuss the tools and methods needed for the diagnosis and management of emerging, reemerging, and endemic infectious diseases. The priority areas of the conference will be malaria, *Mycobacteria*, leishmania, HIV, cholera, hepatitis, opportunistic infections, enteric pathogens, antimicrobial resistance, molecular entomology, and human genetic epidemiology.

For information, see MEEGID5@WWW.CDFD.ORG.IN or contact Altaf A. Lal, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, MS F-22, Atlanta, GA 30333, USA, e-mail: aal1@cdc.gov; Michel Tibayrenc, Centre d'Etudes sur le Polymorphisme des Micro-organismes, IRD, Montpellier, France, e-mail: michel.Tibayrenc@cepm.mpl.orstom.fr; Hooman Momen, Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900, Rio de Janeiro, Brazil, e-mail: hmomen@gene.dbbm.fiocruz.br; or Seyed Hasnain, Center for DNA Fingerprinting and Diagnostics, ECIL Road, Nacharam, Hyderabad 500 076, India, e-mail: ehtesham@www.cdfd.org.in.

Deadline for abstract submission is April 30, 2000; 250-word abstracts should be submitted to Dr. Hasnain at the above address. Registration fee for abstracts submitted by April 30 is US$150.00. Late fee for abstracts submitted by June 30 is US$250.00. Deadline for acceptance for oral and poster presentations is July 31, 2000.

ICEID 2000—Register Now!

Nearly 500 abstracts in 36 categories were submitted for consideration to ICEID 2000. Updated program information will be posted on the conference Web site as it becomes final.

Abstracts of late-breaking research results may be submitted through the Web site beginning in late March, until June 16. Go to the Web site at www.asmusa.org/mtgsrc/iceid99main.htm and follow the links to abstract submission. ICEID 2000 is using the American Society for Microbiology's Web-based Abstract Submission System. If you have ever used the system before, you will be able to reenter the system as a returning user.

Registration is limited! You can register for this important conference through the Web site listed above, or e-mail iceid@asmusa.org to ask for a copy of the printed preliminary program whch contains forms for registration and housing.

Visit our web page at: http://www.cdc.gov/ncidod/EID

Editorial Policy and Call for Articles

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

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

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

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

Instructions to Authors

Manuscript Preparation

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

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

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

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

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

Electronic formats. For word processing, use WordPerfect or MS Word. Send graphics in either .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Helvetica. Convert Macintosh files into one of the suggested formats. Submit slides or photographs in glossy, camera-ready photographic prints.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title in full. List the first six authors followed by "et al."

Tables and figures. Create tables within the word processing program's table feature (not columns and tabs within the word processing program). For figures, use color as needed; send files, slides, photographs, or prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D-61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

Types of Articles

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

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

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

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

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

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

Book Reviews: Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

News and Notes: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.