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The Next Influenza Pandemic: Lessons from Hong Kong, 1997

René Snacken,* Alan P. Kendal,†
Lars R. Haaheim,‡ and John M. Wood§
*Scientific Institute of Public Health Louis Pasteur, Brussels, Belgium; †The Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; ‡University of Bergen, Bergen, Norway; §National Institute for Biological Standards and Control, Potters Bar, United Kingdom

The 1997 Hong Kong outbreak of an avian influenzalike virus, with 18 proven human cases, many severe or fatal, highlighted the challenges of novel influenza viruses. Lessons from this episode can improve international and national planning for influenza pandemics in seven areas: expanded international commitment to first responses to pandemic threats; surveillance for influenza in key densely populated areas with large live-animal markets; new, economical diagnostic tests not based on eggs; contingency procedures for diagnostic work with highly pathogenic viruses where biocontainment laboratories do not exist; ability of health facilities in developing nations to communicate electronically, nationally and internationally; licenses for new vaccine production methods; and improved equity in supply of pharmaceutical products, as well as availability of basic health services, during a global influenza crisis. The Hong Kong epidemic also underscores the need for national committees and country-specific pandemic plans.

Influenza pandemics are typically characterized by the rapid spread of a novel type of influenza virus to all areas of the world, resulting in an unusually high number of illnesses and deaths for approximately 2 to 3 years. Such pandemics occurred in 1918, 1957, and 1968 (Table); in the most severe pandemic (1918-20), at least 20 million people died, most working-age adults (10-12). Most deaths occurred in developing nations—more than 10 million people died in India alone (M. Rammana, pers. comm.). Pregnant women were also severely affected, particularly those from lower socioeconomic groups (13,14). The age distribution of those who died differed from that in later pandemics or epidemics, when deaths were higher in the elderly and lower in other age groups, except possibly in very young children.

Address for correspondence: R. Snacken, Department of Virology, Scientific Institute of Public Health Louis Pasteur, 14 J. Wytsman Street, B-1050 Brussels, Belgium; fax 32-2-642-5654; e-mail: r.snacken@iph.fgov.be.

Novel Influenza Viruses without Pandemics

In addition to true pandemics, false alarms—emergences of a novel strain with few cases and little human transmissibility (Table)—have occurred. Several involved "swine influenza viruses" (4-6) antigenically related to viruses circulating in some pig populations and linked to viruses of the 1918 pandemic (see below). These unusual infections may be more common than reported, as laboratory diagnosis for influenza is rarely undertaken in the absence of unusual illness or association with an outbreak.

Origin of Pandemic Viruses

Before influenza virus could be propagated in a laboratory, retrospective measurement of antibodies to the influenza virus' major surface antigen (hemagglutinin) in persons of different ages was used to identify viruses causing pandemics. Additional use of antibody tests to the second surface antigen (neuraminidase) confirmed earlier ideas that H1N1 subtype viruses resembling classic swine influenza caused the 1918 pandemic (15).

Table. Influenza landmarks in humans this century

Year	Colloquial Name (Subtype)	Source	Impact
Pandemics			
1918 (1)	Spanish flu (H1N1 viruses like swine flu)	Possible emergence from swine or an avian host of a mutated H1N1 virus	Pandemic with >20 million deaths globally
1957 (2)	Asian flu (H2N2)	Possible mixed infection of an animal with human H1N1 and avian H2N2 virus strains in Asia	Pandemic, H1N1 virus disappeared
1968 (2)	Hong Kong flu (H3N2)	High probability of mixed infection of an animal with human H2N2 and avian H3Nx virus strains in Asia	Pandemic, H2N2 virus disappeared
1977 (3)	Russian flu (H1N1)	Source unknown, but virus is almost identical to human epidemic strains from 1950. Reappearance detected at almost the same time in China and Siberia	Benign pandemic, primarily involving persons born after the 1950s. H1N1 virus has cocirculated with H3N2 virus in humans since 1977
Incidents with limited spread			
1976 (4)	Swine flu (H1N1)	United States/New Jersey. Virus enzootic in U.S. swine herds since at least 1930	Localized outbreak in military training camp, with one death
1986 (5)	(H1N1)	The Netherlands. Swine virus derived from avian source	One adult with severe pneumonia
1988 (6)	Swine flu (H1N1)	United States/Wisconsin. Swine virus	Pregnant woman died after exposure to sick pig
1993 (7)	(H3N2)	The Netherlands. Swine reassortant between old human H3N2 (1973/75-like) and avian H1N1	Two children with mild disease. Fathers suspected to have transmitted the virus to the children after having been infected by pigs.
1995 (8)	(H7N7)	United Kingdom Duck virus	One adult with conjunctivitis
1997 (9)	Chicken flu (H5N1)	Hong Kong Poultry virus	18 confirmed human cases, 6 deaths

Molecular biologic analysis of viral nucleic acid supports the hypothesis that animals (particularly birds and pigs) may have been the source for (and possibly are a continuing reservoir of) the hemagglutinin and other genes found in viruses from the above pandemics (16). Some animal viruses containing these genes (e.g., H1, H2, H3) might infect humans directly and become adapted to the human host; alternately, through reassortment of the genes in different animal or human influenza viruses, the genetic information might reappear in an infectious human virus (17). The Hong Kong experience, however, showed that an animal virus with another HA subtype (H5) could directly infect humans and cause illness. The H5 virus, however, did not evolve into a form that is readily transmitted from person to person, and its potential for this kind of transmission remains unknown.

Reports in 1957, 1968, and 1977 indicated China and nearby areas as places where outbreaks of novel viruses often first occur (18). Close contact occurs in such regions between humans and animals (e.g., ducks, pigs) raised for food. Surveillance data show that because of the different seasonality of influenza in northern and southern China, human influenza infections normally occur every month of the year (19). Thus, many opportunities exist in China for viruses to cross-infect different animal species and humans, which may explain why it and nearby areas are the origin of many influenza pandemics.

Avian Influenza Virus in Humans in Hong Kong

In May 1997, a 3-year-old boy in Hong Kong contracted an influenzalike illness, was treated with salicylates, and died 12 days later with complications consistent with Reye syndrome. Laboratory diagnosis included the isolation in cell culture of a virus that was identified locally as influenza type A but could not be further characterized with reagents distributed for diagnosis of human influenza viruses. By August, further investigation with serologic and molecular techniques in the Netherlands

(9, 20, 21) and in the United States (22) had confirmed that the isolate was A/Hong Kong/156/97 (H5N1), which was very closely related to isolate A/Chicken/Hong Kong/258/97 (H5N1). The latter virus was considered representative of those responsible for severe outbreaks of disease on three rural chicken farms in Hong Kong during March 1997, during which several thousand chickens had died. Molecular analysis of the viral hemagglutinins showed a proteolytic cleavage site of the type found in highly pathogenic avian influenza viruses.

Because no further cases of human infection with H5 viruses were seen in Hong Kong during the summer, the case in May was considered an isolated incident, with little or no person-to-person spread. However, surveillance for influenza was increased, and local capability was established to test for H5 subtype among human patients.

As summarized on their Internet disease surveillance site, the Hong Kong Special Administrative Region Department of Health (http://www.info.gov.hk/dh/diseases/flu_1997.htm) detected new cases of human illness caused by H5 virus during November 1997. By late December, the total number of confirmed new cases had climbed to 17, of which 5 were fatal (one in a 13-year-old child and four in adults, 25, 34, 54, and 60 years of age). Including the fatal index case in May, the case-fatality rates were 18% in children and 57% in adults older than 17 years.

Investigation of the circumstances surrounding each case was undertaken by the local authorities with assistance from the World Health Organization Collaborating Centers in the United States and Japan. Except for one doubtful unconfirmed case, all illnesses or laboratory evidence of infection was in patients who had been near live chickens (e.g., in market places) in the days before onset of illness, which suggests direct transmission of virus from chicken to human rather than person-to-person spread. On December 28, 1997, veterinary authorities began to slaughter all (1.6 million) chickens present in wholesale facilities or vendors within Hong Kong, and importation of chickens from neighboring areas was stopped. Subsequently, no more human cases caused by avian influenza virus were detected. Because these cases occurred at the beginning of the usual influenza season in Hong Kong, public health officials were concerned that human strains might cocirculate with the avian influenza to generate human and avian reassortant viruses with capacity for efficient person-to-person spread.

Response to Emerging Influenza Pandemics. Lessons from Hong Kong

Pandemic planning has been proceeding in various countries and at WHO for several years (23). Now, 1 year after the Hong Kong episode ended, a period during which several countries have had severe local outbreaks or epidemics of interpandemic variant A/Sydney/5/97 (H3N2)-like viruses, lessons from Hong Kong could be incorporated in existing or new pandemic response plans.

Improve International Response

When the Hong Kong episode occurred, WHO had been developing formal guidelines for addressing pandemic situations. The draft guidelines were revised after the Hong Kong episode, taking into consideration two strategic steps especially important in the outbreak: risk assessment, which encompasses two components, data collection (investigating the circumstances of the initial infection and subsequent infections, and searching for further evidence of spread) and data evaluation (interpreting and communicating the significance of the threat based on the available data); and risk management, which is a process of continuously considering and updating alternative courses of action as new action is obtained, defining potential risks and benefits of each approach, and selecting the next step, or series of steps, recommended for appropriate authorities.

Having already established a Pandemic Task Force by 1997, WHO was able to initiate technical investigation and evaluation of the Hong Kong situation. Only a very few organizations, from the United States and Japan, rapidly committed staff to join local authorities in collecting information needed for risk assessment. The widespread local and international consequences of the situation in Hong Kong, including impact on commerce and travel, compounded the already large pressures on the investigating team to gather evidence about the risk for an epidemic or pandemic. Further pressure was exerted on the investigating team, WHO Task Force, and collaborating

organizations because much work was urgently needed on a contingency basis to expand capabilities of international surveillance laboratories to detect H5 influenza viruses elsewhere and to support preliminary steps necessary for developing a vaccine against the Hong Kong virus.

Because influenza pandemic threats affect more than one country, facilitating multicountry studies could save critical time in the risk assessment process. Hence, increasing international involvement in both phases of risk assessment is desirable—both to expand resources for investigations and to ensure that all regions of the world, including developing nations, are represented during decision making. Advance commitments could be made to rapidly expand the network of academic, governmental, or other laboratories or disease-investigating organizations that can conduct field investigations and analyze potentially large numbers of isolates and other specimens. We suggest several ways for improving international response. First, the WHO Task Force could develop formal Terms of Reference for its own role and that of its investigating teams. Second, National Health Authorities of WHO member nations might then make these commitments: to invite WHO team(s) to carry out investigations of pandemic threats without delay, agree with the Terms of Reference for the task force and its investigating teams, and designate national organizations to assist investigating teams. Such advance agreements should facilitate the rapid deployment of investigating teams and the acceptance of their work by WHO member nations, regardless of what countries appear to be relevant sites for investigation of a pandemic threat or in what ways the pandemic threat is first identified or affects local interests. However, special questions will be raised regarding leadership, communications, and internal cooperation as more countries become involved, and these issues also should be addressed, if possible, in advance.

In setting Terms of Reference, data collection may be formally separated from risk evaluation and risk management. Such separation would allow technical experts to concentrate on organizing and conducting field and laboratory investigations without being distracted by having to evaluate the significance of findings or recommending responses to a pandemic threat (24). Furthermore, the willingness of some countries to receive WHO investigating teams may be enhanced if the Terms of Reference specify that data collected for the WHO Task Force will be evaluated by an independent advisory group composed of infectious disease and public health experts representing all WHO regions, including developing nations. Such a tiered approach would be consistent with ways many other public health policy decisions are made about epidemics.

Enhance Human and Veterinary Surveillance

Human influenza epidemics may be evaluated through death data (25-27), but weekly illness reports from sentinel primary-care practices, coupled with laboratory diagnosis, provides more timely detection of early isolates as well as epidemics (28,29). First detection of influenza outside the normal influenza season, however, may come from unsystematic sampling—epidemiologic investigations of reports of unusual outbreaks (e.g., most recently among tourists during summer in different parts of the United States [30], the events in Hong Kong in 1997).

The current WHO global influenza program, with the help of four collaborating centers (Atlanta, London, Melbourne, and Tokyo) and 110 national influenza centers, aims to centralize world data, study the epidemiology of the disease, and rapidly obtain new circulating strains to make timely recommendations about the composition of the next vaccine (31). However, many countries have only limited capabilities or resources to systematically search for and investigate unusual occurrences of influenza. The events in 1997 in Hong Kong show the need to expand routine surveillance efforts. Had the H5 virus isolated in May 1997 from a sporadic case not been identified in August, the reagents would not have been available locally to rapidly diagnose the additional human cases of H5 influenza in humans in November and December. Without such diagnoses, and the investigations which they stimulated, authorities might not have addressed the issue of chicken influenza as they did. Transmission of the H5 virus to humans could have continued into the normal influenza season in Hong Kong, possibly developing into a human-transmissible form.

Therefore, priority should be given to the establishment of regular surveillance and investigation of outbreaks of influenza in the most densely populated cities in key locations, particularly in tropical or other regions where urban markets provide opportunities for human-live animal contact (e.g., swine and poultry[and possibly caged birds kept as pets]). Communication and cooperative studies with veterinarians could monitor influenza outbreaks in locations where large numbers of animals are raised, exhibited, or held pending transport or sale, i.e., situations increasing the potential for virus spread. International collaboration with the WHO Collaborating Centers studying human influenza and the WHO Collaborating Center on influenza ecology in lower animals and birds (Memphis, USA) should be enhanced.

Develop Improved, Low-Cost, Laboratory Surveillance Techniques

For many years, influenza viruses have been isolated by injecting clinical samples into embryonated chicken eggs. Viruses have been detected by agglutination of erythrocytes and inhibited by using antisera provided through WHO, thus keeping costs relatively low and methods relatively simple. Laboratories in industrialized countries (including Hong Kong) have the facilities to use tissue culture for virus isolation. However, when the H5 viruses isolated in Hong Kong were injected into chicken eggs, they caused high numbers of deaths, thus making eggs less suitable as the sole host system for surveillance purposes. Thus, developing simple low-cost techniques (with reagents appropriate for the task of detecting circulation of animal influenza viruses) that can be used in places with limited resources needs to be a priority.

Choices must be made whether such tests should be based on isolation of infectious virus (which can immediately provide virus samples for biologic characterization and development of reagents or vaccines) or on antigenic or molecular methods (which may minimize laboratory capabilities needed). In making the choice of tests, it should be remembered that the reported isolation of an atypical virus by one or a very few laboratories may result from contamination of diagnostic specimens by viruses used for research, reagent production, or quality control; molecular techniques may be needed to confirm unrecognized cases of contamination with live viruses (32,33). It is unclear if diagnostic methods based on molecular methods will incur fewer risks from specimen contamination.

Increase Laboratory Safety Capabilities

The episode of H5, a potentially highly pathogenic virus for humans as well as for chickens and other avian species, also raised the issues of how to contain new viruses and protect laboratory workers and the environment. Although the 1918 pandemic strain was extremely pathogenic and was related to classic swine influenza virus, influenza diagnostic laboratories around the world do not use biologic containment procedures (biosafety level 3 or greater) to handle specimens. The Hong Kong experience shows that there can be no absolute certainty about the human pathogenicity or animal transmissibility of any influenza specimen.

Training of laboratory staff in national centers and local laboratories undertaking influenza surveillance, therefore, is needed to ensure that the best practices are routinely used to reduce infection or transmission risk. Contingency plans can be prepared to increase stringency of biological safety procedures, should an unusually pathogenic new influenza subtype again appear. Procedures would need to be appropriate for the technical facilities that actually exist in laboratories in different locations. Authorization to import and maintain supplies of an antiviral agent (e.g., rimantadine) could be organized in advance to protect laboratory workers and others at high risk. Procedures for authorized shipment of potentially hazardous strains to a reference center also can be planned in advance. Experience in 1997 also showed that the same needs may extend to the expanded network of laboratories likely to collaborate in investigations of new influenza viruses, including laboratories using live field strains of the virus for research, vaccine development, or reference material preparation.

Enhance Electronic Communications about Influenza

In 1997, the Hong Kong authorities set a new standard in communications about influenza by providing daily updates on a readily accessible Internet site. Information was also accessible on the FluNet WHO Internet site (http://

www.who.ch/flunet/). Further examples of electronic influenza information systems are the partial European system, which collects and disseminates data from seven countries (34); weekly information from the Centers for Disease Control and Prevention about influenza in the United States (http://www.cdc.gov/ncidod/diseases/flu/weekly.htm); and electronic (e)-mail by the Public Health Service in the United Kingdom, which disseminates up-to-date information on influenza occurrence there. However, these regional or national systems do not obviate the need for a single, universally accessible, global system that would enable national or local public health officials and laboratory workers to monitor influenza without receiving multiple e-mail messages or having to connect to different Internet sites that use varied formats, representations of data, and possibly languages. Such a system could have reduced uncertainty in late 1997 about whether the lack of reports of H5 viruses outside Hong Kong was due to lack of adequate searching for them or lack of their spread. This concern also is hard to address until it becomes possible to receive information electronically from, or provide technical guidance to, most local or national health centers in developing nations undertaking disease investigation and diagnosis.

Accordingly, development of a multifunctional electronic global influenza information exchange system is suggested. (Such a system could also be used to communicate about other important infectious diseases, so long as this does not complicate widespread accessibility for influenza information exchange.) This system would extend current capabilities beyond those of the existing WHO Flu-Net by ensuring the existence of resources (e.g., connection by wired or wireless communication systems) and system management procedures (e.g., authorization passwords and encryption) to allow simple daily access by all national influenza centers; extending access to local scientists and health officials in key cities within participating countries who, because of their surveillance or diagnostic capabilities, may have early information about possibly new influenza virus cases or outbreaks; enabling users to send and receive information rapidly within their own countries, as well as to or from WHO or the collaborating centers; and providing access also to key national and international scientists knowledgeable about occurrences of possible influenza outbreaks in animals. For scientists at a local level to benefit from international electronic information, translation into several major languages may be needed, on line if possible or at international or national Internet sites.

Among other benefits, information from an electronic information exchange system could enable local and national or international scientists to make cooperative decisions about diagnostic sampling and needed epidemiologic information, without the effort and expense of outside experts. Furthermore, operators of public electronic information sites, such as WHO, or a national authority, as was the case in Hong Kong, would be better able to fulfill their task if such a system were in place for them to collect and check information.

Enhance Vaccine Production Capabilities

Pathogenicity of the H5 virus for chickens and chicken eggs complicated the preparation of seed virus for potential production of vaccine, even for supplies for testing in humans; thus, a high-yielding production seed could not be easily adopted. Alternative strategies (e.g., attenuation of the virus by genetic manipulation, expression of the gene coding for the H5 virus into baculovirus-infected insect cells, or use of a nonpathogenic virus antigenically close to the currently isolated strain) were envisaged. However, even now, it is not clear that a practical way to mass-produce vaccine to the H5 Hong Kong virus exists or could be established in a short time, should a similar event occur. Thus, the rules for pandemic planning need revision, recognizing that reliance on existing licensed techniques for vaccine production could entail unacceptably long delays, should a highly pathogenic strain of avian influenza emerge and lead to a strain transmissible in humans (35).

Efforts begun in 1997 to find ways to mass-produce vaccine when the wild virus is highly lethal for eggs should be continued. These include producing vaccine with existing facilities (attenuating the effect of vaccine virus on eggs) and developing alternative techniques (e.g., cell culture grown virus, genetically engineered vaccines). Placing applications to license new methods on the fast track for review by regulatory authorities would be consistent with a basic tenet of pandemic preparedness: the greater the interpandemic production and use of

influenza vaccine, the easier it will be to meet needs should a pandemic occur. Modifying vaccine-control procedures to decrease delays in releasing batches of vaccines in diverse countries with similar requirements in an emergency is also important. (This issue is already being discussed in Europe [J. Wood, pers. comm.].)

Improve Access to Vaccine or Antiviral Agents and Establish Support Systems

During the Hong Kong episode, a rapid local shortage of existing antiinfluenza drugs was observed, and rimantadine was imported. Had vaccines begun to be produced, no process existed for reaching agreements about access by different countries. Waiting until a pandemic strikes to determine access to prophylactic materials inevitably contributes to inequities in supply for countries lacking facilities to produce antiviral agents or vaccines or lacking resources to competitively purchase supplies at a time of scarcity. The issue of equity cannot be resolved by individual governments or manufacturers. Both vaccine and drug industry and international organizations need to discuss how to encourage fair distribution of scarce vaccines or other pharmaceutical drugs before a pandemic crisis arises.

Regardless of vaccine supply issues, vaccines and antiviral agents are unlikely to meet demand, even for industrialized countries able to purchase them. Assuming that people in all countries will be similarly susceptible to the next influenza pandemic virus and even though the elderly usually constitute a smaller percentage of the population in developing than in industrialized countries, during any future pandemic, the absolute number of those dying in the developing world will likely equal or exceed the number of those dying in industrialized countries, as in 1918. Other needs for responding medically must also be considered, including methods to ensure provision of basic nursing support and care when large numbers of people become ill over a few-week period in community after community. During the 1918 pandemic in the United States, for example, the Public Health Service called on the Red Cross to assume responsibility for mobilizing health workers and paying for them during the epidemic and supplying hospitals when local authorities could not (36). Efforts were mounted in many communities, even in remote areas with few facilities for health care. In India, efforts by individual communities without government directive were credited with saving many lives in 1918-19 (M. Rammana, pers. comm.).

Conclusions

One year after concerns were raised in Hong Kong about another influenza pandemic, are we really much further along in establishing the most effective early warning systems and developing the ability to deal with a true pandemic? WHO now has guidelines for responding to a pandemic (24). New helpful relationships, procedures, and scientific knowledge were undoubtedly established in 1997, particularly concerning international efforts for virus surveillance and vaccine production. However, both serious pandemic threats in recent years (1976, United States; 1997, Hong Kong) raised unpredictable new issues related to vaccine supply, which should not stand in the way of planning about the many predictable needs, which extend well beyond producing and using vaccines. For example, had the H5 viruses spread among the human population in Hong Kong (or any other country), national authorities would have rapidly needed to obtain numerous pharmaceutical products, to store and equitably distribute them, to manage demand for basic health-care services, and to maintain social and economic functions during a potential major health crisis (24). Because of the large variety of tasks, the formation of National Pandemic Planning Committees (NPPCs) has been suggested to develop the options for intervention strategies appropriate to each country (37).

Establishment of NPPCs will likely raise procedural matters, such as membership and chain of command. Unless these matters are resolved, valuable time will be lost. As seen in Hong Kong, a pandemic threat arises suddenly and rapidly becomes a public health concern. Yet very few countries have formally established NPPCs and influenza pandemic plans (a process requiring several years). Without increased urgency about this matter, the next pandemic will find most of the world unprepared.

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Dr. Snacken directs the influenza surveillance program at the Scientific Institute of Public Health-Louis Pasteur. He also chairs the European Scientific Working Group on Influenza.(ESWI).

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Disparity in the Natural Cycles of *Borrelia burgdorferi* and the Agent of Human Granulocytic Ehrlichiosis

Michael L. Levin, Franka des Vignes, and Durland Fish
Yale School of Medicine, New Haven, Connecticut, USA

We studied the prevalence of *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis (HGE) among questing nymphal and adult *Ixodes scapularis* ticks of the same generation and the infectivity of wild white-footed mice for ticks feeding on them. The prevalence of *B. burgdorferi* infection in host-seeking ticks increased less than twofold from nymphal (31% to 33%) to adult (52% to 56%) stage, and 52% of white-footed mice were infected. Prevalence of the agent of HGE increased 4.5- to 10.6-fold from nymphal (1.5% to 1.8%) to adult stage (7.6% to 19.0%), while only 18% of mice were infectious to ticks. *B. burgdorferi* infection was more common in mouse-fed ticks than in ticks collected from vegetation, whereas the agent of HGE was half as common in mouse-fed ticks as in ticks collected from vegetation. The different prevalence in nature of these pathogens in ticks suggests that their maintenance cycles are also different.

The agent of human granulocytic ehrlichiosis (HGE) is nearly identical to *Ehrlichia phagocytophila*, which causes tick-borne fever in sheep and goats in Europe (1,2) and is transmitted by the tick *Ixodes ricinus* (3)—the major vector of Lyme disease (4). In the eastern and midwestern United States, the agent of HGE is transmitted by *I. scapularis* (5-7), also a vector of *Borrelia burgdorferi*—the agent of Lyme disease. Both agents are perpetuated in natural cycles between the tick vector and vertebrate hosts (8-10). Neither agent is maintained transovarially (11,12); thus, horizontal transmission involving a susceptible vertebrate host is necessary. The white-footed mouse (*Peromyscus leucopus*) plays an important role as a reservoir for *B. burgdorferi* (13). Animal species that serve as the main source(s) of the agent of HGE for ticks have not been determined.

Rodents serving as natural hosts for the tick species that transmits both agents can be coincidentally exposed to the two agents. Indeed, white-footed mice from Connecticut have been shown to carry antibodies to both agents simultaneously (14). Granulocytic ehrlichia have been found in wild rodents (10,15). Furthermore, the white-footed mouse and several strains of laboratory mice (*Mus musculus)* are susceptible to the agent of HGE in laboratory experiments (5,10,12). Antibodies to the agent of HGE have been detected in various rodent species from California, Colorado, Connecticut, Florida, New Jersey, New York, Maryland, Minnesota, and Wisconsin (14,16-18). These findings allowed the authors to suggest that small rodents, particularly the white-footed mouse, play the same role in perpetuating the agent of HGE in North America that they do in perpetuating *B. burgdorferi* (10,14,16,18,19). We present results of a 2-year field study that provide evidence to the contrary.

The Study

We studied the prevalence of *B. burgdorferi* and the agent of HGE among questing nymphal and adult *I. scapularis* of the same generation, in natural foci with concurrent circulation of both pathogens in Connecticut. In 1996, we collected ticks at two study sites—Bridgeport and Woodbridge—approximately 30 km apart. In 1997, we collected ticks at the Bridgeport site

Address for correspondence: Michael L. Levin, Department of Epidemiology and Public Health, Yale School of Medicine, 60 College Street, #600, P.O. Box 208034, New Haven, CT, 06520-8034, USA; fax: 203-785-4782; e-mail: Michael.Levin@Yale.edu.

only; ticks feeding on white-footed mice and from vegetation were collected and flagged in June (nymphs) and October (adults). Mice were trapped in Sherman live-traps twice per month from June through August and were held for several days in the laboratory in individual wire-mesh cages over water to allow all naturally attached ticks to feed to repletion. While in the laboratory, mice were provided with food and drinking water *ad libitum*. Engorged ticks were collected daily. A serum sample was collected from each mouse before release at the capture site.

Serum samples from 121 mice were tested for specific antibodies to the agent of HGE by indirect immunofluorescence assay (IFA) (Aquila Biopharmaceuticals, Worcester, MA). Antigen derived from human promyelocyte cell culture (HL-60) infected with the agent of HGE obtained from Westchester County, New York. Sera were screened at a dilution of 1:40 in 1X phosphate-buffered saline (pH 7.4). Several studies examined the specificity of IFA for the agent of HGE (including an assay involving the antigen produced by Aquila Biopharmaceuticals) and found no considerable serologic cross-reactivity between the agent of HGE and rickettsial organisms outside the *E. phagocytophila* group at dilutions 1:16 and higher (8,20,21). Thus, screening samples at 1:40 dilution ensures that sera testing HGE-positive were from mice actually exposed to that agent.

Host-seeking nymphal and adult *I. scapularis* collected from vegetation were tested individually by polymerase chain reaction (PCR) for infection with *B. burgdorferi* and the agent of HGE. Engorged ticks were allowed to molt to the next stage and were identified by species. *I. scapularis* nymphs derived from mouse-fed larvae were tested in pools—one pool (up to 10 ticks) per mouse—to assess the infectivity of individual mice for feeding larvae. Adult *I. scapularis* ticks derived from mouse-fed nymphs were tested individually, and the prevalence of each infection among these ticks was compared with the prevalence of each infection among questing adult ticks collected from vegetation at the same site.

For PCR testing, individual adult or nymphal ticks, or pools of nymphs, were placed in sterile 1.5 cc plastic vials, deep-frozen in liquid nitrogen, ground with a sterile plastic pestle, and resuspended in 50 ml of Tris-EDTA buffer. DNA was extracted from samples by using the IsoQuick Nucleic Acid Extraction Kit (ORCA Research Inc., Bothell, WA) to maximize sensitivity (22). Briefly, guanidine thiocyanate, a proprietary extraction matrix, and sodium dodecyl sulfate solution were added to a suspension, and the mixture was incubated at 65°C for 10 min. After separation of phases by centrifugation, DNA was precipitated with sodium acetate and isopropanol and washed with 70% ethanol. The final DNA pellet was resuspended in 50 ml of RNase-free water, and a 2.5-ml aliquot was used for each PCR. Primers EHR521 (5'-TGT AGG CGG TTC GGT AAG TTA AAG-3') and EHR747 (5'-GCA CTC ATC GTT TAC AGC GTG-3') were used to amplify a 247-bp fragment of 16S rDNA from the agent of HGE (6). Primers FLA297 (5'-CGG CAC ATA TTC AGA TGC AGA CAG-3') and FLA652 (5'-CCT GTT GAA CAC CCT CTT GAA CC-3') developed in the laboratory of Dr. Erol Fikrig (Yale School of Medicine) were used to amplify a 378-bp fragment of the flagellin gene of *B. burgdorferi*. The amplification products were visualized in 2% agarose gels.

Prevalence of Infections

The prevalence of *B. burgdorferi* infection in both questing nymphal and adult *I. scapularis* was similar between the two study sites in 1996 and remained stable for 2 consecutive years at the Bridgeport site (Table 1). Within a generation, the proportion of *Borrelia*-infected ticks increased less than twofold from nymphal (32% to 33%) to adult stage (52% to 56%) at both sites and in both years.

Prevalence of ehrlichial infection in *I. scapularis* nymphs was also similar at the two study sites and between years at the Bridgeport site (Table 1), while in questing adult ticks, ehrlichial prevalence varied. In 1996, the

Table 1. Prevalence of infection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis among questing nymphal and adult *Ixodes scapularis* of the same generation, Connecticut

Study site (year)	Stage	Ticks tested	% *Borrelia*-infected (± SE)	% *Ehrlichia*-infected (± SE)
Woodbridge (1996)	Nymphs	442	32.9 ± 2.3	1.6 ± 0.6
	Adults	251	52.6 ± 3.2	8.3 ± 1.9
Bridgeport (1996)	Nymphs	164	31.7 ± 2.2	1.2 ± 0.5
	Adults	48	56.3 ± 3.2	12.5 ± 2.1
Bridgeport (1997)	Nymphs	110	32.7 ± 4.5	1.8 ± 1.3
	Adults	100	55.0 ± 3.8	19.0 ± 3.0

prevalence of the agent of HGE in adult ticks at the Bridgeport site (12.5%) was higher than at the Woodbridge site (8.3%), although not significantly ($p = 0.11$). The proportion of adult ticks infected with the agent of HGE in Bridgeport was significantly higher in 1997 than in the previous year ($p = 1.44 \times 10^{-7}$). Within the same generation, the proportion of *Ehrlichia*-infected ticks increased fivefold in Woodbridge in 1996 and 10.4- to 10.6-fold in Bridgeport in 1996 and 1997, respectively.

An average of 0.7 engorged *I. scapularis* nymphs (0 to 7) and 16.7 (0 to 113) engorged *I. scapularis* larvae were collected per mouse. The highest mean density (± standard error) of nymphal infestation in *P. leucopus* (1.3±0.2) was recorded in late June, with mean larval density peaking in late August (34.6±1.7). Overall, 113 of 121 mice were infested with either nymphal or larval *I. scapularis*. Of 40 mice with nymphs, 32 (80.0%) produced adult ticks infected with *B. burgdorferi*, and 7 (17.5%) yielded ticks infected with the ehrlichial agent. Of 108 mice infested with *I. scapularis* larvae, 56 (51.4%) produced *B. burgdorferi*-infected nymphal ticks, and 20 (18.4%) produced nymphs infected with the agent of HGE. Of the 108 mice, 13 (11.9%) infected feeding ticks with both pathogens. Prevalence of infectivity in mice did not differ by month.

Of 121 mice trapped from June to August 1997 in Bridgeport and tested by IFA, 46 (38.0%) had antibodies against the agent of HGE. The relatively high sera dilution (1:40) used for screening allows the possibility that some samples with low antibody titers were missed and that an even higher proportion of the mouse population had actually been exposed to the agent of HGE.

The proportion of HGE-seropositive mice decreased from 47% (n = 49) in late June and July to 32% (n = 72) in August, perhaps because of a loss of antibody by mice over time, the recruitment of naive young mice into the population, or both. Of the 20 mice that produced nymphs infected with the agent of HGE, 17 were also seropositive. Evidently, these mice remained infectious for ticks, despite the presence of specific antibodies. The other three infectious mice were HGE-seronegative, which most likely indicated recent infection. Thus, only 18.4% of *P. leucopus* were capable of infecting ticks with the agent of HGE, although at least twice as many (as determined by IFA) had been exposed to this agent. Apparently, mice exposed to ticks infected with the agent of HGE may develop an immune response to the pathogen but not become infectious for xenodiagnostic ticks. These findings suggest that a high prevalence of the specific antibody against the agent of HGE in rodent populations does not necessary reflect the scope of rodents' involvement in transmitting the ehrlichial agent.

Prevalence of *B. burgdorferi* infection among adult ticks derived from mouse-fed nymphs was higher than among questing ticks collected from vegetation ($p = 0.0051$) (Table 2). Conversely, prevalence of infection with the agent of HGE in mouse-fed ticks was not quite half that of adult ticks collected from vegetation ($p = 0.0022$). Concurrent infection in mouse-fed ticks was also half that in adult ticks collected from vegetation (Table 2). The same trend was observed when prevalence of ehrlichial infection was compared among *Borrelia*-infected adult ticks. In the *Borrelia*-infected cohort collected from vegetation, 14 (25.5%) of 55 ticks were simultaneously infected with the agent of HGE, but in the cohort collected from mice, only 5 (10.2%) of 49 were simultaneously infected with the agent of HGE.

Mice that collect and feed many nymphs have a high probability of finding an infected tick and becoming infected. Mice that feed large numbers of ticks after infection would increase the rate of pathogen transmission. We tested the hypothesis that mice exposed to or currently infected with the agent of HGE are continuously infested with large numbers of ticks and thus are capable of increasing the prevalence of infection in a natural tick population. When we compared tick densities between mice infectious for ticks, mice seropositive for the agent of HGE, and mice

Table 2. Prevalence of infection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis among adult *Ixodes scapularis* ticks collected as engorged nymphs from wild-caught *Peromyscus leucopus* and from vegetation at the same site (Bridgeport, Connecticut)

Origin of ticks	Ticks tested	% *Borrelia* infected (± SE)	% *Ehrlichia*-infected (± SE)	% Concurrently infected (± SE)
P. leucopus	76	64.5 ± 3.6	9.2 ± 2.2	6.6 ± 2.5
Vegetation	100	55.0 ± 3.8	19.0 ± 3.0	14.0 ± 3.5

without specific antibodies or infected ticks, nymphal and larval infestation densities did not differ significantly (Table 3).

Table 3. Exposure to the agent of human granulocytic ehrlichiosis in white-footed mice, as detected by xenodiagnosis and indirect immunoflorescence assay (IFA), and their infestation with *Ixodes scapularis* nymphs and larvae (Bridgeport, Connecticut)

Status of mice	Mice tested	Mean no. of nymphs (± SE)	Mean no. of larvae (± SE)
Infectious[a]	20	0.7±0.1	19.5±1.6
IFA-positive	46	0.9±0.1	14.9±0.9
IFA-negative, noninfectious	72	0.7±0.1	17.8±1.9

[a]Mice infectious for ticks at the time of study.

Conclusions

Because nymphal *I. scapularis* naturally feed on vertebrate animals (small rodents, medium-sized and large mammals, birds, and reptiles) (23) that may vary in their ability to acquire and transmit a pathogen, the prevalence of infection in different portions of an adult tick population would differ depending on which host species the tick had fed on. Thus, the prevalence of infection for the tick population as a whole is an average resulting from contributions of individual host species. We compared average prevalence to the prevalence of infection in ticks derived from a particular host species to assess the relative contribution of that host species to amplification of a pathogen. For example, the prevalence of *B. burgdorferi* infection in mouse-fed ticks is considerably higher than the average prevalence of the infection in a general tick population, which suggests that the white-footed mouse is an important amplifying reservoir of *B. burgdorferi*. The prevalence of the agent of HGE in mouse-fed ticks is lower than the average, suggesting that the white-footed mouse is not as effective an amplifying reservoir for this agent as are other host species. An important contribution from non-*Peromyscus* host species that infects a large proportion of feeding nymphs with the agent of HGE appears necessary to account for the average prevalence of infection in host-seeking adult ticks.

The prevalence of *B. burgdorferi* infection in ticks increased less than twofold from nymphal to adult stage, while more than 50% of the white-footed mouse population was infected with *B. burgdorferi* and transmitted the pathogen to feeding ticks. At the same time, prevalence of infection with the agent of HGE in ticks regularly showed a 4.5- to 10.6-fold increase from nymphal stage to adult stage, although only 18% of mice were infectious for feeding ticks. This steep increase in prevalence of ehrlichial infection in ticks also suggests the involvement of other susceptible host species that maintain the natural transmission cycle of the agent of HGE at the observed level.

Dissimilarities between two pathogens—in the increase of infection in ticks from nymphal to adult stage and in prevalence of infection in the host-seeking population versus a subpopulation of mouse-fed ticks—suggest that natural cycles of the agents of Lyme disease and HGE differ. They involve the same vector-species, but the principal amplifying hosts for the two pathogens are not the same.

Although the white-footed mouse is susceptible to infection with both agents, this species alone cannot account for the observed prevalence of the agent of HGE in adult ticks. Our data suggest that most nymphal *I. scapularis* acquire the agent of HGE from non-*Peromyscus* hosts.

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Dr. Levin is a postdoctoral associate, Department of Epidemiology and Public Health, Yale School of Medicine. His areas of expertise are ecology and epidemiology of tick-borne zoonoses. His current research focuses on the dynamics of circulation of the agents of Lyme disease and human granulocytic ehrlichiosis in nature, reservoir competence of vertebrate animals, and multiple pathogen interactions.

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Malaria Reemergence in the Peruvian Amazon Region

Javier Aramburú Guarda,* César Ramal Asayag,* and Richard Witzig†
*Loreto Department of Public Health, Iquitos, Peru; and †Centers for Disease Control and Prevention, Atlanta, Georgia,USA

Epidemic malaria has rapidly emerged in Loreto Department, in the Peruvian Amazon region. Peru reports the second highest number of malaria cases in South America (after Brazil), most from Loreto. From 1992 to 1997, malaria increased 50-fold in Loreto but only fourfold in Peru. *Plasmodium falciparum* infection, which has increased at a faster rate than *P. vivax* infection in the last 3 years, became the dominant *Plasmodium* infection in the highest transmission areas in the 1997 rainy season. The vector *Anopheles darlingi* has also increased during this epidemic in Loreto. Moreover, chloroquine and pyrimethamine-sulfadoxine drug-resistant *P. falciparum* strains have emerged, which require development of efficacious focal drug treatment schemes.

The Peruvian Department of Loreto is the last part of the greater Amazon region to report the reemergence of epidemic malaria after the eradication efforts of the 1960s. Loreto, which comprises nearly one-fourth of the land mass of Peru, has the ecologic characteristics of the Amazon lowlands (Figure 1). In central Loreto, the Marañon and Ucayali Rivers combine to form the nascent Amazon River. Iquitos (population 345,000), the only large urban center in Loreto, is accessible only by air or river. The rural population of 474,000 is clustered in towns and villages throughout the Amazon tributary system. Loreto's economy relies on basic agriculture, fishing, lumber, commercial activities, and petroleum.

Epidemiology

Since 1941, the highest number of malaria cases in Peru was 95,000 in 1944, and the lowest was 1,500 cases in 1965 (after the malaria eradication campaign) (1). *Plasmodium falciparum* infections (chloroquine-sensitive) were confined to the northwestern coastal areas of Peru bordering Ecuador, with occasional reports of cases from the north and eastern Loreto border areas with Ecuador, Colombia, and Brazil.

Address for correspondence: Javier Aramburú Guarda, Oficina General de Epidemiología, Ministerio de Salud, Perú, Avenida Camilo Carrillo 402, Jesús María, Lima11, Perú; e-mail: jaramburu@oge.sld.pe.

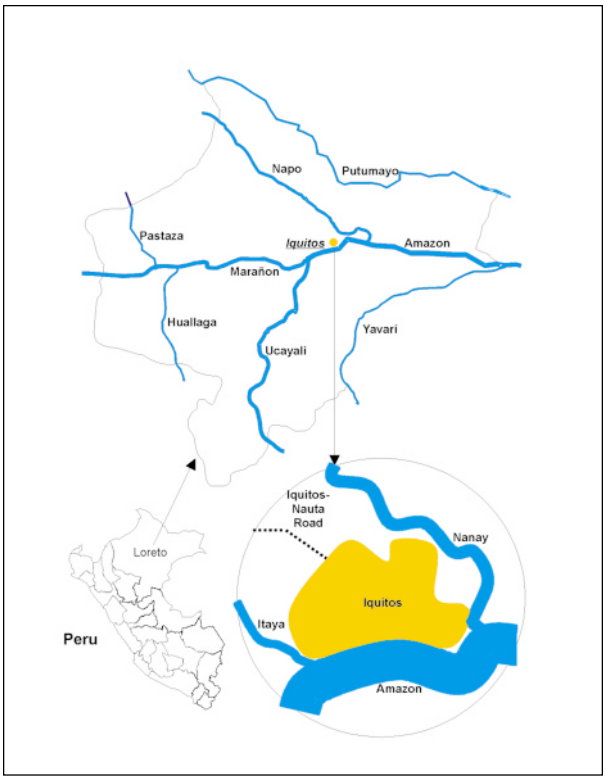

Figure 1: The department of Loreto and the city of Iquitos in Peru.

In 1988, no cases of *P. falciparum* were reported in Loreto. In 1991, 140 cases were reported; the number increased annually until 1997 when 54,290 slide-confirmed *P. falciparum*

cases and 85 deaths were reported (Figure 2). In 1997, a total of 121,268 malaria cases were reported in Loreto; the number indicates an equally dramatic rise in *P. vivax* malaria. *P. malariae* is infrequently reported (44 cases in 1997). An additional 36,864 persons were treated for probable malaria (slide-negative or no available laboratory diagnosis) in 1997. Therefore, the number of malaria cases in 1997 reported by slide or clinical definition was 158,115 (2). More *Plasmodium* infections occurred in males (60.5%) than in females (39.5%). Malaria transmission has been seasonal in the Loreto epidemic, with peaks in the rainy season from November to June (Figure 3).

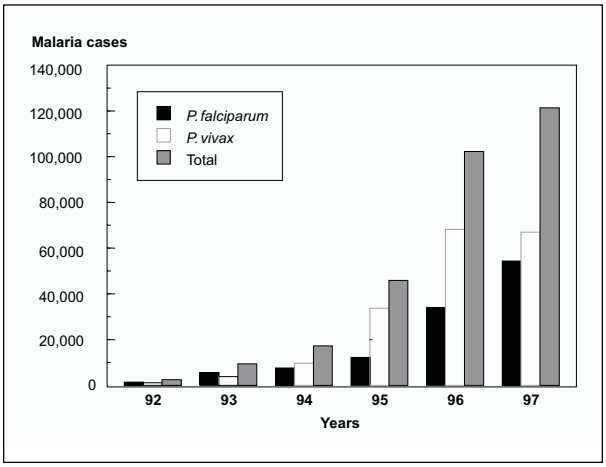

Figure 2: Malaria incidence in Loreto, 1992-1997.

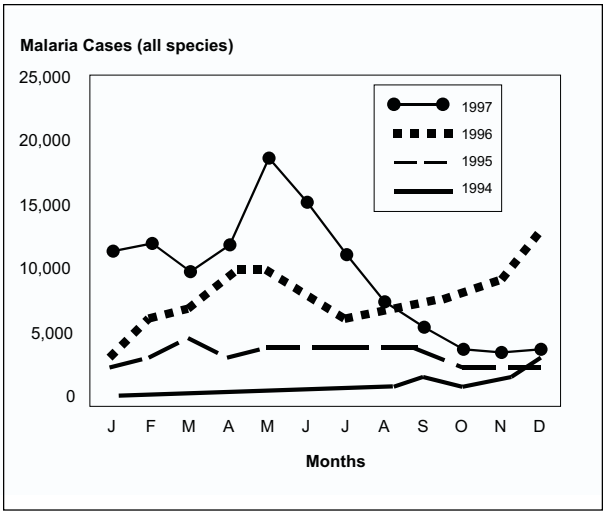

Figure 3. Malaria (all species) incidence in Loreto, by month, 1994–1997.

Malaria around the city of Iquitos accounts for most cases in Loreto. Indigenous *P. vivax* malaria was initially reported in the Iquitos area (Rumococha and Zungarococha) in April 1991, and *P. falciparum* was first detected (Padrecocha) in November 1994. A small number of *P. falciparum* cases have been diagnosed in patients who had not traveled outside Iquitos in the 2 months before illness. Entomologic investigations have confirmed that malaria transmission inside Iquitos is possible. Two high transmission zones surround Iquitos: communities on the Nanay River (which empties into the Amazon after bordering northern and eastern Iquitos) and communities along the first asphalt extension road from Iquitos (the unfinished Iquitos-Nauta road). Two additional high transmission zones include communities on the Yavarí River and the Pastaza River. The annual parasite index (number of malaria cases per 1,000 persons per year), with malaria transmission by districts in Loreto, is shown in Figure 4. The global annual parasite index for Loreto for 1997 was 148 per 1,000 persons.

Age-specific attack rates of *P. falciparum* in most of Loreto are consistent with hypoendemic malaria (defined as parasite or spleen rates less than 10% [Figure 5A]), although in 1997 in the highest transmission areas, mesoendemic malaria measurements were transiently documented in prevalence studies (11% to 50% parasite rates) (3). The high parasitemia rates in adults in Loreto emphasize the environmental

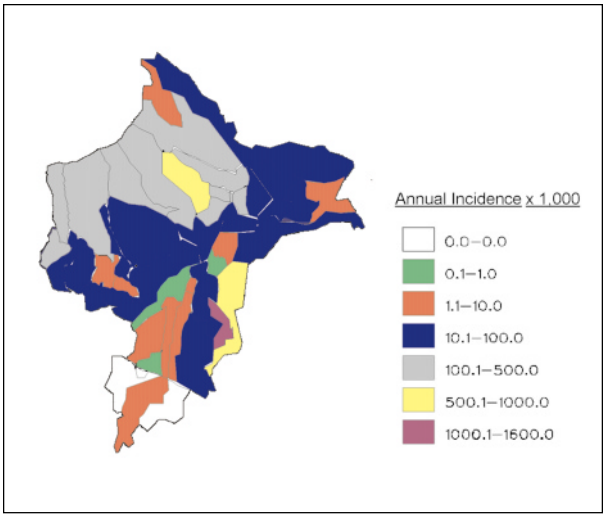

Figure 4: Annual parasite index in Loreto, by district (per 1,000 population).

risks for malaria infection to adults and the lack of immunity in this epidemic. Age-specific death rates have documented a substantial risk for death among those under 5 years of age and among those 60 years of age and older, although the number of deaths in children under 5 years declined in 1997 (Figure 5B). The death rate from *P. falciparum* malaria has been decreasing (from 1.8 to 1.3 per 1000) in the last 4 years (1994 to 1997).

Because of the current epidemic, Peru reports the second highest number of malaria cases in South America (after Brazil)—most from Loreto (in 1997, Loreto reported 67.2% of the malaria cases in Peru) (4). From 1992 to 1997, malaria increased 50-fold in Loreto and fourfold in Peru. In 1992, 1.6% of malaria cases in Peru were due to *P. falciparum*; in 1997 (in Loreto), 44.8% of malaria cases were due to *P. falciparum* (5,6).

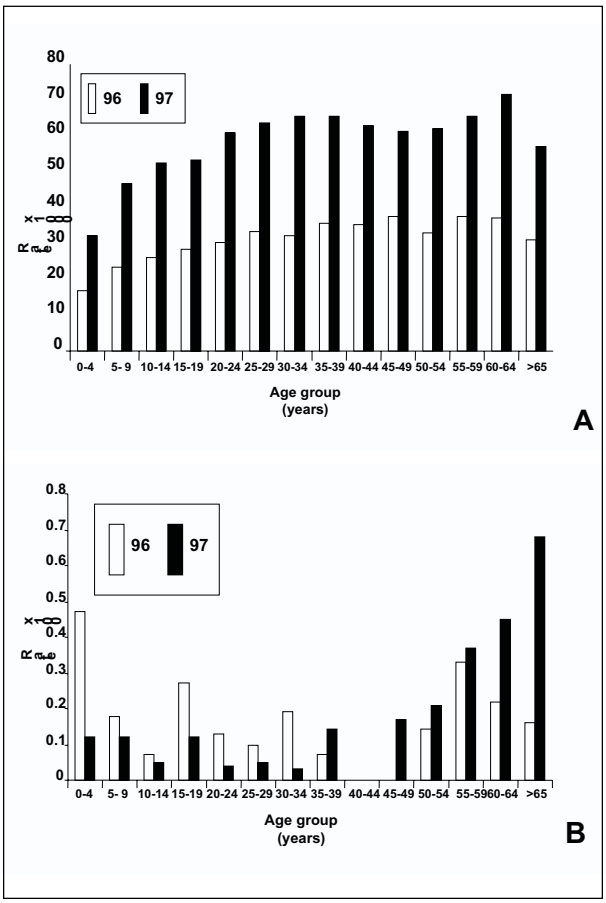

Figure 5. A. Age-adjusted incidence rates for *Plasmodium falciparum* malaria in Loreto, 1996-1997. B. Age-adjusted death rates for *Plasmodium falciparum* malaria in Loreto, 1996-1997.

Vector Dynamics

Paralleling the malaria epidemic has been an increase in the highly competent and anthropophilic malaria vector, *Anopheles darlingi*, the principal *P. falciparum* vector in the Brazilian Amazon (7,8). *An. darlingi* was not found in the Iquitos area in 1991 (9) when indigenous *P. vivax* was detected but has now been found in all areas of Loreto and even within Iquitos (10-12). The abundance of *An. darlingi,* already established when *P. falciparum* was first reported from the peri-Iquitos area in 1994, is clearly linked to the marked rise in *P. falciparum* transmission.

An. darlingi larvae habitats around Iquitos include small pools on cleared land, fish hatcheries, areas of poor sanitation, swamps, and the edges of small rivers. *An. darlingi* in the Iquitos area bite indoors or outdoors from dusk to midnight, with a small dawn peak. *An. darlingi* represent more than 90% of the anophelines in the peri-Iquitos area in the rainy season; they decrease but remain the major anopheline species in the dry season.

An. benarrochi is the dominant malaria vector in western Loreto, while *An. triannulatus* is the dominant vector in eastern Loreto. Other *Anopheles* species in Loreto known to be malaria vectors are *An. oswaldoi, An. nuneztovari,* and *An. rangeli.*

Climatic Associations

Loreto has a climate typical of the Amazon region, with a rainy season from November to May and a second precipitation peak in September in the dry season. The El Niño phenomenon in 1997 extended the dry season in Loreto, but caused torrential rains along coastal Peru.

The level of the Amazon River in Iquitos varies 10 meters annually (from 108 to 118 meters above sea level); flooding is usual on the smaller tributaries but infrequent on the main Amazon River. The mean annual temperature is 28°C, the warmest months are September and October, and the humidity is persistently higher than 87% (2).

Climatic indexes of river level, rainfall, temperature, and humidity at Iquitos were analyzed to determine association with malaria prevalence. The height of the Amazon River at Iquitos depends on rainfall on the eastern side of the Andes Mountains as well as precipitation in Loreto. The height of the Amazon is related to malaria prevalence; the highest river level

precedes the malaria prevalence peak by 2 months, and the lowest level precedes the malaria prevalence low by 2 months (Figure 6A). Precipitation peaked twice in 1997: 3 months before and the same month that malaria cases reached their highest number (Figure 6B). The mean temperature was 27°C to 29°C and was inversely associated with malaria cases (Figure 6C). Relative humidity was 87% to 93% and was not associated with malaria prevalence.

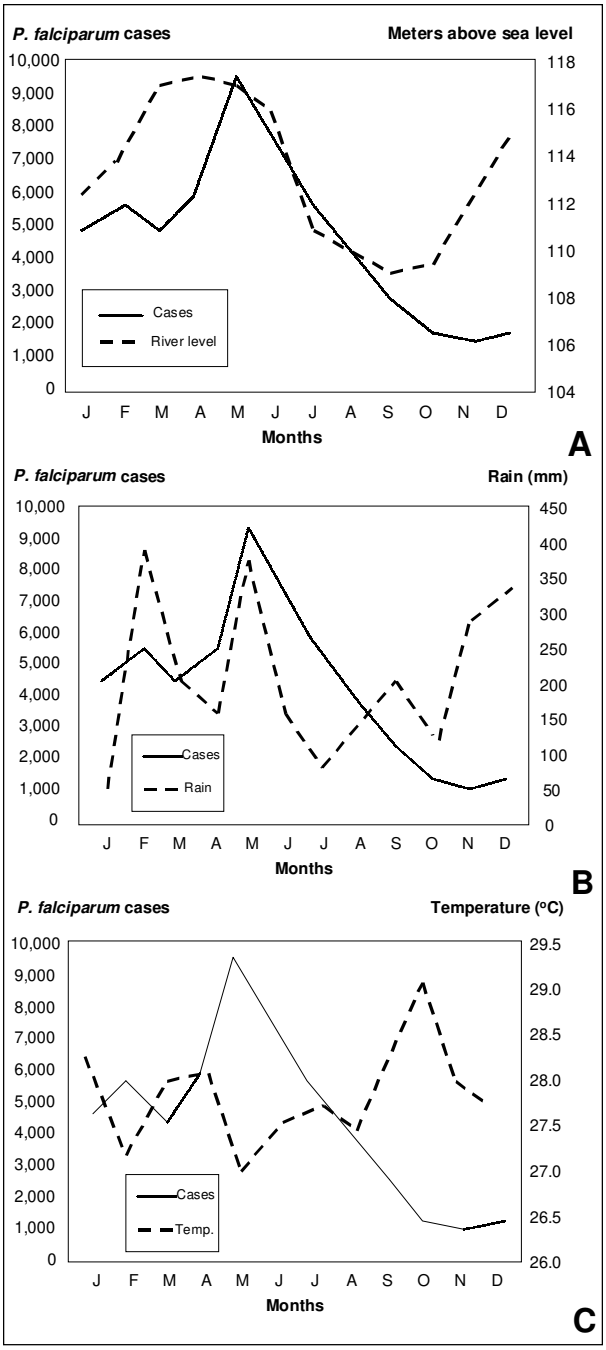

Figure 6: *Plasmodium falciparum* malaria incidence in Loreto. A. Average Amazon River level at Iquitos, by month. B. Precipitation at Iquitos, by month. C. Average temperature (°C) at Iquitos, by month.

Drug Resistance and Drug Treatment

Clinical resistance of *P. falciparum* parasites to chloroquine and to the combination antifolate drug pyrimethamine-sulfadoxine is a growing public health problem in Peru. In an epidemic characterized by limited population immunity, parasitologic resistance (persistence or reemergence of parasites) inevitably leads to clinical resistance (reemergence of malarial symptoms).

Multiple regional in vivo drug susceptibility testing for chloroquine (10% to 70% resistance) and pyrimethamine-sulfadoxine (10% to 63% resistance) has documented significant focal differences in resistance patterns (13-15). Three *P. falciparum* strains have converged on Loreto and Iquitos: (Brazilian-pyrimethamine-sulfadoxine and chloroquine, complete resistance; Loretana–pyrimethamine-sulfadoxine, variable resistance; chloroquine resistant; and Pastazan/coastal-chloroquine susceptible). These strains were likely introduced and disseminated in different ways (e.g., routine travel, illegal narcotrafficking), but the increased abundance of *An. darlingi* in Loreto has allowed them to thrive. The highest percentage of strains resistant to multiple drugs (both chloroquine and pyrimethamine-sulfadoxine–resistant) have been found in the border areas of Colonia Angamos on the Yavarí River, communities on the Blanco River, and in Santa Clara near Iquitos.

From July through November 1996, we performed in Iquitos two prospective studies of *P. falciparum* drug resistance in patients from periurban and rural areas in Loreto. Twenty-eight day in vivo drug susceptibility testing of *P. falciparum* to pyrimethamine-sulfadoxine was performed in 62 patients. The WHO in vitro microtest method was used to evaluate *P. falciparum* susceptibility to mefloquine, chloroquine, pyrimethamine-sulfadoxine, and quinine in pretreatment specimens from the same patients (16). Results showed significant pyrimethamine-sulfadoxine resistance in Loreto (31% in vivo and 67% in vitro) and documented in vitro resistance of chloroquine (78% resistance). *P. falciparum* parasites were susceptible to mefloquine and quinine (17).

A recent study in Loreto showed that in vivo resistance to pyrimethamine-sulfadoxine correlated with specific point mutations in *P. falciparum* dihydrofolate reductase at amino acid positions 51, 108, and 164, and dihydropteroate synthase mutations at positions 437, 540, and 581 (18). Further studies mapping the molecular resistance patterns of *P. falciparum* in Loreto are under way.

Malaria diagnosis and treatment are provided free of charge by the National Malaria Program. Focal treatment schemes have been designed depending on the local resistance patterns as documented by in vivo methods. Depending on local efficacy, first-line treatment of chloroquine, second-line of pyrimethamine-sulfadoxine, or third-line of quinine with clindamycin or tetracycline is administered (Figure 7). In 1998, treatment with mefloquine was initiated in selected communities with

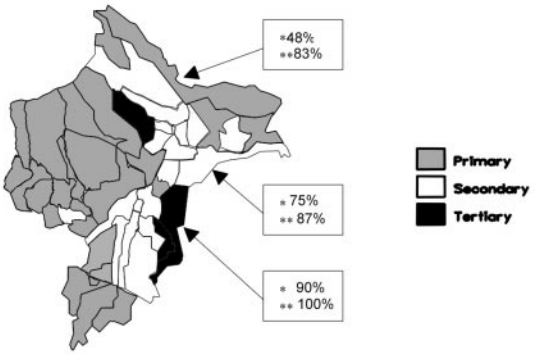

Figure 7: Initial *Plasmodium falciparum* malaria treatment schemes in Loreto by district, 1998. Treatment efficiency* (cases cured/cohort number x 100) and efficacy** (cases cured/[cases cured + resistant cases] x 100) shown for each treatment scheme region. Treatment schemes: Primary-chloroquine; secondary-pyrimethamine-sulfadoxine; tertiary-quinine.

tertiary treatment schemes. All treatment lines include one dose of primaquine to eradicate *P. falciparum* gametocytes.

Control Schemes

Control strategies used by the Loreto Public Health Department and the National Malaria Program have included source, chemical, and biologic reduction strategies. Source reduction has consisted of community participation in identifying and filling in larval sites. Chemical control includes the use of insecticides in spatial fogging and domiciliary spraying and on bed nets. Pyrethroids (e.g., cyfluthrin) are used for fogging and domiciliary residual spraying. Since 1988, DDT has not been used in Loreto. An important challenge is that many Amazonian houses have open eaves and windows that allow mosquitoes to enter and exit without touching surfaces (except for a person's skin). *An. darlingi* behavioral studies have not yet been performed in Loreto to determine if the mosquitoes rest inside or outside after feeding. Larvaciding is performed with temephos (tetramethyl-thiodiphenylene phosphorothioate) placed at the surface of the larval breeding sites. Biologic control has been investigated with *Bacillus sphaericus* and *thuringiensis* in larval breeding pools. These bacteria have a short residual effect of 7 to 15 days in Loreto and require optimal conditions of shade for maximum efficacy.

Bed nets (*mosquiteros*) are ubiquitous in Loreto because of the many biting mosquito species. Local bed nets are made principally of cotton and sell for US$10 to $15. Insecticides were used in 1997 in the form of spraying (with cyfluthrin or deltamethrim) and impregnation (with either permethrim or deltamethrim) (19). Preliminary results show a decrease in malaria prevalence where insecticide-treated bed nets are in use.

Conclusions

Malaria began to reemerge in the upper Pastaza River and eastern border areas of Loreto in the early 1990s. *P. falciparum* exceeded *P. vivax* infections in the early part of the Loreto epidemic (1992-1993). The abundance of *An. darlingi* starting in 1993 set the stage for explosive growth of both *P. falciparum* and *P. vivax* transmission, especially around Iquitos, which had previously not been affected. The increase in the number of malaria cases and the erosion of *P. vivax* dominance over *P. falciparum* occurred in spite of governmental interventions (treatment schemes, fumigation, larviciding, and domiciliary spraying). While *P. vivax* infections decreased slightly from 1996 to 1997, *P. falciparum* cases continued to increase by 60%. However, the rate of increase in total malaria cases slowed—from a factor of 2.23 from 1995 to 1996 to 1.19 in 1996 to 1997. The predominance of malaria infection in men is

likely the result of occupational risks (e.g., working in recently cleared areas, logging, and night fishing and hunting).

The death rate of epidemic *P. falciparum* in Loreto appears to be low. The oft-quoted 1% death rate for *P. falciparum* infection (3) has been reduced seven times, perhaps because of determined efforts at the regional and national levels to provide rapid and appropriate treatment in malarious areas. Future efforts are planned to continue and expand coverage to further reduce deaths due to *P. falciparum*.

Although positive correlations were found between malaria transmission periods and Amazon River level and rainfall, the negative correlation with temperature may not be as significant as in other areas where higher temperatures are positively correlated with malaria cases (20). The mean temperature in the rainy season in Loreto is still within the optimal range for anopheline development, and so the observed temperature correlation may have little biologic influence on malarial prevalence.

Analysis of environmental factors indicates that the optimal timing of malaria control activities in 1998 was 2 months before the rainy season. Integrated malaria control activities began in the dry season with the strategy to intervene before high transmission season. In 1998, slide-documented malaria cases decreased 30.7% to 84,059, with *P. vivax* cases decreasing 15.3% to 56,710 and *P. falciparum* cases decreasing 49.6% to 27,336. The longer dry season in 1997, apparently caused by El Niño, may have assisted the Loreto Public Health Department and National Malaria Program efforts to control malaria in 1998.

Danger exists for further expansion of malaria in Loreto, especially through expansion of *An. darlingi* (into areas where it is not yet the dominant vector) and evolution of *P. falciparum* drug resistance. Consequently, more malaria control research is needed, particularly environmental control program studies, *Anopheles* behavioral studies, drug resistance testing, and community bed net trials.

Acknowledgments

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Dr. Javier Aramburú Guarda is chairman of Surveillance of Infectious Diseases, General Office of Epidemiology, Ministry of Health of Perú. His research focuses on antimalarial resistance, integrated malaria control, and malaria control policies.

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Enteropathogenic *E. coli*, *Salmonella*, and *Shigella*: Masters of Host Cell Cytoskeletal Exploitation

Danika L. Goosney, Derek G. Knoechel, and B. Brett Finlay
University of British Columbia, Vancouver, British Columbia, Canada

Bacterial pathogens have evolved numerous strategies to exploit their host's cellular processes so that they can survive and persist. Often, a bacterium must adhere very tightly to the cells and mediate its effects extracellularly, or it must find a way to invade the host's cells and survive intracellularly. In either case, the pathogen hijacks the host's cytoskeleton. The cytoskeleton provides a flexible framework for the cell and is involved in mediating numerous cellular functions, from cell shape and structure to programmed cell death. Altering the host cytoskeleton is crucial for mediating pathogen adherence, invasion, and intracellular locomotion. We highlight recent advances in the pathogenesis of enteropathogenic *Escherichia coli, Salmonella* Typhimurium, and *Shigella flexneri*. Each illustrates how bacterial pathogens can exert dramatic effects on the host cytoskeleton.

Enteropathogenic *Escherichia coli* (EPEC): A Model for Studying Bacterial Attachment and Effacement

Pathogenic *E. coli* strains remain a leading cause of severe and persistent infant diarrhea in developing countries. Although EPEC is recognized as a major diarrheal pathogen, until recently our understanding of how it causes disease lagged behind that of other pathogenic *E. coli*, such as enterotoxigenic *E. coli* or enteroinvasive *E. coli*.

EPEC is one of a class of pathogens identified as causing attaching and effacing (A/E) lesions on intestinal cells (1). A/E pathogens typically reside on a pedestal on the surface of the host epithelial cell and ultimately cause severe disruption of the microvilli brush border (Figure 1A). Other pathogens displaying similar histopathologic features include *Hafnia alvei*, *Citrobacter rodentium* (formerly *C. freundii* biotype 4280), and enterohemorrhagic *E. coli,* the causative agent of hemolytic uremic syndrome.

Address for correspondence: B. Brett Finlay, Biotechnology Laboratory and the Departments of Microbiology and Immunology and Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3; fax: 604-822-9830; e-mail: bfinlay@unixg.ubc.ca.

Bacterial Factors Involved in EPEC-Induced A/E Lesion Formation

The interactions between EPEC and host cells have been divided into three stages. Initial adherence to cultured epithelial cells is mediated by the formation of type IV fimbriae known as bundle forming pili (BFP) (2). While not essential for forming the characteristic A/E lesions, initial adherence helps bring the bacteria in close contact with the host cell. BFP mediate bacterial-bacterial interactions in a human intestinal organ culture model (3).

All the genes necessary for the formation of A/E lesions by EPEC are contained within a 35-kbp pathogenicity island termed the locus of enterocyte effacement (LEE) (Figure 1B) (4,5). These include the *esp*s (*E. coli*-secreted protein), *esc*s (*E. coli* secretion), *sep* (secretion of *E. coli* proteins), *eae* (*E. coli* attaching and effacing that encodes intimin), and *tir* (translocated intimin receptor) genes (6).

The second stage of EPEC pathogenesis involves the secretion of bacterial proteins, some into the host cell, including EspA, EspB, and EspD (7,8). The expression of these proteins is maximal at the host body temperature (9) and at conditions similar to those found in the gastrointestinal tract (10), which implies that they may be involved in virulence. The translocation of these proteins is essential for

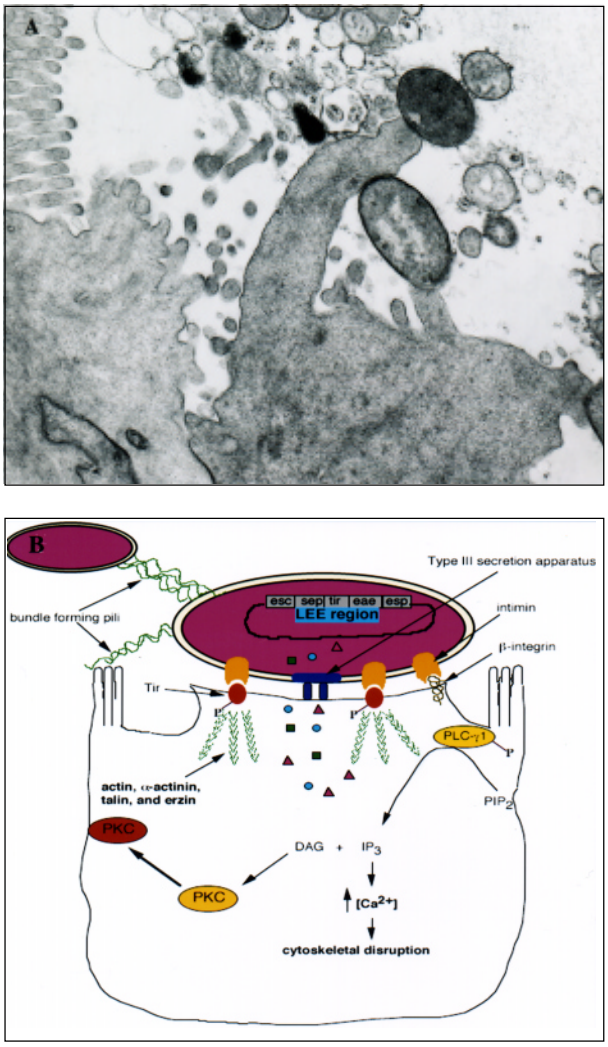

Figure 1. A. Transmission electron micrograph of an A/E lesion formed by rabbit enteropathogenic *Escherichia coli* (EPEC) infecting rabbit intestinal epithelial cells (micrograph provided by Dr. Ursula Heczko, Biotechnology Laboratory, University of British Columbia). B. Effects of EPEC infection on host intestinal epithelial cells. EPEC initially adheres to the host cell by its bundle-forming pili, which also mediate bacterial aggregation. Following initial attachment, EPEC secretes several virulence factors by a type III-secretion system. Signal transduction events occur within the host, including activation of phospholipase C (PLC) and protein kinase C (PKC), inositol triphosphate (IP_3) fluxes, and Ca^{2+} release from internal stores. The bacterium intimately adheres to the cell by secreting its own receptor, Tir, into the host and binding to it with its outer membrane ligand, intimin. Intimin can also bind ß1-integrins. Several cytoskeletal proteins are recruited to the site of EPEC attachment, including actin, α-actinin, talin, and ezrin. Cytoskeletal rearrangements occur following Tir-intimin binding, resulting in the formation of a pedestal-like structure upon which the pathogen resides.

activating a number of signal transduction pathways (7), although their precise role in pathogenesis is not well defined. EspA makes filamentous appendages outside the bacterium and may be part of the translocation machinery involved in delivering other virulence proteins (11). EspB is translocated into the host cytosol and membrane, where it may effect changes in the host cell's signaling pathways (12). All of these effector proteins are secreted by a type-III secretion system encoded by the *esc* and *sep* genes (6). Type-III secretion systems also play an important role in other gram-negative pathogenic bacteria such as *Yersinia*, enabling virulence factors to be translocated directly from the bacterial cytoplasm to the host-cell membrane or cytoplasm (13).

The third stage of EPEC infection is characterized as intimate attachment with the host cell. Intimin, a 94-kDa outer membrane protein encoded by the *eae* gene (14), binds to a 90-kDa tyrosine phosphorylated protein in the host membrane (15). This receptor, originally thought to be a host protein, has recently been found to be of bacterial origin and has been designated as the translocated intimin receptor (Tir) (16). As the name suggests, Tir is translocated from the bacterial cell into the host membrane, where it becomes phosphorylated on one or more tyrosine residues and functions as a receptor for its binding partner, intimin. The resultant tight association is accompanied by the formation of actin pedestals up to 10 µm in length (15). Purified intimin also binds ß1 integrins, which suggests that intimin may be binding more than one receptor on the epithelial cell (17). Although integrins are not present on the apical surface of enterocytes, they are located on the apical surface of microfold cells found in Peyer's patches along the intestinal lumen (18).

Host-Cell Factors Involved in A/E Formation

The host cell undergoes a number of changes during infection by EPEC (Figure 1B). The most striking change in the cellular structure of the host cell is the formation of characteristic actin pedestals. Within 3 hours of infection by EPEC, host-cell actin, α-actinin, talin, erzin, and villin accumulate directly under the bacteria (19,20). The latter four cytoskeletal components are involved in cross-linking of actin microfilaments. Localized actin accumulation is so distinct that it

forms the basis of an in vitro diagnostic test for EPEC, which uses fluorescein-tagged phalloidin to detect actin accumulation within infected cells (21). The actin pedestals are not static; instead they lengthen and shorten, resulting in apparent movement of EPEC along the host-cell surface (20). The pedestals resemble microvilli in the distribution of actin and villin (20). Microtubule and intermediate filament structures are not affected by EPEC virulence factors (19).

Intracellular calcium levels also seem to play a role in EPEC pathogenesis. EPEC-infected HEp-2 cells show significant elevation of intracellular calcium levels (22), and buffering of these levels can prevent or delay the formation of A/E lesions (23). Increases in intracellular calcium levels can result in the depolymerization of actin by villin (a calcium-dependent microvillus protein) and a breakdown of the host cytoskeleton not unlike that seen in EPEC-infected cells (24). Inositol triphosphate (IP_3) is involved in the release of Ca^{2+} from intracellular stores, and increased levels of IP_3 (25) and inositol phosphate fluxes (26) have been observed in EPEC-infected cells. EPEC interactions with PLC-γ1 HeLa epithelial cells activate a number of proteins, including phospholipase C-γ1 (PLC-γ1) (27). Phosphorylation of PLC-γ1 leads to the IP_3 and Ca^{2+} fluxes mentioned above, underscoring the importance of this signaling event. Cytosolic protein kinase C also gets activated upon EPEC infection and translocates to the plasma membrane (28).

Despite the dramatic changes induced by EPEC in the cytoskeleton, there appears to be little involvement of the Rho family of small GTP-binding proteins normally involved in cytoskeletal rearrangements (29). Inhibition of Rho, Rac, and Cdc42 by compactin and *Clostridium difficile* ToxB, as well as dominant negative alleles, had no effect on pedestal formation by EPEC, which suggests that this pathogen uses a nontraditional mechanism to rearrange actin.

Salmonella Typhimurium: A Model for Studying Bacterial Invasion

S. Typhimurium is a gram-negative bacterium that causes a variety of diseases, from gastroenteritis in humans to typhoid fever in mice. *S.* Typhimurium infections are contracted by oral ingestion and penetration into the intestinal epithelium before induction of systemic (invasive) disease. Invasion into the host intestinal cells results in dramatic morphologic changes to the cell that are due to exploitation of the host cytoskeleton.

Once in close contact with the epithelium, *Salmonella* induces degeneration of enterocyte microvilli (30). Loss in microvillar structure is followed by profound membrane ruffling localized to the area of bacterial-host cell contact (Figure 2A) (29-31). Membrane ruffling is accompanied by profuse macropinocytosis, which leads to the internalization of bacteria into the host cells (32). The entire process occurs within minutes and when completed, *Salmonella* resides within membrane-bound vesicles, and the cytoskeleton returns to its normal distribution (33).

Bacterial Factors Involved in *Salmonella* Invasion

Salmonella entry into nonphagocytic epithelial cells requires several chromosomal genes (*inv/spa*) clustered in a pathogenicity island termed SPI1 (*Salmonella* pathogenicity island 1) (34). Like EPEC, SPI1 encodes a type III-secretion system and several potential virulence factors secreted by this machinery. The type III-secretion system is activated upon host-cell contact and allows export of virulence determinants directly into the host cell, where they effect bacterial uptake (35,36). Recently, SptP, a bacterial protein encoded within SPI1, has been shown to be translocated into the host epithelial cell, where it modulates the host actin cytoskeleton through its tyrosine phosphatase activity (37) (Figure 2B). Disruption of a critical Cys residue in the catalytic domain of SptP results in loss of phosphatase activity (38). It is hypothesized that SptP may function in disrupting host actin stress fibers, thereby facilitating membrane ruffling and subsequent bacterial uptake into host cells.

Other bacterial factors are not encoded next to the secretion apparatus but instead on the genome of a cryptic bacteriophage found in the *Salmonella* chromosome. Recently, a virulence factor encoded within this genome, SopE, has been shown to be required for efficient bacterial entry into host cells (39). SopE requires the type III-secretion system to be translocated into the host cell, where it can directly stimulate actin cytoskeletal rearrangements. It acts as a guanidine exchange factor for members of the

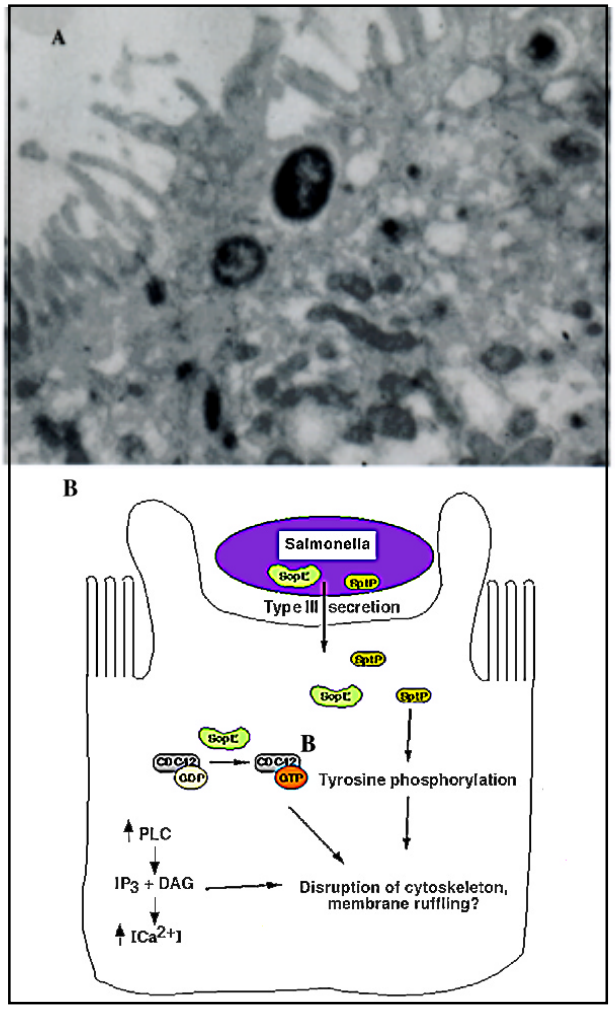

Figure 2. A. Transmission electron micrograph of *Salmonella*-induced membrane ruffling in polarized Caco-2 epithelial cells. B. *Salmonella* invasion into host epithelial cells. *Salmonella* secrete virulence proteins, including SopE and SptP, into host cells by the type III-secretion system. SopE functions as a guanidine exchange factor for small GTP-binding proteins, probably mediating the exchange of GDP for GTP on a Rho subfamily member, CDC42. SptP is a tyrosine phosphatase required for invasion, probably by disrupting the cytoskeleton. Invasion also stimulates phospholipase C (PLC) activity, leading to inositol triphosphate (IP_3) and Ca^{2+} fluxes, which in turn may be involved in cytoskeletal rearrangements leading to membrane ruffling and *Salmonella* internalization.

Rho subfamily of small GTPases. *sopE* mutants exhibit less extensive actin cytoskeletal rearrangements upon entry into epithelial cells than do wild-type *Salmonella* (40). This discovery clearly illustrates how pathogens (which contain no primary sequence homology with host proteins) can craftily subvert the host's own signaling machinery within the cell by mimicking host proteins.

Host Factors Involved in *Salmonella* Invasion

The massive restructuring of the host cytoskeletal components during *Salmonella* entry requires many host factors. A Rho subfamily member, Cdc42, is needed for mediating bacterial uptake through membrane ruffling (41). It is believed that the guanidine exchange activity of SopE is responsible for the stimulation of Cdc42 in the host. The pathogen also activates host PLC upon bacterial contact, leading to the production of two second messengers, which further initiate signaling events (42). As a consequence, the host cell's Ca^{2+} levels are altered to trigger cytoskeletal rearrangements resulting in *Salmonella* invasion. Although EPEC and *Salmonella* use some of the same signaling components (PLC, Ca^{2+} fluxes), the cytoskeletal changes induced in the host cell by each pathogen are quite different. This could be the result of different upstream or downstream effectors in the signaling pathway. Several cytoskeletal components involved in invasion have been identified. These include α-actinin, tropomyosin, ezrin, and talin (19). The specific roles of these proteins in *Salmonella* invasion are not defined.

Shigella flexneri: A Model for Intracellular Motility

S. flexneri, a gram-negative bacillus that causes bacillary dysentery in humans, directs its own uptake into the colonic mucosa through membrane ruffling and macropinocytosis in a manner similar to *Salmonella* uptake (43,44). After engulfment, the pathogen is surrounded by a membrane-bound vacuole within the host. Unlike *Salmonella*, however, *Shigella* rapidly lyses the surrounding vacuole and is released into the cytosol, where it grows and divides (45). Once the microbe has escaped from the vacuole, it quickly becomes coated with filamentous actin and ultimately forms an actin tail at one pole of the bacterium (Figure 3A) (46,47). This actin polymerization propels the bacterium through the cytoplasm at speeds reaching 0.4 μm/sec (48). When the pathogen reaches the plasma membrane of the cell, it forms a long protrusion

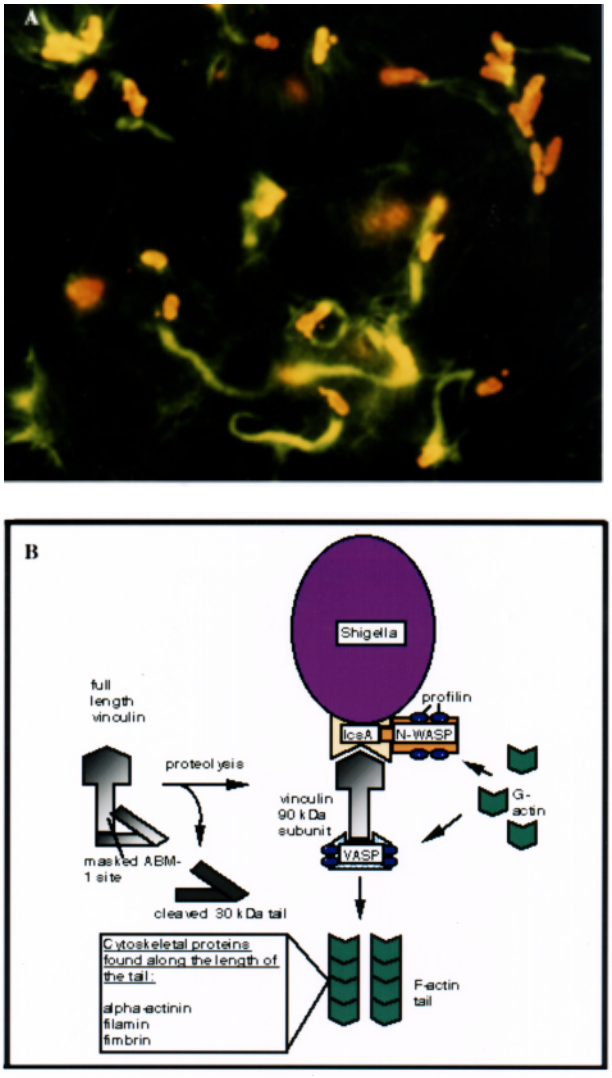

Figure 3. A. Immunofluorescence micrograph showing *Shigella* (red) propelling itself through the cytoplasm by polymerizing actin (green) (Philippe Sansonetti, Institut Pasteur, reprinted with permission from Trends in Microbiology, 1996). B. *Shigella*-mediated cytoskeletal rearrangements. The outer membrane protein, IcsA, is sufficient to drive actin-based motility of *Shigella*. IcsA directly binds two proteins, vinculin and neural-Wiskott-Aldrich Syndrome protein (N-WASP). Vinculin undergoes proteolysis within the host cell upon *Shigella* infection, producing a 90-kDa fragment that can bind to IcsA and to the vasodilator-stimulated phosphoprotein (VASP). VASP in turn can recruit profilin to the bacterial surface, which can provide actin for tail construction. N-WASP binding of IcsA can also recruit profilin to the bacterial surface and may be another means of obtaining monomeric actin for tail formation and subsequent bacterial motility.

into the neighboring cell, which subsequently internalizes the microbe (49). The bacterium again breaks out of the vacuole, thereby starting a new cycle of infection in a new host cell (50). This process allows *Shigella* to move from cell to cell without ever contacting the extracellular milieu.

Bacterial Factors Involved in *Shigella* Motility

Analysis of mutants deficient in intracellular motility and cell-to-cell spread has identified a bacterial gene, *icsA*, necessary for *Shigella* locomotion (46,51,52). IcsA (also called VirG) is a 120-kDa outer membrane protein that hydrolyzes ATP and is localized to one pole of the bacterium, at the junction between the microbe and the actin tail (Figure 3B) (53). IcsA expression on the surface of *Shigella* is sufficient to direct actin-based motility (54,55). In fact, *E. coli* expressing IcsA can synthesize actin tails in cytoplasmic extracts (54,55).

During infection, IcsA is also detected as a 95-kDa amino-terminal fragment of the 120-kDa full-length protein (53). This proteolytic cleavage of IcsA is due to a bacterial protease, SopA (IcsP) (56,57). Cleavage is required for polarized distribution of IcsA on the bacterial surface and for proper actin-based motility of *Shigella* in infected cells (56-58).

Host Factors Involved in *Shigella* Motility

IcsA expression on the *Shigella* surface promotes rapid accumulation of actin around the bacterium. Following bacterial division and IcsA polarization, actin tails begin to form on one end of the bacterium. Several host cytoskeletal proteins are involved in tail formation, including α-actinin (48), filamin (59), fimbrin (59), vasodilator-stimulated phosphoprotein (VASP) (60), vinculin (49,61), and neural-Wiskott-Aldrich syndrome protein (N-WASP) (63). Of these proteins, only vinculin and N-WASP are able to directly bind IcsA (61,62).

Shigella infection results in the cleavage of intact vinculin (120 kDa) to produce a 90-kDa fragment (63). This proteolysis unmasks an actin-based motility 1 site on vinculin, which contains a polyproline region capable of binding VASP. VASP recruitment to the bacterial surface in turn allows the recruitment of other

cytoskeletal proteins, such as actin and profilin, and forms the basis of an actin-based motor for *Shigella* movement.

Recently, N-WASP was shown to be required for *Shigella* motility (62); like vinculin, it can bind IcsA directly. It is possible that N-WASP, in addition to VASP, can recruit profilin and actin to the surface of *Shigella*, thereby mediating actin polymerization. Furthermore, N-WASP contains an actin depolymerization factor/cofilin homologous region, which could be used for severing actin filaments at the pointed ends and increasing the monomeric actin concentration. The precise mechanisms involved in *Shigella*-driven actin polymerization, however, are unclear.

Conclusions

Bacterial pathogens have evolved several mechanisms to hijack host-cell signaling machinery and disrupt the cytoskeleton. EPEC mediates its effects on the host cell from the cellular surface. It secretes its own receptor, Tir, into the host and then binds intimately to it by its outer membrane protein, intimin. Tir-intimin binding results in a dramatic reorganization of the cytoskeleton to form the pedestal upon which EPEC resides. *Salmonella*, on the other hand, actively invades intestinal epithelial cells by inducing membrane ruffling and macropinocytosis. Invasion is dependent on the secretion of virulence proteins, including SptP and SopE, into the host cell, and mediates its effects on the host from within a membrane-bound vesicle. *Shigella* is also an invasive pathogen but lyses the phagocytic vacuole and initiates intracellular actin-based locomotion to spread from cell to cell in the cytoplasm. This motility is dependent on the bacterial outer membrane protein IcsA, which recruits several actin-associated proteins to the bacterial surface. Despite the outward differences between each mode of pathogenesis, EPEC, *Salmonella*, and *Shigella* have effectively managed to subvert the host cytoskeleton for their own purposes and cause substantial diarrheal disease.

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Danika Goosney is a Ph.D. candidate in Dr. B. Brett Finlay's laboratory in the Department of Microbiology and Immunology and the Biotechnology Laboratory at the University of British Columbia. She is currently investigating EPEC-induced cytoskeletal rearrangements in cultured epithelial cells.

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Bacterial Toxins: Friends or Foes?

Clare K. Schmitt, Karen C. Meysick, and Alison D. O'Brien
Uniformed Services University of the Health Sciences,
Bethesda, Maryland, USA

Many emerging and reemerging bacterial pathogens synthesize toxins that serve as primary virulence factors. We highlight seven bacterial toxins produced by well-established or newly emergent pathogenic microbes. These toxins, which affect eukaryotic cells by a variety of means, include *Staphylococcus aureus* α-toxin, Shiga toxin, cytotoxic necrotizing factor type 1, *Escherichia coli* heat-stable toxin, botulinum and tetanus neurotoxins, and *S. aureus* toxic-shock syndrome toxin. For each, we discuss the information available on its synthesis and structure, mode of action, and contribution to virulence. We also review the role certain toxins have played in unraveling signal pathways in eukaryotic cells and summarize the beneficial uses of toxins and toxoids. Our intent is to illustrate the importance of the analysis of bacterial toxins to both basic and applied sciences.

Since diphtheria toxin was isolated by Roux and Yersin in 1888 (1), microbial toxins have been recognized as the primary virulence factor(s) for a variety of pathogenic bacteria. Bacterial toxins have been defined as "soluble substances that alter the normal metabolism of host cells with deleterious effects on the host" (2). Indeed, the major symptoms associated with disease caused by *Corynebacterium diphtheriae* (diphtheria), *Bordetella pertussis* (whooping cough), *Vibrio cholerae* (cholera), *Bacillus anthracis* (anthrax), *Clostridium botulinum* (botulism), *Clostridium tetani* (tetanus), and enterohemorrhagic *Escherichia coli* (bloody diarrhea and hemolytic uremic syndrome) are all related to the activities of the toxins produced by these organisms. With the recognition of the central role of toxin in these and other diseases has come the application of inactive toxins (toxoids) as vaccines. Such toxoid vaccines have had an important positive impact on public health.

In this review, we provide a summary overview (Table) of a variety of bacterial toxins categorized according to mode of action: damaging cell membranes, inhibiting protein synthesis, activating second messenger pathways, inhibiting the release of neurotransmitters, or activating the host immune response. We also describe in detail seven toxins: *Staphylococcus aureus* α-toxin, Shiga toxin (Stx), cytotoxic necrotizing factor type 1 (CNF1), *E. coli* heat-stable toxin (ST), botulinum and tetanus neurotoxins, and toxic-shock syndrome toxin (TSST) produced by *S. aureus*. We emphasize these toxins because they are produced by emerging (Stx of enterohemorrhagic *E. coli*) or reemerging (α-toxin of multidrug-resistant *S. aureus*) pathogens or illustrate different structures or modes of action (ST, CNF1, neurotoxins, and TSST).

Address for correspondence: Alison D. O'Brien, Uniformed Services University of the Health Sciences, Department of Microbiology and Immunology, 4301 Jones Bridge Road, Bethesda, MD 20814, USA; fax: 301-295-3773; e-mail: aobrien@mxb.usuhs.mil.

When It Rains, It Pores

Many bacterial exotoxins have the capacity to damage the extracellular matrix or the plasma membrane of eukaryotic cells. The damage not only may result in the direct lysis of cells but also can facilitate bacterial spread through tissues. Toxins that mediate this cellular damage do so by either enzymatic hydrolysis or pore formation. Bacterial hyaluronidases, collagenases, and phospholipases have the capacity to degrade cellular membranes or matrices. Specific examples of these types of toxins include the α-toxin of *Clostridium perfringens,* which has phospholipase C activity; *Streptococcus pyogenes* streptokinase, which can hydrolyze plasminogen to plasmin and dissolve clots; and the clostridial collagenases (3-5). Pore-forming toxins, as the

Table. Characteristics of bacterial toxins[a]

Organism/toxin	Mode of action	Target	Disease	Toxin implicated in disease[b]
Damage membranes				
Aeromonas hydrophila/aerolysin	Pore-former	Glycophorin	Diarrhea	(yes)
Clostridium perfringens/ perfringolysin O	Pore-former	Cholesterol	Gas gangrene[c]	?
Escherichia coli/hemolysin[d]	Pore-former	Plasma membrane	UTIs	(yes)
Listeria monocytogenes/ listeriolysin O	Pore-former	Cholesterol	Foodborne systemic illness, meningitis	(yes)
Staphyloccocus aureus/α-toxin	Pore-former	Plasma membrane	Abcesses[c]	(yes)
Streptococcus pneumoniae/ pneumolysin	Pore-former	Cholesterol	Pneumonia[c]	(yes)
Streptococcus pyogenes/ streptolysin O	Pore-former	Cholesterol	Strep throat, Sf[c]	?
Inhibit protein synthesis				
Corynebacterium diphtheriae/ diphtheria toxin	ADP-ribosyltransferase	Elongation factor 2	Diphtheria	yes
E. coli/*Shigella dysenteriae*/ Shiga toxins	N-glycosidase	28S rRNA	HC and HUS	yes
Pseudomonas aeruginosa/ exotoxin A	ADP-ribosyltransferase	Elongation factor 2	Pneumonia[c]	(yes)
Activate second messenger pathways				
E.coli				
CNF	Deamidase	Rho G-proteins	UTIs	?
LT	ADP-ribosyltransferase	G-proteins	Diarrhea	yes
ST[d]	Stimulates guanylate cyclase	guanylate cyclase receptor	Diarrhea	yes
CLDT[d]	G2 block	Unknown	Diarrhea	(yes)
EAST	ST-like?	Unknown	Diarrhea	?
Bacillus anthracis/edema factor	Adenylate cyclase	ATP	Anthrax	yes
Bordetella pertussis/				
dermonecrotic toxin	Deamidase	Rho G-proteins	Rhinitis	(yes)
pertussis toxin	ADP-ribosyltransferase	G-protein(s)	Pertussis	yes
Clostridium botulinum/C2 toxin	ADP-ribosyltransferase	Monomeric G-actin	Botulism	?
C. botulinum/C3 toxin	ADP-ribosyltransferase	Rho G-protein	Botulism	?
Clostridium difficile/				
toxin A	Glucosyltransferase	Rho G-protein(s)	Diarrhea/PC	(yes)
toxin B	Glucosyltransferase	Rho G-protein(s)	Diarrhea/PC	?
Vibrio cholerae/cholera toxin	ADP-ribosyltransferase	G-protein(s)	Cholera	yes
Activate immune response				
S. aureus/				
enterotoxins	Superantigen	TCR and MHC II	Food poisoning[c]	yes
exfoliative toxins	Superantigen (and serine protease?)	TCR and MHC II	SSS[c]	yes
toxic-shock toxin	Superantigen	TCR and MHC II	TSS[c]	yes
S. pyogenes/pyrogenic exotoxins	Superantigens	TCR and MHC II	SF/TSS[c]	yes
Protease				
B. anthracis/lethal factor	Metalloprotease	MAPKK1/MAPKK2	Anthrax	yes
C. botulinum/neurotoxins A-G	Zinc-metalloprotease	VAMP/synaptobrevin, SNAP-25, syntaxin	Botulism	yes
Clostridium tetani/tetanus toxin	Zinc-metalloprotease	VAMP/synaptobrevin	Tetanus	yes

[a]Abbreviations: CNF, cytotoxic necrotizing factor; LT, heat-labile toxin; ST, heat-stable toxin; CLDT, cytolethal distending toxin; EAST, enteroaggregative *E. coli* heat-stable toxin; TCR, T-cell receptor; MHC II, major histocompatibility complex class II; MAPKK, mitogen-activated protein kinase kinase; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosomal-associated protein; UTI, urinary tract infection; HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome; PC, antibiotic-associated pseudomembranous colitis; SSS, scalded skin syndrome; SF, scarlet fever; TSS, toxic-shock syndrome.

[b]Yes, strong causal relationship between toxin and disease; (yes), role in pathogenesis has been shown in animal model or appropriate cell culture; ?, unknown.

[c]Other diseases are also associated with the organism.

[d]Toxin is also produced by other genera of bacteria.

name suggests, disrupt the selective influx and efflux of ions across the plasma membrane by inserting a transmembrane pore. This group of toxins includes the RTX (repeats in toxin) toxins from gram-negative bacteria, streptolysin O produced by *S. pyogenes*, and the *S. aureus* α-toxin (described below).

S. aureus α-toxin can be considered the prototype of oligomerizing pore-forming cytotoxins. The α-toxin gene resides as a single copy on the chromosome of most pathogenic *S. aureus* strains, and its expression is environmentally regulated at the transcriptional level by the staphylococcal accessory gene regulator (*agr*) locus (6,7). The α-toxin is synthesized as a 319 amino acid precursor molecule that contains an N-terminal signal sequence of 26 amino acids. The secreted mature toxin, or protomer, is a hydrophilic molecule that lacks cysteine residues and has a molecular mass of approximately 33 kDa (6-8). Recently, the crystallographic structure of the fully assembled α-toxin pore was solved (9). On the plasma membrane, seven toxin protomers assemble to form a 232-kDa mushroom-shaped heptamer comprising three distinct domains (Figure 1A) (9,10). The cap and rim

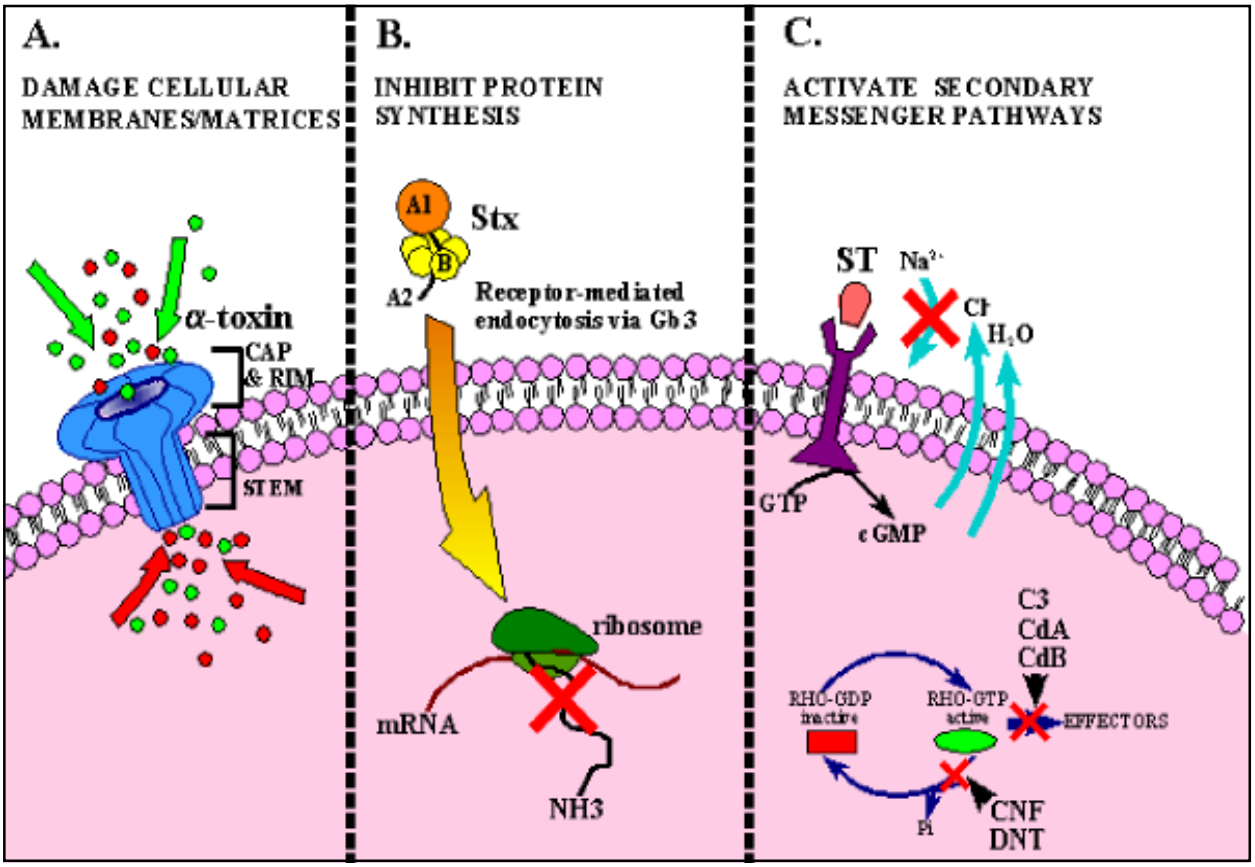

Figure 1. Diagrammatic representation of the mode of action of several bacterial toxins. A. Damage to cellular membranes by *Staphylococcus aureus* α-toxin. After binding and oligomerization, the stem of the mushroom-shaped α-toxin heptamer inserts into the target cell and disrupts membrane permeability as depicted by the influx and efflux of ions represented by red and green circles. B. Inhibition of protein synthesis by Shiga toxins (Stx). Holotoxin, which consists of an enzymatically active (A) subunit and five binding (B) subunits, enters cells through the globotriasylceramide (Gb3) receptor. The N-glycosidase activity of the A subunit then cleaves an adenosine residue from 28S ribosomal RNA, which halts protein synthesis. C. Examples of bacterial toxins that activate secondary messenger pathways. Binding of the heat-stable enterotoxins (ST) to a guanylate cyclase receptor results in an increase in cyclic GMP (cGMP) that adversely effects electrolyte flux. By ADP-ribosylation or glucosylation respectively, the C3 exoenzyme (C3) of *Clostridium botulinum* and the *Clostridium difficile* toxins A and B (CdA & CdB) inactivate the small Rho GTP-binding proteins. Cytotoxic necrotizing factor (CNF) of *E. coli* and the dermonecrotic toxin (DNT) of *Bordetella* species activate Rho by deamidation.

domains of the α-toxin heptamer are situated at the surface of the plasma membrane, while the stem domain serves as the transmembrane channel.

Alpha-toxin is cytolytic to a variety of cell types, including human monocytes, lymphocytes, erythrocytes, platelets, and endothelial cells (6,8). For α-toxin to damage cellular membranes, three sequential events are required. Toxin protomers must first bind to target membranes by either unidentified high-affinity receptors or through nonspecific absorption to substances such as phosphotidylcholine or cholesterol on the lipid bilayer (6-8). Second, membrane-bound protomers must oligomerize into a nonlytic prepore heptamer complex. Third, the heptamer must undergo a series of conformational changes that create the stem domain of the toxin, which is then inserted into the membrane (9,10). The α-toxin pore allows the influx and efflux of small molecules and ions that eventually lead to the swelling and death of nucleated cells and the osmotic lysis of erythrocytes. Pore formation has also been shown to trigger secondary events that could promote development of pathologic sequelae. These events include endonuclease activation, increased platelet exocytosis, release of cytokines and inflammatory mediators, and production of eicosanoids (6,8). Several animal models have demonstrated that α-toxin is required for *S. aureus* virulence in these systems (6,8); however, the precise role of α-toxin in staphylococcal diseases in humans remains unclear.

Stop, in the Name of Toxin

A second class of toxins intoxicates target cells by inhibiting protein synthesis. Substrates for toxins in this group are elongation factors and ribosomal RNA. Diphtheria toxin and *Pseudomonas* exotoxin A act by ADP-ribosylating elongation factor 2 (EF2) (11,12). The modified EF2 is no longer able to function in protein synthesis. Stxs, also called verotoxins, are produced by *Shigella dysenteriae* serotype 1 and the emerging pathogens designated Stx-producing *E. coli* (STEC). Stxs inactivate ribosomal RNA (by a mechanism described below) so that the affected ribosome can no longer interact with elongation factors (13,14). The inhibition of protein synthesis by this group of toxins ultimately results in death of the target cell.

Stxs are potent cytotoxins that can be divided into two antigenically distinct groups that share 50% to 60% homology: Stx/Stx1 and Stx2 (15-17). Stx and Stx1 are elaborated by *S. dysenteriae* serotype 1 and *E. coli*, respectively, and differ at only one amino acid. Stx2-type toxins have been found only in *E. coli* isolates and are quite diverse. While Stx2 is considered the prototype of this group, variants have been found that differ antigenically, in receptor specificity and in activation by intestinal mucus. Some of these attributes are the result of only one or two nucleotide differences in the toxin genes.

The *stx* of *S. dysenteriae* is invariably chromosomally located. The genes that encode Stx1 and Stx2 are carried chromosomally or by lysogenic bacteriophages. The genes that code for the A and B subunits of Stxs, *stxA* and *stxB*, are organized within an operon. The operator region of Stx/Stx1 (but not Stx2) contains a consensus fur box that is responsible for the iron-regulation of Stx and Stx1 production. Neither iron nor any other environmental factors examined affect the expression of Stx2. However, intestinal mucus enhances the activity of some Stx2 variants (18). The Stxs, which carry typical N-terminal leader sequences, are not actively secreted from the bacterial cell and are thought to be released into the milieu during cell lysis.

Stxs display an AB-toxin structure; an enzymatically active A subunit is noncovalently associated with a binding, or B, component. The crystal structures of the Stx1 B pentamer (19) and the Stx holotoxin have been solved (20) (Figure 2). Other toxins that share this AB structure are the *E. coli* heat-labile toxin (21), cholera toxin, and pertussis toxin (22) (Figure 2). The molecular masses of mature Stx A and B monomeric subunits are approximately 35 kDa and 7.5 kDa, respectively, although holotoxin contains five B subunit molecules. The B subunit pentamer directs the binding of the holotoxin to sensitive eukaryotic cells via specific glycolipid receptors. Once internalized, the A polypeptide is cleaved into an enzymatically active A_1 portion and an A_2 portion; these fragments remain associated through a disulfide bond. The A_2 portion serves to link the A_1 fragment and the B pentamer.

The enzymatic A subunit acts as a specific *N*-glycosidase to cleave a single adenine residue from 28S ribosomal RNA (13,14). This

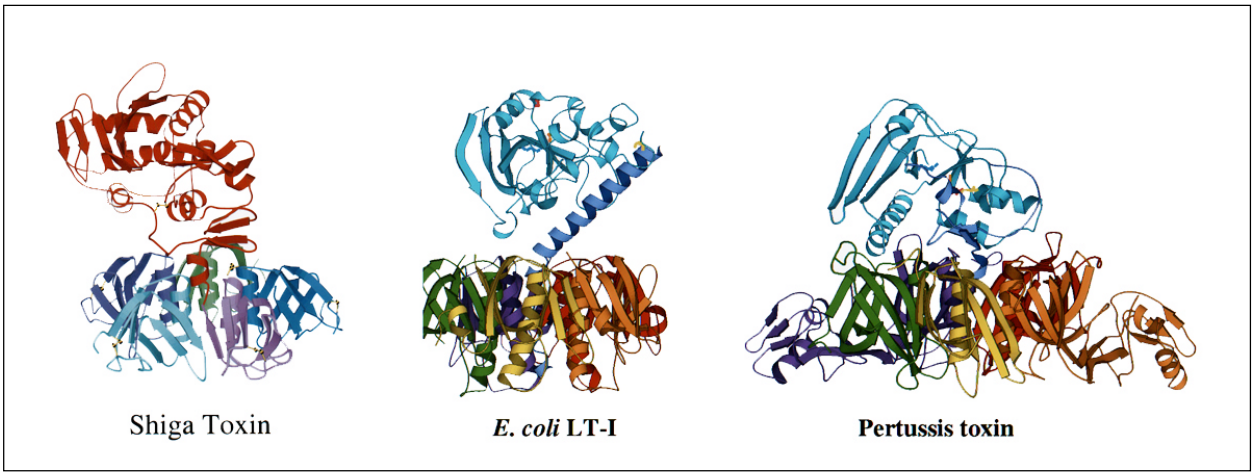

Figure 2. Ribbon crystal structures of *Shigella dysenteriae* Shiga toxin (20), *Escherichia coli* heat-labile toxin I (LT-I) (21), and pertussis toxin (22). The Shiga toxin figure was contributed by Marie Frasier. The LT-I and pertussis figures were contributed by Ethan Merritt. The figures were drawn in MOLSCRIPT (75).

depurination ultimately results in the inhibition of protein synthesis within intoxicated cells (Figure 1B). Prokaryotic ribosomes are as sensitive to the *N*-glycosidase activity of Stx as eukaryotic ribosomes (23).

STEC are considered emerging pathogens (24) because they were first described less than 20 years ago, during a 1983 outbreak of hemorrhagic colitis associated with undercooked hamburger (25,26). STEC O157:H7 causes approximately 20,000 cases of hemorrhagic colitis each year in the United States (27). Approximately 1,000 cases of the life-threatening sequelae hemolytic uremic syndrome and approximately 100 deaths are also attributed to *E. coli* O157:H7 annually in the United States (27).

Don't Shoot the Messenger

Bacterial toxins can also target and alter the function of a variety of cellular proteins without directly killing the intoxicated cell. Toxin activation or modification of secondary messengers can cause dramatic alterations to signal transduction pathways critical in maintaining a variety of cellular functions. To demonstrate the diversity among the toxins that belong to this category, we will describe CNF type 1 and the heat-stable enterotoxins.

Cytotoxic Necrotizing Factor (CNF)

CNF types 1 and 2 (CNF1/2) from *E. coli* belong to a group of bacterial toxins that modify Rho, a subfamily of small GTP-binding proteins that are regulators of the actin cytoskeleton (28,29). Most members of this toxin family, which includes the large clostridial cytotoxins and the C3 exoenzyme of *C. botulinum*, inactivate Rho (29). CNF1, CNF2, and the dermonecrotic toxins from *Bordetella* species form a unique subset in this family, since these toxins have the capacity to activate Rho (Figure 1C) (29-32). CNF1 and CNF2 share 99% amino acid similarity; however, we will discuss only CNF1 in detail because of its association with extraintestinal *E. coli* infections in humans, most notably urinary tract infections.

The gene for CNF1 is chromosomally encoded and resides on a pathogenicity island in uropathogenic *E. coli* (33,34). The toxin is synthesized as a hydrophilic polypeptide of approximately 115 kDa that remains primarily cytoplasmic because of the lack of a signal sequence (33). Recent structure and function analysis of CNF1 indicates that the toxin has distinct binding and enyzmatic domains (35). The N-terminal half of CNF1, which includes two potential transmembrane domains, contains the cellular binding domain. This region of the molecule shows amino acid similarity to the *Pasteurella multicoda* toxin, a potent mitogen thought to be the etiologic agent of progressive atrophic rhinitis in pigs (33,35). The C-terminal portion of CNF1 represents the toxin's enzymatic domain and shows homology with dermonecrotic toxins in a 100-amino acid stretch that may represent the active site of the toxin (33,35).

Eukaryotic cells intoxicated with CNF1 exhibit membrane ruffling; the formation of focal adhesions and actin stress fibers; and DNA replication in the absence of cell division, a phenomenon that results in enlarged multinucleated cells (Figure 3). The drastic changes apparent in CNF1-treated cells are a result of the toxin's capacity to modify Rho (29,30,32). This modification has recently been identified as a deamidation of the glutamine residue at position 63 of Rho to a glutamic acid. This amino acid change produces a dominant active Rho protein unable to hydrolyze bound GTP (30,32). In vivo, CNF1 causes necrosis in rabbit skin following intradermal injection and persistent inflammation in a mouse footpad assay (36). Epidemiologic data support the role of CNF1 as a virulence factor in human extraintestinal infections, although direct proof of the toxin's role in disease remains to be determined (29,37).

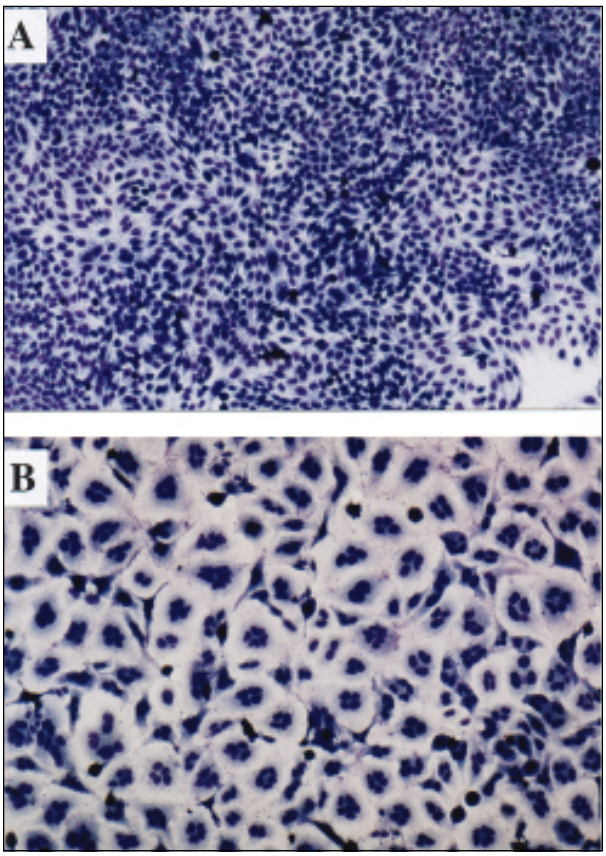

Figure 3. The effect of cytotoxic necrotizing factor type 1 (CNF1) on eukaryotic cells. A. HEp-2 cells, magnification 10X. B. HEp-2 cells intoxicated with CNF1, magnification 10X.

Heat-Stable Toxin (ST)

Two families of diarrheagenic STs have been described: STa (or STI) and STb (or STII). Distinct STas are produced by a variety of enteric pathogenic organisms: enterotoxinogenic *E. coli* (ETEC) (the focus of this section), *V. cholerae*, *Vibrio mimicus*, *Yersinia enterocolitica*, *Citrobacter freundii*, and *Klebsiella*.

Strains of ETEC associated with human disease may produce either STa, heat-labile toxin I, or both. STas from ETEC isolates are related but distinct toxins (38). STh is produced by strains of human origin, while STp is found predominantly in porcine strains. The STa genes (*estA*) of ETEC are encoded within a transposable element and have been found on a variety of replicons (39,40). STa is translated as a precursor molecule of 72 amino acids and undergoes two cleavage events before the secretion of the mature form into the culture supernatant. Mature STs are small peptides that range from 17 to 53 amino acids. STh and STp contain 19 and 18 residues, respectively. STas share a conserved C-terminal region of 13 amino acids essential for toxicity and the heat-stable nature of the toxin. Six cysteine residues are present within this domain, and the three disulphide bonds formed between the cysteine residues are necessary for toxicity of the molecule. Binding of STa to its cellular receptor results in the stimulation of membrane-bound guanylate cyclase, which in turn leads to an increase in intracellular cyclic GMP (Figure 1C) (41). This increase in cyclic GMP affects electrolyte flux in the bowel; sodium absorption is inhibited and chloride secretion is stimulated. These ion flux changes result in the secretory diarrhea characteristic of ETEC infection. ETEC cause traveler's diarrhea and are a major source of childhood diarrhea in many parts of the world.

The Nerve of Some Toxins

The *C. botulinum* neurotoxins (BoNTs, serotypes A-G) and the *C. tetani* tetanus neurotoxin (TeNT) constitute another category of bacterial toxins on the basis of similarities in structure, enzymatic activity, and the targeting to cells of the nervous system. BoNTs are most commonly associated with infant and foodborne botulism and exist in nature as large complexes comprised of the neurotoxin and one or more associated proteins believed to provide protection

and stability to the toxin molecule while in the gut (42,43). TeNT, which is synthesized from vegetative *C. tetani* in wounds, does not appear to form complexes with any other protein components (42,43).

The BoNTs and TeNT are either plasmid encoded (TeNT, BoNTs/A, G, and possibly B) or bacteriophage encoded (BoNTs/C, D, E, F), and the neurotoxins are synthesized as inactive polypeptides of 150 kDa (44). BoNTs and TeNT are released from lysed bacterial cells and then activated by the proteolytic cleavage of an exposed loop in the neurotoxin polypeptide (45). Each active neurotoxin molecule consists of a heavy (100 kDa) and light chain (50 kDa) linked by a single interchain disulphide bond (42,45). The heavy chains of both the BoNTs and TeNT contain two domains: a region necessary for toxin translocation located in the N-terminal half of the molecule, and a cell-binding domain located within the C-terminus of the heavy chain (45,46). The light chains of both the BoNTs and TeNT contain zinc-binding motifs required for the zinc-dependent protease activities of the molecules (45,46).

The cellular targets of the BoNTs and TeNT are a group of proteins required for docking and fusion of synaptic vesicles to presynaptic plasma membranes and therefore essential for the release of neurotransmitters. The BoNTs bind to receptors on the presynaptic membrane of motor neurons associated with the peripheral nervous system. Proteolysis of target proteins in these neurons inhibits the release of acetylcholine, thereby preventing muscle contraction (47,48). BoNTs/B, D, F, and G cleave the vesicle-associated membrane protein and synaptobrevin, BoNT/A and E target the synaptosomal-associated protein SNAP-25, and BoNT/C hydrolyzes syntaxin and SNAP-25 (42,45,46). TeNT affects the central nervous system and does so by entering two types of neurons. TeNT initially binds to receptors on the presynaptic membrane of motor neurons but then migrates by retrograde vesicular transport to the spinal cord, where the neurotoxin can enter inhibitory interneurons (45,47). Cleavage of the vesicle-associated membrane protein and synaptobrevin in these neurons disrupts the release of glycine and gamma-amino-butyric acid, which, in turn, induces muscle contraction (47,48). The contrasting clinical manifestations of BoNT or TeNT intoxication (flaccid and spastic paralysis, respectively) are the direct result of the specific neurons affected and the type of neurotransmitters blocked (45-47).

Bacterial Superantigens: Too Much of a Good Thing

Several bacterial toxins can act directly on the T cells and antigen-presenting cells of the immune system. Impairment of the immunologic functions of these cells by toxin can lead to human disease. One large family of toxins in this category are the pyrogenic toxin superantigens (PTSAgs), whose hallmark biological activities include potent stimulation of the immune cell system, pyrogenicity, and enhancement of endotoxin shock (49-51). These stable, secreted toxins of 22 kDa to 30 kDa include staphylococcal enterotoxins serotypes A-E, G, and H; group A streptococcal pyrogenic exotoxins serotypes A-C and F; group A streptococcal superantigen; and staphylococcal TSST-1, which we discuss below.

All PTSAgs share common biological activities, but TSST-1 is the most divergent member of the toxin family, with less than 30% amino acid homology to other family members (52-54). TSST-1 is chromosomally encoded, and the *tst* gene is located in a variable genetic element in *S. aureus* (49,52,55). The toxin is synthesized as a precursor molecule of 234 residues with the first 40 amino acids acting as a signal sequence that is cleaved to generate the mature 22 kDa toxin (49). Expression of TSST-1 depends on oxygen, temperature, pH and glucose levels, and is regulated by the *S. aureus agr* locus (49,51). On the basis of crystallographic analysis, TSST-1 appears structurally similar to several other PTSAgs in that the toxin consists of two distinct domains; however, unlike other family members, TSST-1 does not require a zinc cofactor (51-54). Domain A of TSST-1 (amino acid residues 1-17 and 90-194) exists as a ß-grasp motif, and domain B consists of a five-stranded ß-barrel motif that forms an oligosaccharide/oligonucleotide binding fold.

In general, the potent immunostimulatory properties of PTSAgs are a direct result of toxin binding to distinct regions outside the peptide binding cleft of the major histocompatibility class II molecules (expressed on the surface of antigen-presenting cells) and to specific Vß elements on the T-cell receptor. In particular, the domain B of TSST-1 binds primarily to the α-chain of human leukocyte antigen-DR1

molecules, while domain A specifically binds to human T-cell receptor Vß2 elements (51-53,56). Binding of TSST-1 to Vß2 T-cell receptor elements results in a massive proliferation of up to 20% of peripheral T cells, an event that drastically skews the T-cell Vß repertoire (53,56). T cells that undergo this expansion can subsequently exist in a state of anergy or undergo apoptosis (56). Concomitant to T-cell proliferation is a massive release of both lymphocyte (interleukin [IL]-2, tumor necrosis factor ß, gamma interferon)-derived and monocyte (IL-1, IL-6, tumor necrosis factor α)-derived cytokines (51,56). These cytokines serve as mediators of the hypotension, high fever, and diffuse erythematous rash that are characteristic of toxic-shock syndrome. Long established as a key substance in causing staphylococcal toxic-shock syndrome, TSST-1 has more recently been linked with Kawasaki syndrome, a leading cause of acquired heart disease in children in the United States (50,54).

Dr. Jekyll or Mr. Hyde?

Some of these powerful disease-causing toxins have been exploited to further basic knowledge of cell biology or for medical purposes. For example, cholera toxin and the related labile-toxin of *E. coli*, as well as *B. pertussis* toxin, have been used as biologic tools to understand the mechanism of adenylate cyclase activation and the role of cyclic AMP as a second messenger in the eukaryotic cell (57-59). Derivatives of some of these toxins, cholera toxin and labile toxin, have also been incorporated into human vaccines because of the adjuvant properties of these molecules (60,61).

Similarly, the activities of several potent cytotoxins have been harnessed as potential therapies for certain cancers. Such toxins can either be used directly in treatment or as components of immunotoxins (62-64). For example, Stx binds to the cell surface glycolipid CD77, which is expressed by B cells in certain B-cell lymphomas (65,66). This finding led to studies that showed that Stx can purge murine (and potentially human) bone marrow of malignant $CD77^+$ B cells before an autologous bone marrow transplant (67). Other toxins that inhibit protein synthesis, such as diphtheria toxin, *Pseudomonas* exotoxin A, or the plant toxin ricin, are frequently engineered as the cell-killing component of immunotoxins. These "magic bullets," hybrids of the enzymatically active portion of a toxin molecule and monoclonal antibodies (or a receptor), are in clinical trials for the treatment of persons with B-cell lymphomas, leukemia, and bone marrow transplants.

Several clinical applications have also been found for the powerful botulinum neurotoxin type A (BoNT/A) (46,68). The disorders that respond to BoNT/A involve muscle hyperactivity. A minuscule amount of purified toxin injected into specific sites results in paralysis of the target muscle and ablation of the muscle spasm. Therapy must be continual since the effect of the toxin usually lasts for no more than several months. The first maladies treated with BoNT/A were eye movement abnormalities (69). However, the therapeutic value of BoNT/A has been shown for many other disorders including cervical and laryngeal dystonia, writer's cramp, hemifacial spasm, tremors, and tics (46,68). BoNT/A is also used cosmetically to reduce deep wrinkles caused by the contraction of facial muscles (70).

Another toxic bacterial product with medical applications is streptokinase, a potent plasminogen activator produced by several pathogenic streptococcal strains. The proteolytic activity of streptokinase is used to clear blocked arteries in patients who have heart attacks (71,72).

Vaccinate, Don't Procrastinate

Vaccines directed at the toxic component of bacterial pathogens have proven quite effective in preventing certain diseases. Most licensed toxoid vaccines are relatively crude, but effective, preparations. These vaccines consist of partially purified toxin preparations obtained from culture supernatants of bacteria such as *C. diphtheriae*, *C. tetani*, or *B. anthracis*. Formaldehyde treatment is used to detoxify the diphtheria and tetanus toxins for vaccine formulation. The anthrax vaccine contains the protective antigen and small amounts of the lethal factor and edema factor toxins. The current botulinum vaccine is an investigational drug composed of crude preparations of five botulinum toxoids and is distributed by the Centers for Disease Control and Prevention to researchers that work with the toxin or organism. Acellular pertussis vaccines that contain pertussis toxoid, alone or as one of several components, are as effective as killed whole-cell vaccines but less reactogenic (73);

such vaccines have recently been approved for use in infants as well as older children.

New vaccines aimed at toxins are in various stages of development: research and development, preclinical, phase I, phase II, or phase III (74). The next generation of toxoid vaccines falls into three general categories: purified toxoids that have been inactivated by chemical or genetic means; live, attenuated strains of the causative agent that produce a genetically derived toxoid; or live, attenuated unrelated bacterial vector strains, such as *V. cholerae* or *Salmonella,* that produce the target toxoid. Examples of each of these approaches and progress in development of specific toxoid vaccines are described annually in the Jordan Report (74).

Antitoxins raised against diphtheria, tetanus, and botulinum toxoids have also been used for many years to treat seriously ill patients. Antiserum specific for the Stx toxins produced by *E. coli* O157:H7 and other STEC is under development for the treatment and prevention of hemolytic uremic syndrome, a life-threatening sequela of these infections.

Summary

Microbial toxins capable of interrupting or hyperstimulating many essential functions and pathways of eukaryotic cells have evolved along with the carrier bacterium. Presumably these toxins confer some benefit to the bacterium, either during a stage of the host-parasite interaction or in some environmental niche encountered by the bacterium. Certain bacterial toxins act on the target cell surface to irreparably damage the cell membrane or alter normal cellular signal transduction. Other toxins exhibit enzymatic activity once the molecule has gained access to the cytoplasm of the sensitive cell by endocytosis. Yet other bacterial toxins act by either turning off or locking on a normal host cell function.

Although detrimental to the susceptible host during an infection, the activities of several bacterial toxins have been exploited as probes of eukaryotic cellular pathways and for medicinal applications. Thus, research on a microbial toxin produced by an established, emerging, or reemerging pathogen is likely to yield novel information about the role of that toxin in disease as well as the properties of host cells that are subverted by the toxin.

Acknowledgments

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Dr. Schmitt is a research assistant professor in the Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA. Research interests include virulence factors and pathogenesis of disease caused by Shiga toxin-producing *E. coli* and regulation of *Salmonella* virulence.

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Clonal Differences among Erythromycin-Resistant *Streptococcus pyogenes* in Spain

Emilio Perez-Trallero,* José María Marimón,* Milagrosa Montes,* Beatriz Orden,† and Manuela de Pablos‡
*Universidad del País Vasco, San Sebastián, Spain; †Centro Especialidades "Argüelles," Madrid, Spain; ‡Hospital Txagorritxu, Vitoria, Spain

The aim of this study was to determine whether the high levels of erythromycin resistance in *Streptococcus pyogenes* found in Spain are due to the introduction and spread of one or more clones. Phenotypic and genotypic techniques were used to characterize all erythromycin-resistant *S. pyogenes* (ErR) isolated in Gipuzkoa, Spain, in the last 10 years and 128 ErR isolated in Vitoria and Madrid during 1996. Of 437 ErR, 97% had the M phenotype; all 283 of the strains studied had the *mef*A determinant of resistance. After biotyping, T serotyping, *emm* typing, and genotyping, four major clones were detected. Clones B (biotype I, type T4, *emm*4, pulsed-field gel electrophoresis [PFGE] II) and D (biotype V, type T8.25, *emm*75, PFGE IV) comprised 78.8% of all ErR. The resistance of *S. pyogenes* to erythromycin was mainly due to an efflux mechanism of resistance (M phenotype); few clones were responsible for it.

The Lancefield group A streptococci (*Streptococcus pyogenes*), major causative agents of human disease (1), can produce both mild (e.g., pharyngitis) and severe (e.g., life-threatening "toxic shock-like syndrome," necrotizing fasciitis) infections. During the last few years, erythromycin-resistant *S. pyogenes* (ErR) has been reported in different parts of the world (2-4). Two distinct mechanisms of erythromycin resistance are described among group A streptococci. One consists of target-site modification by *erm* methylase (5,6) strains that express the MLS_b phenotype of resistance; the other (recently described) consists of an active drug efflux that pumps 14- and 15-membered macrolides out of the cell (7). This novel mechanism of macrolide resistance is encoded by the gene *mef*A (8), and strains show the M phenotype. In the last few years, increased resistance to erythromycin in *S. pyogenes* has been detected in Spain (9-11). Therefore, we performed an epidemiologic investigation to determine the biotypes, serotypes (T-agglutination patterns), *emm* types, and pulsed-field gel electrophoresis (PFGE) patterns of chromosomal DNA and their relationship to macrolide resistance.

Address for correspondence: Emilio Perez-Trallero, Servicio de Microbiología, Apartado 477, 20080 San Sebastián, Spain; fax: 34-943-007112; e-mail: labmikro@teleline.es.

The Study

Sources of Bacterial Isolates

From 1988 to 1997, 2,561 nonduplicated isolated strains of *S. pyogenes* were collected from throat swabs and extratonsilar samples at the Nuestra Señora de Aránzazu Hospital and at primary-care centers in two districts of Gipuzkoa (approximately 300,000 residents). In 1996, two other samples were collected and included in this study; 33 ErR strains from Vitoria (Hospital Txagorritxu) and 95 from Madrid (Centro de Especialidades Argüelles). Gipuzkoa Province (San Sebastian is its capital) is located in the northeastern area of the Basque country of Spain, bordered by the Cantabric Sea and France to the north; Madrid is located in the center of Spain (415 km from San Sebastian); and Vitoria (in Alava Province) is located in the north of Spain (110 km from San Sebastian).

Identification, Susceptibility, Typing, and Clone Definition

Group A streptococci were identified by colony morphology, beta-hemolysis on blood

agar, and commercial latex-agglutination techniques (Streptex, Wellcome, Dartford, UK, or Phadebact, Boule Diagnostics AB, Huddinge, Sweden). The confirmation of *S. pyogenes* and biotyping were done with a commercially available identification system: rapid ID 32 STREP (BioMerieux, La Balme-les-Grottes, France). Biotyping was performed according to Bouvet et al. (12).

All *S. pyogenes* were tested for susceptibility to erythromycin and other antibiotics by broth microdilution. ErR strains were restudied by agar dilution and by agar diffusion (erythromycin induction of resistance) to determine macrolide and lincosamine resistance phenotypes (11).

T-protein types were determined by slide agglutination of trypsin-digested suspensions of bacteria with rabbit type-specific antiserum (SEVAC, Prague)(13).

The *emm* gene type of T-serotyped strains was determined by polymerase chain reaction (PCR)-enzyme-linked immunosorbent assay (ELISA), as described by Saunders et al. (14). Capture probes for the *emm* gene not described by Saunders et al. were selected from the DNA sequences encoding the N-terminal hypervariable region of strains of types *emm*2, *emm*9, *emm*48, and *emm*75 (GenBank accession nos. X56608, U12002, U11961, and U11993).

PFGE was done (15) with the following modifications. Cells were resuspended to an optical density $(OD)_{560nm}$ = 1.0, and 4 ml of the adjusted suspension was centrifuged. Pelleted cells were resuspended in 200 µl of Pett IV buffer (10 mM Tris, pH 7.2, 20 mM NaCl, 50 mM EDTA) and mixed with 100 µl of 2% low-melting agarose; incubation of plugs with lysozyme solution (1 mg/ml) was reduced to 3 hours. Slices of plugs were digested at 50ºC for 16 hours with 30 UI of the enzyme *Sfi*I since it has proven satisfactory in differentiating DNA fragments of *S. pyogenes* (15,16). Digested inserts were electrophoresed by using a CHEF-DR III apparatus (BioRad), along with DNA size standards (BioRad) under the following conditions: 22 hours with an initial switch time of 20 seconds, rising on linear ramp to 75 seconds at 6V/cm, with an included angle of 120ºC. Gels were stained with ethidium bromide and visualized under UV light with Imagestore 5000 ver.7.12 (Ultra-Violet Products Ltd, Cambridge, England). Similarities among PFGE patterns were established using the Dice coefficient and Lane-Manager 2.1 (TDI, Madrid, Spain) commercial computer software.

A clone was defined as a group of strains expressing both the same characteristic phenotype and genotype (PFGE pattern similarity ≥ 90%).

Erythromycin Resistance

We studied 2,561 strains of *S. pyogenes* isolated from 1988 to 1997 in Gipuzkoa; 309 (12.1%) were resistant to erythromycin. A report of ErR in Gipuzkoa from 1984 to 1996 (11) showed that until the end of 1990, erythromycin resistance in *S. pyogenes* was low (1.2%, 13 of 1,060); after 1990, resistance increased, reaching 34.8% (87 of 250) of all *S. pyogenes* isolated in 1995. In 1997, resistance decreased to 13.7% (57 of 417). In Madrid and Vitoria, resistance to erythromycin in 1996 was 22.4% (126 of 563) and 31.6% (43 of 136), respectively.

Among the ErR strains isolated in Gipuzkoa, two phenotypes of resistance were found: 8 (2.6%) strains showed the classic MLS_b phenotype, while the other 301 (97.4%) strains expressed the M phenotype, as shown by susceptibility testing and confirmed by the presence of the *mef*A gene (11). Of the 128 ErR isolated in Madrid and Vitoria, 5 (3.9%) strains (all from Madrid) displayed the MLS_b phenotype, while the remaining 123 (96.1%) showed the M phenotype. The presence of the *mef*A gene was searched for in 283 ErR with the M phenotype; it was detected in all.

Biotyping, T Serotyping, *emm* Typing, and PFGE of M-Phenotype ErR

Among the 424 ErR with the M phenotype, only four biotypes (of 10 possible) were identified—biotypes I, II, III, and V. In Gipuzkoa, biotype III was the only biotype found until 1990; between 1991 and 1997, biotypes I and V comprised 275 (93.8%) of the 293 resistant strains. Seven T-agglutination patterns were found in Gipuzkoa, each one correlating with an *emm* type except for TB3264 (T1 *emm*1, T2 *emm*2, T4 *emm*4, T8.25 *emm*75, T12 *emm*12, and T28 *emm*28). TB3264 biotype III was *emm*2, but TB3264 biotype I was not typeable with any of the 14 *emm* types assayed. Until 1990, type T12 *emm*12 was the only type found. Between 1991 and 1997, type T4 *emm*4 and T8.25 *emm*75 comprised 92.2% (270 of 293) of all isolates with

the M phenotype of resistance. In Vitoria and Madrid, T4 *emm*4 and T8.25 *emm*75 types were also the most frequently found.

Fifteen different PFGE patterns were found among the 424 M phenotype ErR; 92% of these strains belonged to four patterns (clones A-D) (Table, Figure 1). Among ErR with the MLS_b phenotype, eight PFGE patterns were found. Each biotype/T-serotype/*emm*-type combination corresponded with one PFGE pattern except on three occasions. Three different PFGE patterns that could be established among ErR belonged to biotype III/T12/*emm*12, two PFGE patterns belonged to I/T4/*emm*4, and another two patterns belonged to V/T8.25/*emm*75. The types of other clones, their annual distribution, and a dendrogram showing their similarities are given in the Table and Figure 2.

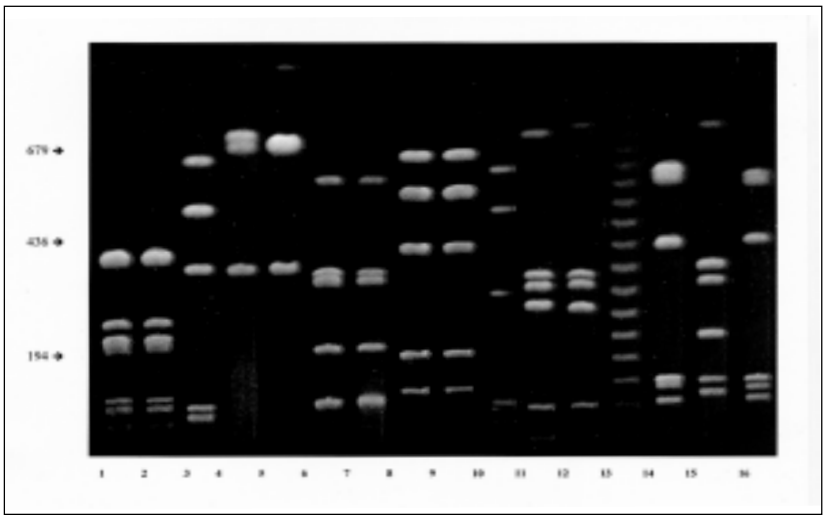

Figure 1. Pulsed-field gel electrophoresis of *Sfi*I restriction fragments of *Streptococcus pyogenes* DNAs of erythromycin-resistant S. *pyogenes*. Lanes 1-2, MLS_b phenotype strains; lane 3, clone F (biotype I, type T28, *emm*28); lanes 4-5, clone A (biotype III, type T12, *emm*12); lanes 6-7, clone B (biotype I, type T4, *emm*4); lanes 8-9, clone C (biotype I, type T4, *emm*4); lane 10, clone D (biotype V, type T8.25, *emm*75); lanes 11-12, clone E (biotype I, type T1, *emm*1); lane 13, DNA size standards (lambda ladder, 48.5 to 1,018 kb); lanes 14 and 16, clone H (biotype III, type TB3264, *emm*2); and lane 15, clone G (biotype I, type TB3264 *emm* not typeable).

Table. Annual distribution of clones of M-phenotype erythromycin-resistant *Streptococcus pyogenes*

Year	Total M-phenotype	Clones[a] A	B	C	D	E-O (biotype/T type/*emm* type)
Gipuzkoa 1988-1990	8	7				
Gipuzkoa 1991-1994	80		76			1 E (I/T1/*emm*1)
						1 F (I/T28/emm28)
Gipuzkoa 1995	83		31	18	29	1 G (I/TB3264/*emm* nt[b])
						1 H (III/TB3264/emm2)
Gipuzkoa 1996	74		19	23	25	2 H
						1 E
						1 I (I/T4/emm4)
Gipuzkoa 1997	56	1	4	3	40	2 H
						2 J (V/T8,25/emm75)
						1 K (II/T2/emm2)
						1 L (III/Tnt/emm3)
Madrid 1996	90		12	2	68	6 M (III/T12/*emm*12)
						2 N (III/T12/emm12)
Vitoria 1996	33	1	30		1	1 O ((III/Tnt/*emm*3)
Total	424[c]	9	172	46	163	23

[a]Clone A: biotype III, type T12 *emm*12, pulsed-field gel electrophoresis (PFGE) pattern I; clone B: biotype I, type T4*emm*4, PFGE pattern II; clone C: biotype I, type T4 *emm*4, PFGE pattern III; clone D: biotype V, type T8.25 *emm*75, PFGE pattern IV.
[b]nt: nontypeable.
[c]Eleven strains were not typed.

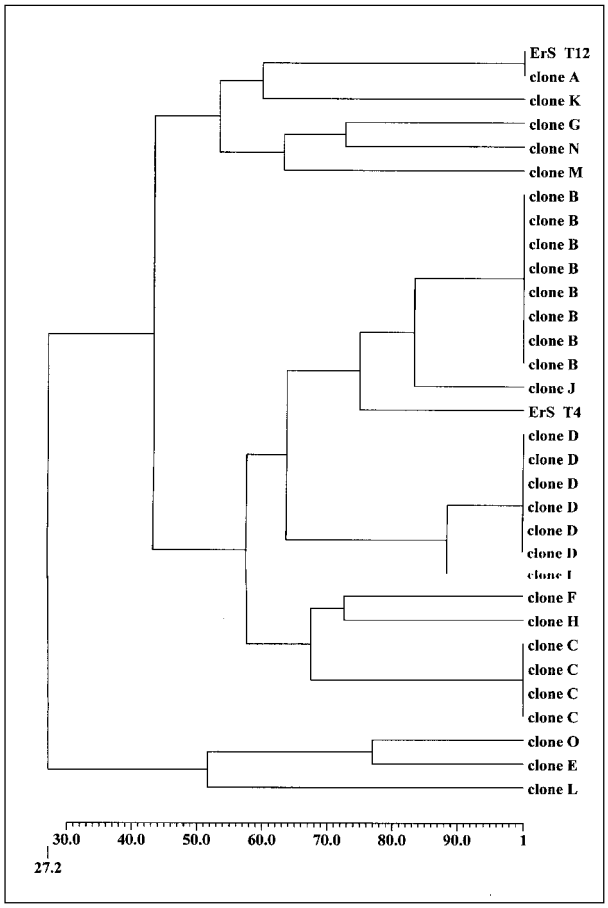

Figure 2. A dendrogram showing the genetic relationship of 15 clones of M-phenotype erythromycin-resistant *Streptococcus pyogenes* and two erythromycin-sensitive strains (ErS) established from pulsed-field gel electrophoresis patterns obtained after *Sfi*I digestion by using the Dice coefficient and UPGMA and Lane Manager 2.1 software. Clone A (III/T12/*emm*12[a]); Clone B (I/T4/*emm*4); Clone C (I/T4/*emm*4); Clone D (V/T8,25/*emm*75); Clone E (I/T1/*emm*1); Clone F (I/T28/*emm*28); Clone G (I/ TB3264/*emm* nt[b]); Clone H (III/TB3264/*emm*2); Clone I (I/T4/*emm*4); Clone J (V/T8,25/*emm*75); Clone K (II/T2/*emm*2); Clone L (III/T nt/ *emm* nt); Clone M (III/T12/*emm*12); Clone N (III/T12/*emm*12); Clone O (III/Tnt/*emm* nt); ErS T12 (III/T12/*emm*12); ErS T4 (I/T4/*emm*4).
[a]Biotype/T-serotype/*emm* type.
[b]nt = nontypeable.

Genetic Relationship of ErR and Erythromycin-Sensitive *S. pyogenes*

The genetic relationship of ErR and erythromycin-sensitive *S. pyogenes* with the same biotype and T serotype was analyzed by PFGE. In a sample of 360 erythromycin-sensitive *S. pyogenes,* biotypes I and III were the most frequent (66.7%); T1 and T28 were the most frequent T serotypes (30.6%). Only 8 (2.2%) erythromycin-sensitive strains of biotype I, serotype T4 were found. None of the 19 T8.25 erythromycin-sensitive strains found were biotype V (18 biotype II, *emm* 75 and 1 biotype I, *emm* nontypeable). Infrequent biotype and T-serotype combinations among ErR, such as I/T1 and I/T28, were frequently found among erythromycin-sensitive strains. Similarities between PFGE patterns of most of the erythromycin-sensitive and -resistant strains with the same biotype and T-serotype combination was less than 75%. Exceptions to this were several III/T12, I/T1, and I/T28 erythromycin-sensitive strains that had a close similarity (>90%) with ErR of the same biotype and T-serotype combination.

Four major clones of ErR were detected: clone A (T12, *emm*12, biotype III, PFGE I), which was present in Gipuzkoa until 1990; clone B (T4, *emm*4, biotype I, PFGE II), which was introduced in 1991; and clones C (T4, *emm*4, biotype I, PFGE III) and D (T8,25, *emm*75, biotype V, PFGE IV), which were introduced in 1995 and persisted during 1996 and 1997. In Madrid and Vitoria, 89.4% (110) of the 123 M-phenotype strains isolated in 1996 belonged to clones B and D.

Conclusions

In 1990, a new phenotype of erythromycin resistance (first designated NR and later M) was found in group A streptococci in Finland (7,17). ErR with the M phenotype had a low level of erythromycin resistance (8 mg/l to 16 mg/l) and showed cross-resistance with the 14- and 15-membered macrolides; however, they showed the same susceptibility to the 16-membered macrolides and to clindamycin as the erythromycin-susceptible strains (11). This M phenotype was prevalent among *S. pyogenes* in Europe and was the predominant phenotype of resistance among ErR isolated in Finland (18,19), Sweden (20), Austria (21), and Spain (9-11). In Italy, a high prevalence of erythromycin-resistant *S. pyogenes* was reported, but the prevalence of the M-phenotype strains among ErR varied by geographic area (22-24). Among the 437 ErR in our study in Spain, 424 (97%) showed the M phenotype; the *mef*A gene was detected and studied in 283 of these strains.

The epidemiologic surveillance of *S. pyogenes* can be done by using phenotypic or genotypic

methods or both (as we did). Biotyping does not need specialized personnel or equipment, and it is useful, in combination with serotyping methods, for a first approximation of the epidemiologic characterization of *S. pyogenes*. In this study, the most prevalent T serotypes among ErR were infrequent among erythromycin-sensitive strains and vice versa. M serotyping is a classic typing method with at least 74 types recognized, but because it is a very specialized method and reagents are not available commercially, it is restricted to a few reference centers. A rapid PCR-ELISA to determine the *emm* gene type was an accessible alternative to serology for M-antigen typing.

Although the discriminatory power of biotyping and serotyping is considered poor because different genotypes may express the same phenotypic characteristics (15,25,26), these tests were of great value in our epidemiologic surveillance. The biotype and T-serotype combination was able to discriminate between ErR and erythromycin-sensitive strains and delimited most clones among ErR.

Genomic typing methods have rarely been used in characterizing the epidemiology of noninvasive *S. pyogenes*. Among these methods, restriction endonuclease analysis of genomic DNA (REA), random amplified polymorphic DNA (RAPD), ribotyping, PFGE of chromosomal DNA, and DNA sequence analysis have been used with varying degrees of success (15,16,19,26-28). PFGE patterns confirmed the results of biotyping and serotyping and further distinguished among isolates within the same biotype and T-serotype combination. However, PFGE is a complex method—results take at least 4 days to obtain—and expensive equipment and specialized personnel are needed.

Although the polyclonal nature of the ErR strains was established in this study and previously (18), most ErR belonged to only a few clones. In Finland, 91% of the ErR isolated in 1994 were serotype T4 M4 and 88% constituted one clone by RAPD and REA (19). In our study, many clones were detected during the 10-year period. Two of the four main clones comprised 78.8% of ErR. The clonal distribution of ErR in Spain could be due to the introduction and spread of ErR from other locations or to mutations in *S. pyogenes* previously present in our environment. ErR with the M phenotype already existed in Gipuzkoa before 1990, and serotype T12 was the only one found. From 1980 to 1988, serotype T12 was predominant among ErR with the M phenotype in Sweden (20). In Gipuzkoa, the first serotype T4 ErR strain was isolated in 1991; until the end of 1994, 93.8% of ErR isolated belonged to the same clone (clone B: biotype I, type T4, *emm*4, PFGE II). Only three biotype I serotype T4 erythromycin-sensitive strains were detected before 1991. Clone B probably did not emerge in Gipuzkoa from a mutation of one of these uncommon sensitive strains. Apart from the strains described in Finland, Sweden, and Spain, strains with M phenotype were isolated in Great Britain before 1990 (4,29). Among these British and Finnish strains, type T4 M4 was frequently isolated (4,18,29). In a 1986 outbreak of 10 associated cases in Somerset, Great Britain, isolates were group A, type M4 and resistant to erythromycin (MIC 8 mg/l) but sensitive to clindamycin (29). We do not know whether the strains of clone B isolated in Spain are the same as the ErR type T4 M4 found in Great Britain and Finland before 1991, but it is probable. In Italy, serotypes T4 and T8.25 were found among M-phenotype strains (22), which suggests that a few clones have spread across Europe and caused a regional epidemic. No erythromycin-sensitive strains were detected in Gipuzkoa with the same biotype/T-type/*emm* type combination as clone D.

We believe that clones B and D, the most frequent among ErR in Spain, were of epidemic origin and that clone B probably came from northern or western Europe.

Dr. Perez-Trallero is a clinical microbiologist and infectious disease consultant. He is head of the Microbiology Department at Complejo Hospitalario Donostia and assistant professor of Preventive Medicine and Public Health at the Facultad de Medicina at the Basque Country University. His research focuses on antimicrobial resistance, epidemiology of transmissible diseases, and methods for their prevention and control.

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Air Evacuation under High-Level Biosafety Containment: The Aeromedical Isolation Team[1]

George W. Christopher and Edward M. Eitzen, Jr.
U.S. Army Medical Research Institute of Infectious Diseases,
Fort Detrick, Maryland, USA

Military contingency operations in tropical environments and potential use of biological weapons by adversaries may place troops at risk for potentially lethal contagious infections (e.g., viral hemorrhagic fevers, plague, and zoonotic poxvirus infections). Diagnosis and treatment of such infections would be expedited by evacuating a limited number of patients to a facility with containment laboratories. To safely evacuate such patients by military aircraft and minimize the risk for transmission to air crews, caregivers, and civilians, the U.S. Army Medical Research Institute of Infectious Diseases maintains an aeromedical isolation team. This rapid response team, which has worldwide airlift capability designed to evacuate and manage patients under high-level containment, also offers a portable containment laboratory, limited environmental decontamination, and specialized consultative expertise. This article also examines technical aspects of the team's equipment, training, capabilities, and deployments.

Air evacuation of patients with potentially lethal, contagious infections poses unique challenges and risks to air crews and medical personnel. Evacuation of such patients is relevant to military contingency operations because troops may be placed at risk for hemorrhagic fevers and other infections during deployment to tropical environments or by adversaries' use of biological warfare agents.

Evacuation of patients to the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) would afford the immediate availability of biosafety level 4 laboratories (designed for the study of pathogens requiring maximum biological containment for laboratory safety) and facilitate rapid diagnosis of diseases due to pathogens posing extraordinary laboratory safety hazards. Furthermore, USAMRIID has the only fixed patient-care suite in the world designed for medical care under maximum biological containment. To safely evacuate a limited number of patients to the containment-care suite and provide medical care while minimizing the risk for transmission to air crews, caregivers, and civilians, USAMRIID maintains an aeromedical isolation team (1-3).

Address for correspondence: George W. Christopher, USAMRIID, ATTN: MCMR-UIZ-T, 1425 Porter Street, Fort Detrick, MD, 21702-5011, USA; fax: 301-619-2312; e-mail: george_christopher@detrick.army.mil.

The Aeromedical Isolation Team

The purpose of the isolation team is to safely transport patients with potentially lethal communicable diseases for which no effective vaccines, chemoprophylaxis, or specific therapies exist. These would include patients with an unknown disease pending identification of the pathogen, patients with viral hemorrhagic fevers (notably those due to filoviruses and arenaviruses), and those suspected of being affected by a biological attack (Table 1) (3). Etiologic diagnosis and medical care would be provided at USAMRIID.

USAMRIID can simultaneously deploy two teams, each consisting of one physician, one registered nurse, and four to six medics. Each team can transport and manage one patient. In addition, the team can deploy a portable containment laboratory with rapid diagnostic

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Table 1. Infections and conditions requiring containment care during transport

Arenavirus infection
Argentine hemorrhagic fever (Junin virus)
Bolivian hemorrhagic fever (Machupo virus)
Brazilian hemorrhagic fever (Sabiá virus)
Lassa fever
Venezuelan hemorrhagic fever (Guanarito virus)
Bunyavirus infection
Congo-Crimean hemorrhagic fever
Filovirus infection
Ebola
Marburg
Orthopoxvirus infection
Monkeypox
Variola
Pneumonic plague until sputum cultures are negative
Any unknown, virulent, communicable disease pending diagnosis
Suspected biological-warfare–caused infection

Figure 1: Aeromedical isolation team members in field-protective suits equipped with battery-powered HEPA-filtered respirators transporting the stretcher isolator, a light-weight unit designed for initial patient retrieval. The team trains on several types of military aircraft, including the C-130 transport shown in the background.

assays, including enzyme-linked immunosorbent assays and polymerase chain reaction (PCR), as well as standard clinical laboratory support, for all agents listed in Table 1 (in development: Machupo, Sabiá, and Guanarito viruses).

Deployable on rotary and fixed-wing military aircraft, the team conducts in-flight training and can deploy within 6 to 12 hours of notification. Although a military asset, the team has been mobilized for situations involving civilians. Requests may be forwarded through local and state health departments to the Centers for Disease Control and Prevention, through the Federal Emergency Management Agency, or through the Federal Bureau of Investigation and are then reviewed by the Directorate of Military Support. Evacuation of non-U.S. citizens from other countries to the United States would require coordination through the Department of State, Bureau of Political-Military Affairs.

Biosafety Containment under Field Conditions

Maximum biological containment is designed to prevent transmission of highly hazardous pathogens and is accomplished in two steps. First, the health-care worker wears an impermeable suit consisting of a lightweight polyvinyl chloride (PVC) coverall, a separate hood, and vinyl boots (Figure 1). A HEPA-filtered respirator powered by a rechargeable battery supplies air under positive pressure for breathing and cooling. HEPA filters are certified to remove 99.7% of particles 0.03 μm to 3.0 μm diameter; each filter is tested with particulate aerosol challenge studies before delivery. Air enters at a rate of 170 L/min through an intake port near the top of the hood and exits through an exhaust valve at its base. Two-way radios permit communication between team members and patients. The suit and respirator ensemble has been tested by the manufacturer by particulate aerosol challenge and meets the standards of the National Institute of Occupational Safety and Health and the Occupational Safety and Health Administration for working in environments with respiratory hazards.

Second, the patient is isolated within a sealed container under negative air pressure maintained by a battery-powered HEPA-filtered ventilation system providing five air exchanges per hour (Figure 1). Two isolators are used: the stretcher isolator, a lightweight unit for initial patient retrieval (Figure 1), and the Vickers aircraft transport isolator (VATI), a larger unit for definitive transport and in-flight care (Figure 2; Table 2).

The design and construction of the isolators are similar to that of transparent flexible PVC isolators for gnotobiotic animals in biomedical research; the isolators were adapted both for in-patient and transport use (4-7). Challenge studies have demonstrated the efficacy of containing aerosolized T-2 bacteriophage during

Figure 2: The Vickers aircraft transport isolator (VATI), designed for prolonged patient transportation and in-flight care.

Table 2. Dimensions of portable isolators

Isolator	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Stretcher	221	69	86	45
Vickers aircraft transport	221	91	152	112

both hypobaric and isobaric conditions (8). The HEPA filters are certified to remove 99.7% of all particles 0.3 μm to 3 μm in diameter. Isolators have been used to treat in-patients with suspected Ebola, Lassa, and Marburg hemorrhagic fevers (5,6). The utility and safety of these isolators for in-patient care have been questioned (9), and their use in hospitals is not recommended (10,11). However, transport isolators, the only available technical means of reliably maintaining airborne isolation in a military transport aircraft, have been successfully used for the aeromedical evacuation of patients with suspected Ebola fever (6) and suspected (7) and proven Lassa fever (9).

Both isolators feature transparent PVC envelopes suspended from metal frames by detachable plastic rings. Both envelopes include gloved sleeves, transfer and docking ports for patient entry, and transfer and supply ports for introducing supplies. Electrical current is supplied by rechargeable batteries or the aircraft electrical system. Both isolators can be equipped with portable oxygen tanks, intravenous fluids and tubing, medication, and portable defibrillators.

Aeromedical Evacuation Process

The patient must be evaluated and stabilized before transport to ensure survival en route. Only patients likely to survive transport would be evacuated. The physiologic effects of altitude, effect of confinement on patient-care delivery, and psychologic effect of confinement within the isolator must be considered. Mechanical ventilation cannot be provided in the VATI, and suction capabilities are limited; therefore, acute respiratory failure and presence of gas trapped within closed body cavities that may pressurize at high altitudes (e.g., pneumothorax or intestinal gas due to ileus or bowel obstruction) contraindicate evacuation. Evacuation of patients with conditions requiring special in-flight management, e.g., hemodynamic instabilty and severe anemia (<2.5 million erythrocytes/cc or <7.0 g hemoglobin/100 ml) (12), may also be contraindicated.

The patient is placed inside the stretcher isolator and carried to a transfer point near the aircraft. There the stretcher isolator and team members are decontaminated with a 5% hypochlorite solution. During the decontamination procedure, the patient breathes portable oxygen from a mask, and the ventilation intake port is sealed to prevent chlorine gas from entering the isolator. The portals of the isolators are then connected with an airtight sleeve, and the patient is transferred to the VATI (Figure 3). The sleeve is clamped at two points before being heat-sealed and cut, maintaining air-tight seals throughout the transfer. The cut ends are decontaminated and covered with PVC seals, which are then attached to the isolators with

Figure 3: Patient's view of an aeromedical isolation team member providing care through a half-suit in the Vickers aircraft transport isolator (VATI).

pressure-sensitive tape. Both isolators are maintained under negative air pressure until decontaminated at USAMRIID. Equipment is removed, placed in bags, and returned to USAMRIID for decontamination of respirators and radios and disposal or decontamination of coveralls.

The patient is transported on standard military transport aircraft (C-130 or C-141), which maintain an internal cabin atmosphere equivalent to approximately 8,000 feet above sea level while at altitude (26,000 feet to 35,000 feet). This level of air pressure is considered adequate to protect commercial airline passengers (13) and results in an arterial blood hemoglobin oxygen saturation of approximately 90% in healthy persons. However, because the VATI maintains negative air pressure, the partial pressure of oxygen inside the VATI is lower than that of ambient atmosphere. This lower pressure would be hazardous for persons with respiratory failure or chronic obstructive pulmonary disease. In addition, rapid decompression could place the patient at further risk. Accordingly, the VATI is deployed with portable oxygen tanks, tubing, and masks capable of delivering 100% of needed oxygen.

Design features of the VATI that facilitate in-flight care include its larger size, additional glove ports, two half-suits, 12 cones at the base of the envelope for introducing wires and tubing, two sleeves for intravenous therapy, and two large pockets for placing waste supplies.

Diagnosis and therapy, which can be delivered in the VATI, include monitoring cardiac function, blood-pressure, and oxygen saturation of the blood; providing oxygen supplementation, intravenous therapy, and phlebotomy; and determining hemoglobin and hematocrit levels and serum electrolytes (by using a portable hand-held laboratory analyzer) (Figure 3). Because the use of glove ports limits manual dexterity, team members practice these skills on each other during on-ground and in-flight training exercises. Endotracheal intubation, manual ventilatory assistance with a bag and valve device, and cardiopulmonary resuscitation are practiced on mannequins in the isolators. To minimize the risk of puncturing the isolator, no glass bottles or instruments with rough or sharp edges are used. Phlebotomy is minimized, and a needleless intravenous system is used.

After arriving at USAMRIID, the patient is transferred from the VATI into the containment-care suite through a plastic sleeve connected to a port on an outside wall.

Aeromedical Isolation Team Deployments

The first of several team deployments occurred during the October 1989 epizootic of Ebola hemorrhagic fever among cynomolgus monkeys (*Maccaca fascicularis*) imported from the Philippines and held at a primate quarantine facility in Reston, Virginia (14-16). Because Ebola virus had been isolated only in association with epidemics of human disease in Africa, which had death rates of 53% to 88%, potential transmission of Ebola to animal handlers in the facility and secondary transmission to other members of the community were of concern. Aeromedical isolation team and additional personnel from USAMRIID were deployed. Animal handlers were trained in the use of suits and respirators, containment methods, decontamination, and waste disposal; 450 monkeys were humanely euthanized; and team members obtained specimens of blood and tissue for histopathologic and virologic studies and sealed and decontaminated the facility by paraformaldehyde fumigation followed by conventional disinfectants (16).

Respiratory transmission was suggested by the epizootic spread among monkeys housed in separate cages (with no opportunity for physical contact [16]) and by subclinical human infections. Serologic evidence of recent Ebola infection developed in four of the five animal handlers; only one had percutaneous blood exposure. None became ill, which suggested that the epizootic strain was not virulent for humans (17), and none of the 42 USAMRIID personnel participating became infected. The Ebola isolates from infected primates represented a newly described strain, Ebola Reston, genetically and taxonomically distinct from related human pathogens identified in Africa.

In another episode, an aeromedical isolation team member was deployed to Linkoping, Sweden, in January 1990 to assist in implementing biosafety containment for a patient suspected of having a viral hemorrhagic fever after returning from eastern Africa (1).

During November 1995, the team was deployed with a senior medical advisor after construction workers at Wright-Patterson Air

Force Base, near Dayton, Ohio, uncovered a buried cache of biological munitions. Some of the munitions, produced during the U.S. offensive biological warfare program (1942–1969) (18), were intact, but most were perforated due to corrosion of the munition casings. The munitions were brought inside a bunker by the U.S. Army Technical Escort Unit and sampled inside the VATI. Samples of liquid bomb fill and adjacent soil samples were transported in sealed containers on ice packs in accordance with U.S. Department of Transportation regulations by military aircraft to USAMRIID, the Naval Medical Research Institute, Bethesda, Maryland, and the Armed Forces Institute of Pathology, Washington, D.C. The bomb fill contained nonviable gram-negative bacteria (identified as *Brucella suis* by strain-specific PCR); soil samples tested positive for *Brucella* DNA and antigens and cultures yielded normal commensal flora but no growth of *Brucella* sp. Background soil and groundwater tested negative for *Brucella* DNA and antigens. All munitions were drained, and the fill and casings were sterilized by autoclave before disposal. Documents later retrieved confirmed that the munitions were bomblets filled with *B. suis* and used at the base from June to October 1954 to train personnel in viability testing and handling of biological weapons. After training was completed, the munitions were heated in a ground portable heater with an ambient temperature of 104°C for 4 hours each of 2 days, with the temperatures of the innermost munitions reaching 70°C to 74°C, and then buried.

Although not deployed, the team was on alert during 1994 for a laboratory-acquired Sabiá virus infection (Brazilian hemorrhagic fever) at Yale University (19) and during the 1995 Ebola epidemic in the former Zaire.

Lt. Colonel Christopher is chief of the Containment Care Medicine Department, Operational Medicine Division, USAMRIID. His interests include medical care delivery under biological containment and the formulation of medical practice guidelines for the care of biological warfare casualties.

Colonel Eitzen is chief of the Operational Medicine Division, USAMRIID. He directs the division in multiple activities to enhance medical biological defense, including medical education programs; collaboration of basic scientific, medical, public health, and operational communities; and consultative expertise for military and other government agencies.

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emm Typing and Validation of Provisional M Types for Group A Streptococci[1]

R. Facklam,* B. Beall,* A. Efstratiou,† V. Fischetti,‡ D. Johnson,§ E. Kaplan,§ P. Kriz,¶ M. Lovgren,# D. Martin, B. Schwartz,* A. Totolian,†† D. Bessen,‡‡ S. Hollingshead,§§ F. Rubin,¶¶ J. Scott,## and G. Tyrrell¶**

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Central Public Health Laboratory, London, United Kingdom; ‡The Rockefeller University, New York, New York, USA; §University of Minnesota, Minneapolis, Minnesota, USA; ¶National Institute of Public Health, Prague, Czech Republic, #National Center for Streptococcus, Edmonton, Alberta, Canada; **Communicable Disease Group, ESR, Porirua, New Zealand; ††Institute of Experimental Medicine, St. Petersburg, Russia; ‡‡Yale University, New Haven, Connecticut, USA; §§University of Alabama, Birmingham, Alabama, USA; ¶¶National Institutes of Health, Bethesda, Maryland, USA; and ##Emory University, Atlanta, Georgia, USA

This report discusses the following issues related to typing of group A streptococci (GAS): The development and use of the 5' *emm* variable region sequencing (*emm* typing) in relation to the existing serologic typing system; the designation of *emm* types in relation to M types; a system for validation of new *emm* types; criteria for validation of provisional M types to new M-types; a list of reference type cultures for each of the M-type or *emm*-type strains of GAS; the results of the first culture exchange program for a quality control testing system among the national and World Health Organization collaborating centers for streptococci; and dissemination of new approaches to typing of GAS to the international streptococcal community.

The Lancefield M-typing system, a typical serologic system based on antigen-antibody reactions, is dependent on the preparation of type-specific antisera and extraction of a protein identified as M protein on the surface of group A streptococci (GAS) (1). The antisera against the M-protein antigens are produced with whole-cell streptococcal vaccines used to immunize rabbits. Acceptable antisera contain specific-precipitin antibodies and type-specific antibodies that must enhance the phagocytosis of the strain used to immunize the rabbit (2,3). The precipitin antibodies are made specific by absorption of the serum with streptococcal cells to remove the carbohydrate group antibodies and any cross-reactive precipitin antibody to heterologous M-type strains. Each rabbit antiserum is tested for reaction with antigens of all known M types.

Approximately half of GAS strains produce an apoproteinase, an enzyme that causes mammalian serum to increase in opacity. This reaction is called the serum opacity factor reaction, and the responsible enzyme is referred to as opacity factor (OF). The OF enzymes are OF type-specific because each M type that produces OF can induce type-specific OF antibodies that can be used in OF inhibition tests (3,4). Preparation of the OF antisera and specific details of the OF tests are described elsewhere (3,4). Some laboratories have used OF typing to predict M types in epidemiologic investigations. Even though it is not uniformly agreed that OF typing antisera results should be reported as M or provisional M-typing results, anti-OF antisera in many situations predict the M type in a type-specific manner. For reporting purposes, if

Address for correspondence: Richard R. Facklam, Centers for Disease Control and Prevention, 1600 Clifton Road, Mail Stop C02, Atlanta, GA 30333, USA; fax: 404-639-1379; e-mail: rrf2@cdc.gov.

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cultures are identified with M- or OF-typing antisera, they should be identified as M type or OF type.

Reference strains used to prepare the M antisera (several of which were originally described by Griffith in 1935) were historically obtained from the Lancefield collection, Rockefeller University, New York; however more recently, reference strains have been available from other reference laboratories. M-types 1 through 51 were designated in the laboratory of Dr. Rebecca Lancefield between 1928 and 1945. M-types 52 through 81 were submitted by various investigators to reference laboratories in Atlanta, London, New York, and Prague between 1965 and 1976 for confirmation. Some laboratories believe that certain strains in the Lancefield collection do not adequately express M protein and are unsuitable for the production of M-type antiserum. Preparing M-type antiserum or a typing system accurately related to the Lancefield system requires documented reference strains from one of the internationally recognized reference laboratories.[2]

In the Lancefield typing system, strains representing types 7, 16, 20, and 21 (originally described as Griffith) are not GAS but belong to groups C and G. M-type 10 is the same serotype as M12, M-type 24 is the same as M45, and M-type 35 is the same as M49; thus, designations of serotypes 7, 10, 16, 20, 21, 35, and 45 are not included in the Lancefield M-typing system 1 to 81 for GAS.

The *emm* gene of *S. pyogenes* is the gene that encodes the M protein. The M protein, responsible for the bacterium's capacity to resist phagocytosis, is a major virulence factor in GAS. The 5' ends of *emm* genes are highly heterogeneous and encode for the serotype specificity used for the M-typing system developed by Dr. Lancefield in 1928 (1). Producing type-specific M-typing antisera is difficult and specialized; no attempt has ever been made to produce them commercially, and only a few international reference laboratories prepare them. The resurgence of rheumatic fever cases in the United States and the emergence of severe infections (streptococcal toxic shock and necrotizing fasciitis) caused by GAS in the 1980s and 1990s indicated the need to reassess typing strategies for GAS. Since production of M-type precipitating antisera is very expensive and labor-intensive, the potential usefulness of a nonserologic typing system for GAS sequencing the 5' end of the M protein (*emm*) gene toward a molecular-based typing system was examined.

emm Typing System for GAS

Before an *emm* genotype-based typing scheme was developed for GAS, the nucleotide sequence at the 5' ends of *emm* genes had been reported for many strains representing M-types 1-81 and several provisional M types (PT) (5,6). However, it was not always evident that true reference strains had been used. The knowledge gained from these studies provided the impetus for exploring the feasibility of an *emm*-based genotyping system. Subsequent studies based on sequencing the 5' *emm* genes from GAS reference strains and clinical culture specimens have now been published (7,8). In all studies involving *emm* sequence typing, 160 to 660 bases were sequenced from the 5' terminal end of the *emm* gene. The methods of *emm* sequencing and *emm* gene amplicon profiling restriction pattern techniques have been described (7,8). Two isolates are regarded as sharing the same *emm* sequence type if they are ≥ 95% identical over their 5' end 160 nucleotides (includes approximately 50 bp of the moderately conserved leader peptide-coding region), allowing for one frame shift or in-frame insertion/deletion of no more than seven codons (8). The results of *emm* gene sequencing of Dr. Lancefield's reference strains types 1 to 51 and reference strains of M-types 52 to 81, submitted to the Centers for Disease Control and Prevention (CDC) Streptococcus Reference Laboratory as potential new M types from 1967 to 1976 by various international investigators, are summarized below.

The 5' end *emm* sequences of the following CDC M-type reference strains matched the following sequences in GenBank that were submitted by other investigators: Types 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 19, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 33, 36, 37, 39, 41, 43, 44, 46,

[2]Centers for Disease Control and Prevention, Atlanta, Georgia, USA; Central Public Health Laboratory, London, United Kingdom; The Rockefeller University, New York, New York, USA; University of Minnesota, Minneapolis, Minnesota, USA; National Institute of Public Health, Prague, Czech Republic, National Center for Streptococcus, Edmonton, Alberta, Canada; Communicable Disease Group, ESR, Porirua, New Zealand.

47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 72, 73, 74, 75, 76, 77, 78, 80, 81. The accession numbers for these and many other *emm* sequences deposited in GenBank are listed in references 5-8 and at http://www.cdc.gov/ncidod/biotech/infotech_hp.html.

The 5' *emm* sequences from the CDC reference strains of GAS for the following M types were submitted to GenBank: M-types 13 (AF025950), 32 (L47325), 34 (L47324), 38/40 (L46817), 42 (L46799), 67 (AF025949), 68 (AF025948), 69 (AF035838), 70 (AF035838), 71 (L46652). The 5' *emm* sequences that were different from those submitted to GenBank by previous investigators (6) are M-types 13, 67, 68, and 79. For M-type 13, the *emm* sequence in GenBank was not from a recognized Lancefield typing strain; *emm* sequence AF025950 should be considered the *emm* sequence for Lancefield M-type 13. For M-types 67 and 68, the *emm* sequences in GenBank did not match those from CDC. The reasons for this are unknown; however, *emm* sequence AF025949 (M-type 67) and *emm* sequence AF025948 (M-type 68) were obtained from the reference strains submitted to CDC by the investigators who originally described these M types. For M-type 79, the *emm* sequence obtained by the CDC investigators matched the *emm* sequence in GenBank labeled M-type 80. Personal communication with the investigators who submitted the *emm* sequences for M-types 79 and 80 indicated a transcription error during submission of the sequences. Sequence U12004 is the correct sequence for M-type 79. The *emm* sequences from the CDC reference strains representing these four M types were confirmed by an independent laboratory.

CDC has additional data on *emm* typing of more than 1,500 GAS isolates from population-based studies, as well as random cultures from the United States and several other countries. Nearly 100% of the cultures could be genotyped by the *emm* typing system. In addition to determining the *emm* types of reference strains of M-types 1 to 81, several provisional type strains as well as new sequence type strains were typed by the *emm* typing procedure. Only one of more than 1,500 GAS isolates could not be *emm* typed by current methods. The *emm* sequence type of clinical isolates representing 35 distinct serotypes match the *emm* sequence type of the corresponding reference strain—included in this set of analyses are clinical isolates that underwent M or OF serotyping at one of the internationally recognized reference laboratories or elsewhere (8;9; B. Beall and D. Bessen, unpub. data).

The historical correlations between T type, OF reaction, and M type are largely unchanged when *emm* sequence type is substituted for M type (3,4,7,8). Because this observation is based upon the analysis of >3,000 clinical isolates, the T type and OF reactions together constitute an invaluable second-tier method for further confirmation of the grouping of closely related isolates, as well as for resolution of unrelated sets of organisms (R. Beall and R. Facklam, unpub. data).

When comparing a phenotypic-based typing scheme to a genotypic-based scheme, complete concordancy is not expected. Although for most types the M serotype is paired with a unique *emm* sequence type, there are several discrepancies. In five well-documented instances, 5' end *emm* sequences are >95% identical for two distinct M serotypes: M-types 27L and 77, 38 and 40, 44 and 61, 50 and 62, and 65 and 69 (10). The molecular basis for a lack of concordancy can differ for each unique M-type/*emm*-type pair. A few critical nucleotide substitutions at the 5' *emm* region could result in new, dominant antigenic epitopes that lead to the generation of a distinct serologic type. Alternatively, *emm* genes can occasionally undergo horizontal exchange and move onto a new genetic background, as appears to be the case for *emm*44 and *emm*61 (6); in this instance, additional dominant, polymorphic antigens may exist that can also be detected by M-typing sera. For the five examples cited above, introduction of a second typing scheme (T-agglutination and OF reaction) has proven useful in distinguishing between pairs displaying identical *emm* sequence types, but distinct M serotypes.

The discriminatory power of the genotypic *emm* sequence-typing scheme approximates that of the phenotypic M-serotyping scheme. For most M serotypes, there is a one-for-one relationship with a unique *emm* sequence type. The selection of 95% sequence identity as the cutoff value for defining the *emm* sequence type is based on empirical measures that best match the level of resolution achieved by M serotyping. In several examples, the *emm* sequence of one *emm*-type/M-type pair displays a relatively high

level of sequence identity (but <95%) to a second, unique *emm*-type/M-type pair. For example, the *emm*3 and *emm*31 sequences share 91.3% identity over their first 160 bases; *emm*2 and *emm*73 are 89% identical over their first 160 bases of 5' end sequence, and this similarity is increased to 92.3% identity over their first 326 5' bases. Conceivably, certain genetic changes, such as a single bp insertion in the *emm* hypervariable region followed by a single bp deletion much farther downstream, could alter the reading frame of the gene and hence the antigenic structure and serotype of the *emm* gene product; however, this kind of variant has rarely been encountered in CDC surveys. Other genetic changes, such as synonymous substitutions, have no effect on phenotype. In some instances a single deletion or insertion of seven or fewer codons within the hypervariable 5' end 160 bp had no effect on the predicted M serotype (B. Beall, unpub. data). Therefore, an *emm* gene with less than 95% sequence identity to other *emm* genes may confer a new M serotype specificity. Thus, two isolates are regarded as sharing the same *emm* sequence type if they are ≥95% identical over their first 160 nucleotides, allowing for one frame shift or in-frame insertion/deletion of no more than seven codons (8). The 95% identity cutoff is not expected to match perfectly what can be achieved by serologic methods.

Most of the GAS isolates that are deemed nontypable by serologic methods can be genotyped through *emm* sequence determination. Furthermore, the M serotype does not always match the *emm* sequence-type (9,11). However, among the hundreds of strains analyzed by the streptococcal reference laboratory at CDC no discrepancies were observed between M serotype and *emm* sequence type. The full extent of putative M-serotype/*emm* sequence-type discordancies is not known, and the explanations for such a lack of congruency are numerous. A more complete understanding of the basis for discrepancies will be forthcoming as the *emm* sequence-typing method becomes more widely implemented.

The *emm*-typing system is a useful and reliable epidemiologic tool for subdividing GAS. Because it is independent of *emm* gene expression and can often discriminate between biologically distinct isolates that may be only weakly antigenic or nontypeable, *emm* sequence typing has the potential to classify isolates that have been difficult to type by serologic methods.

Designation of M, Provisional M, and *emm* Sequence Types

GAS strains fall into three categories: Validated M types, provisional M types, and *emm* sequence types. If a laboratory has prepared antiserum to an unknown strain and the serum has type-specific precipitating antibodies, as well as bactericidal antibodies directed to that strain, verified by one of the six original reference laboratories, an M-type designation can be assigned to that strain. If two laboratories (at least one being one of the six reference laboratories) produces type-specific precipitating and bactericidal antiserum to the strain, the strain also qualifies as a new M type.

When a laboratory has prepared antiserum as described above to an unknown strain but the specificity of this antiserum has not yet been confirmed by a second reference laboratory, that strain is designated a provisional type. The requirements for conventional validation of new M types will be described elsewhere. A third category are sequence types or *emm* types, which are strains typed by sequencing the *emm* gene. If cultures are identified by *emm* sequencing, they should be reported as *emm* type.

Validation Procedures and Nomenclature of New *emm* Sequence Type Strains

Published *emm* sequences from studies conducted in New Zealand and Australia included several new *emm* sequences included in GenBank (12,13). In addition, data from the CDC studies (B. Beall and R. Facklam, http://www.cdc.gov/ncidod/biotech/infotech_hp.html) indicated that more than 30 unknown *emm* sequences were identified among the 1,500 isolates of GAS that had been *emm* sequenced. A working group of representatives of each of the six international reference centers in Canada, New Zealand, Czech Republic, United Kingdom, and United States, was charged with establishing a definitive protocol both for submission of new *emm* sequences and for subsequent validation of new *emm* types. As an interim measure, unique 5' end *emm* sequences proposed as new *emm* types must be confirmed by a second laboratory; at least one of the two laboratories should be the streptococcal reference laboratory at CDC. If the uniqueness of the *emm* sequence

can be confirmed by the second laboratory, the original investigator or one of the confirming laboratories will submit the findings to the Working Group, which will determine whether the strain should be assigned a new *emm* type number (e.g., *emm*94). In addition to sequence uniqueness, additional factors may be considered by the working group when making this decision; for example, previous requirements for assigning regular M- and provisional M-type numbers to strains were restricted to strains of particular clinical significance or to those occurring in a population "with significant frequency." Another remaining unresolved issue is whether or not all new *emm* reference strains should actively express M protein. A lack of surface expression of this *emm* gene product will preclude any possibility for correlation of *emm* type with classic serologic type or subsequent evaluation of biological significance. The relationship of *emm* sequence to biological function needs to be further explored.

CDC has validated six *emm* sequences submitted to GenBank by Australian investigators (12-14) and one *emm* sequence from a strain submitted to CDC by an investigator in the United States (15); these sequences should all be considered for official status as new *emm* types. Four additional isolates were examined at the CDC laboratory for which *emm* sequences had been submitted to GenBank (12-14). Strains STBSB75, ST1293, ST87/156, and STNS27 were shown to have the same *emm* types as M-type 70, M-type 76, PT2110, and PT5757, respectively. Therefore, these four *emm* sequences should not be accepted as new *emm* types, and their sequences should be reidentified in GenBank.

CDC has identified 10 new *emm* types from population-based studies of GAS invasive disease (7,8, unpub. data). In addition, eight new *emm* sequences from Brazil, six from Malaysia, three from Papua New Guinea, three from India, two from Ethiopia, two from Gambia, and one each from New Zealand and Chile have been confirmed by a second laboratory for *emm* sequence uniqueness, for 36 potentially new *emm* types.

List of Reference Strains

The WHO Collaborating Laboratory for Reference and Research on Streptococci in Prague has prepared a database of the reference type strains to be used for research and antiserum production from information provided by the six international Reference Centers in Canada, New Zealand, Czech Republic, United Kingdom, and United States. Although all the reference strains on the list at one time had demonstrated survival in the in vitro bactericidal test (presumably reflecting functional M protein), the reference strains should be retested for survival in the bactericidal test before use in research. Dr. Lancefield's strains types 1 to 50 are listed at http://www.rockefeller.edu/vaf/

Because listing of type strains may be slightly different for each culture collection, when cultures are obtained, the strains should be properly identified. The strains for reference types 1 to 50 should be traced to the Lancefield collection, from which the M-typing system was derived. In the past, if a reference strain lost the capacity to express M protein on the bacterial cell surface, that strain was passaged in vivo and selected for the increased presence of M protein; therefore, many derivatives of the original reference cultures are in use by the reference laboratories, and none are known to have undergone change in their *emm* gene nucleotide sequence. The American Type Culture Collection (ATCC) has Lancefield's strains 1 to 50, the UK National Collection of Type Cultures has types 1 to 81, as well as the provisional types, and the Czech Culture Collection in Prague also has types 1 to 81 on deposit. Reference cultures 51 to 81 have been deposited in the ATCC by the CDC investigators who will also deposit the provisional type strains shortly. Plans are to continue the deposition of cultures of new *emm* types as they are confirmed.

Additionally, the CDC Streptococcus Laboratory has established an *emm*-type database at http://www.cdc.gov/ncidod/biotech/infotech_hp.html for use as a sequence comparison tool and additional documentation of all of the verified M and *emm* types. The database contains detailed information on the CDC method of *emm* typing and provides additional background on each reference strain and most known *emm* sequence types. Sequence comparisons can be done by using BLAST (Basic Local Alignment Search Tool). The data are prereviewed for errors and discrepancies, and all newly deposited *emm*-types are first verified directly by the CDC reference laboratory. At this time, until a complete validation protocol is established, investigators who discover new

emm sequence types should submit their isolates to the CDC Streptococcus Laboratory to confirm uniqueness. As a first step, investigators should search the CDC database for *emm* sequences; Genbank can be used as a second step, since it is always possible that neither database will necessarily have all known *emm* sequences of GAS at any given time. Furthermore, the CDC database will include the accepted *emm* type designation for those types, whereas a possible incorrect *emm* sequence may have been submitted to GenBank by an investigator.

Validation of Provisional M Types to New M and *emm* Types

The Table lists the provisional M-type strains and the status of validation. Collaborative investigations involving the six reference laboratories included several other provisional M-type strains and has confirmed the following: PT179 fulfills all the phenotypic criteria for an M type; however, only anti-OF serum has been prepared. Therefore, the status of this strain as a potential new M type remains on hold until further supporting data can be provided. PT4854, Colindale Laboratory, United Kingdom (UK), had a closely matching *emm* type as M-type 43; this finding correlated to serologic tests at CDC and UK laboratories as M-type 43 that demonstrate that both strains reacted with M43 typing antiserum. PT3800, Prague, Czech Republic (CZ), has the same *emm* type as M-type 65, which correlated to serologic tests performed at the National Streptococcus Laboratory in Edmonton, Canada, showing that both strains reacted with M65 typing antiserum. PTYE327 (CZ) has the same *emm* type as PT2841(UK), which correlates to serologic tests performed in both the Colindale and Prague laboratories. PT1437, Porirua Laboratory, New Zealand (NZ), has the same *emm* type as PT4245 (UK), which correlates to serologic tests performed in both the UK and NZ laboratories. ST2974.95 (CDC) has the same *emm* type as PT5118 (NZ). ST2974.95 was shown to be PT5118 in serologic tests performed in the Porirua Laboratory. In summary, PT4854, PT3800, PTYE327, PT1437, and ST2974.95 should be identified as *emm*43 (M43), *emm*65 (M65), *emm*87 (M87), *emm*89 (M89), and *emm*92, (M92) respectively. The sources of most provisional types were first documented in 1985 (16).

Table. Recommended action for M and *emm* designation for provisional M types[a]

Provisional type	ID number	CDC ID number	New *emm* or M type
PT180(UK)[b]	NCTC12062	SS-1395	82
PT2110(UK)	NCTC12064	SS-1400	83
PT2233(UK)	R75/2233	SS-1449	84
PT2612(UK)	R76/2612	SS-1447	85
PT2631(UK)	R76/2631	SS-1448	86
PT2841(UK)	NCTC12065	SS-1399	87
PT3875(UK)	R67/3875	SS-1455	88
PT4245(UK)	NCTC12067	SS-1397	89
PT4931(UK)	NCTC12068	SS-1396	90
PT5757(UK)	NCTC12056	SS-1398	91
PT5118(NZ)		SS-1460	92
PTPotter41(UK)	R76/2631	SS-1493	93

[a]For definitions of M and *emm* designations, see section in text, Designation of M, Provisional M, and *emm* Sequence Types.
[b]Abbreviations; UK= United Kingdom, NZ= New Zealand.

The following *emm* types were isolated from patients with severe invasive disease in the United States; *emm*82, *emm*83, *emm*86, *emm*87, *emm*88, *emm*89, and *emm*92. These isolates comprised 12.7% of all isolates identified during these studies. Types *emm*82, *emm*85, *emm*87, and *emm*89 were associated with epidemic investigations in the United States. Many of these new *emm* types were also identified from other countries, including Argentina, Brazil, Bulgaria, Chile, Colombia, Denmark, India, Korea, Malaysia, New Guinea, and Poland. Other reference laboratories have reported these new M types (by using provisional type identifiers) in a variety of infections, including rheumatic fever and acute glomerulonephritis (17,18).

An External Quality Assurance Typing Program among International Reference Laboratories

At the request of the World Health Organization (19), a quality assurance program on GAS typing has been established by the Central Public Health Laboratory, London, United Kingdom. Ten GAS isolates were examined by six different laboratories, and the results of the first distribution showed a very good correlation between laboratories. *Emm* typing by one laboratory correlated very well with M typing by the other five laboratories. Only minor differences were noted among the T- and OF-typing results; no errors were found among the M-typing results. Responsibility for

sending 10 cultures to the other five centers twice a year would be rotated among the six reference centers and a report of the quality assurance program will be presented at the next International Lancefield Society meeting in New Zealand in the fall of 1999.

Dr. Richard Facklam is chief of the Streptococcus Laboratory, Division of Bacterial and Mycotic Diseases Division, National Center for Infectious Diseases, CDC. His major fields of interest include improvement in laboratory procedures for the diagnosis of acute respiratory tract infections, taxonomy of streptococci and related gram-positive cocci, identification of virulence factors associated with bacterial respiratory pathogens, and development of new systems for epidemiologic study of the transmission of bacterial respiratory pathogens.

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Rapid Molecular Genetic Subtyping of Serotype M1 Group A *Streptococcus* Strains

Nancy Hoe,* Kazumitsu Nakashima,* Diana Grigsby,* Xi Pan,* Shu Jun Dou,* Steven Naidich,† Marianne Garcia,‡ Emily Kahn,‡ David Bergmire-Sweat,‡ and James M. Musser*

*Baylor College of Medicine, Houston, Texas, USA; †eGenomics, Inc., New York, New York, USA; ‡Texas Department of Health, Austin, Texas, USA

Serotype M1 group A *Streptococcus,* the most common cause of invasive disease in many case series, generally have resisted extensive molecular subtyping by standard techniques (e.g., multilocus enzyme electrophoresis, pulsed-field gel electrophoresis). We used automated sequencing of the *sic* gene encoding streptococcal inhibitor of complement and of a region of the chromosome with direct repeat sequences to unambiguously differentiate 30 M1 isolates recovered from 28 patients in Texas with invasive disease episodes temporally clustered and thought to represent an outbreak. Sequencing of the *emm* gene was less useful for M1 strain differentiation, and restriction fragment length polymorphism analysis with IS*1548* or IS*1562* as Southern hybridization probes did not provide epidemiologically useful subtyping information. Sequence polymorphism in the direct repeat region of the chromosome and IS*1548* profiling data support the hypothesis that M1 organisms have two main evolutionary lineages marked by the presence or absence of the *speA2* allele encoding streptococcal pyrogenic exotoxin A2.

Molecular genetic approaches that differentiate isolates of a pathogenic microbial species have revolutionized contemporary epidemiologic investigations of putative disease outbreaks. The human gram-positive bacterium group A *Streptococcus* (GAS) has more than 80 M-protein serotypes, but isolates expressing the M1 serotype are disproportionately represented among invasive disease episodes in most case series (1). M1 organisms also commonly cause pharyngitis. For reasons that are unknown, M1 isolates and organisms expressing other M serologic types can undergo rapid temporal variation in disease frequency and severity (1). Serotype M1 isolates have been studied by several molecular typing approaches, including multilocus enzyme electrophoresis; pulsed-field gel electrophoresis; rRNA gene polymorphism typing (ribotyping); random amplified polymorphic DNA analysis; and sequencing of the genes encoding streptokinase, C5a peptidase, M protein, hyaluronidase, and pyrogenic exotoxin A, B, and C (1-5). The common theme of these analyses is that most M1 isolates cultured from patients with invasive disease episodes are closely allied in overall chromosomal relationship as a consequence of sharing a recent common ancestor (1,3,5). Lack of readily detectable chromosomal variation has limited insights on the molecular origin of new virulent strains, velocity of strain spread in human populations, and association of genetic subtypes with certain clinical syndromes, including necrotizing fasciitis and acute rheumatic fever.

Recently, Akesson et al. (6) identified a GAS extracellular protein made by M1 strains that inhibits human complement. This streptococcal inhibitor of complement (Sic) protein is incorporated into the membrane-attack complex (C5b-C9) and inhibits target cell lysis by an undetermined mechanism. Analysis of molecular diversity among 16 M1 GAS isolates from patients with pharyngitis identified seven alleles of the *sic* gene (7). The high level of *sic* polymorphism was unanticipated, given that other methods of molecular analysis had failed to identify substantial variation among M1 isolates

Address for correspondence: James M. Musser, Institute for the Study of Human Bacterial Pathogenesis, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA; fax: 713-798-4595; e-mail: jmusser@bcm.tmc.edu.

(1-5). Subsequently, Stockbauer et al. (8) analyzed 165 M1 isolates from diverse localities, identified 62 alleles, and documented a uniquely high level of allelic variation in this gene. The molecular features of *sic* variation indicated that structural change in Sic is mediated by natural selection (8). Moreover, study of 70 M1 isolates from two temporally distinct epidemics of streptococcal infections in the former East Germany suggested that variation in *sic* contributed to fluctuations in GAS disease frequency and severity (8).

The observation that the polymorphism in the *sic* gene greatly exceeded that for all other genes examined in serotype M1 isolates suggested that *sic* sequencing could be used as a rapid strategy to differentiate organisms thought to be epidemiologically linked. A recent statistically significant increase in cases of invasive GAS in Texas presented an opportunity to test this hypothesis. We also tested whether molecular variation in a region of the chromosome with multiple direct repeat (DR) nucleotide sequences and restriction fragment length polymorphism (RFLP) analysis with insertion elements IS*1548* (9) and IS*1562* (10) would differentiate M1 isolates.

Brief Overview of the GAS Epidemiology

Statistics gathered by the Texas Department of Health indicated that from December 1, 1997, through March 5, 1998, 117 invasive episodes of GAS (and 26 deaths) had occurred statewide. Sixty of these cases and 14 deaths were in central Texas (population 1.4 million). Concern was raised by community physicians, lay individuals, and the media that an unusually virulent strain was causing a disease outbreak. (A complete description of the epidemiology of this outbreak will be presented elsewhere.) For molecular analysis of the GAS causing recent cases, 100 isolates were sent to the laboratory of J.M.M. at Baylor College of Medicine, Houston, TX. On receipt, the bacteria were checked for purity by visual inspection and were confirmed to contain beta-hemolytic organisms with a colony morphology consistent with GAS. Chromosomal DNA was isolated as described (5).

Sequence Analysis of *emm*

To determine whether one or a few unusually virulent strains might account for most of the invasive episodes, we sequenced the hypervariable part of the *emm* gene encoding M-type specificity (5,11). After the sequence data were edited electronically, they were used to search an *emm* database maintained in the laboratory that contains at least one sequence of all known M-protein serotypes and provisional serotypes (11). The database also contains 33 *emm1* allelic variants identified among serotype M1 organisms from global sources (1,5,12) (Figure 1).

	10	20	30	40	50	60	70	80	90	100
*emm*1.0	TVLGAGFANQ	TEVKANGDGN	PREVIEDLAA	NNPAIQNIRL	RHENKDLKA-	------RLEN	AMEVAGRDFK	RAEELEKAKQ	ALEDQRKDLE	TKLKELQQDY
*emm*1.1	.......T..	......N..R	S.D.T.EI..	..TTV.....	.N...N...-	------....	..N.....	....	....	....
*emm*1.2	.......T..	......N..R	S.D.T.EI..	..TTV.....	.N...N...K	NEDLEA....	..N.....	....	....	....
*emm*1.3	....	.........S	....	....	.........-	------....	....	....	....	....
*emm*1.4	..V.....	......ENVG	..D.VKE.VE	KD.VL..K..	.S..QK..E-	------S...	.RD.....	....	...A......	....
*emm*1.5	....	....	......G...	....	.........-	------....	....	....	....	....
*emm*1.6	....	....	....	....	.Y.......-	------....	....	....	....	....
*emm*1.7	....	....	.---------	------....	.Y.......-	------....	....	....	....	....
*emm*1.8	....	....	..D.......	....	.........-	------....	....	....	....	....
*emm*1.9	....	....	....M.....	....	.........-	------....	....	....	....	....
*emm*1.10	....	....	.....G....	....	.........-	------....	....	....	....	....
*emm*1.11	....	....	...A......	....	.........-	------....	....	....	....	....
*emm*1.12	....	....	..K.......	....	.........-	------....	....	....	....	....
*emm*1.13	....	....	S.........	....	.........-	------....	....	....	....	....
*emm*1.14	....	....	L.........	....	.........-	------....	....	....	....	....
*emm*1.15	....	....	....	....	.........-	------....	....	....	.......Y..	....
*emm*1.16	....	....	....	....T.....	.........-	------....	....	....	....	....
*emm*1.17	....	....	....	....	....G....-	------....	....	....	....	....
*emm*1.18	....	....	....	....V.....	.........-	------....	....	....	....	....
*emm*1.19	....	....	....	....	.........-	------....	.......G..	....	....	....
*emm*1.20	....	....	..G.......	....	.........-	------....	....	....	....	....
*emm*1.21	....	....	.......F..	....	.........-	------....	....	....	....	....
*emm*1.22	....	....	....	....	.........-	------K...	....	....	....	....
*emm*1.23	....	....	.....K....	....	.........-	------....	....	....	....	....
*emm*1.24	....	....	....V.....	....	.........-	------....	....	....	....	....
*emm*1.25	....	....	....	....	....--...-	------....	....	....	....	....
*emm*1.26	....	....	....Y.....	....	.........-	------....	....	....	....	....
*emm*1.27	....	....	....	....	.R.......-	------....	....	....	....	....
*emm*1.28	....	....	....	..T.......	.........-	------....	....	....	....	....
*emm*1.29	....	....	....	....	....R....-	------....	....	....	....	....
*emm*1.30	....	....	....	..S.......	.........-	------....	....	....	....	....
*emm*1.31	....	....	....	...V......	.........-	------....	....	....	....	....
*emm*1.32	....	....	....T.....	....	..G......-	------....	....	....	....	....
*emm*1.33	....	....	....	....	.....N...-	------....	....	....	....	....

Figure 1. Alignment of inferred N-terminal amino acid sequences of 33 alleles of *emm1*. The region shown represents amino acids 27 through 110 (GenBank accession number X07860). Six of the *emm1* alleles were identified in this study, several were described previously (1,5,12), and others were from ongoing analysis of *emm1* in M1 strains from global sources. Amino acid residues identical to those encoded by *emm1.0* are represented by periods.

The most common M type identified was M1 (n = 30 isolates) (Table). Five *emm1* alleles were identified in the 30 M1 isolates, including four (*emm1.13, emm1.18, emm1.19,* and *emm1.24*) not previously described (Figure 1). Twenty-three Texas isolates had allele *emm1.0*, the most common *emm1* allele in M1 isolates globally (5). Three isolates had allele *emm1.19*, two organisms had allele *emm1.24*, and one isolate each had allele *emm1.13* and *emm1.18* (Table). Compared with the *emm1.0* allele encoding variant M1.0, each of these alleles is characterized by single nucleotide changes resulting in single amino acid substitutions in the resulting M1 protein (Figure 1). The additional 70 isolates were a heterogeneous array of M types, including M3, M4, M5, M6, M12, M18, and many others. A more detailed description of the bacteriologic features will be presented elsewhere.

Analysis of *speA* Encoding Pyrogenic Exotoxin A

Because M1 isolates were a prominent cause of the invasive disease episodes, we sought to determine the extent of genotypic heterogeneity among the 30 M1 GAS isolates. First, polymerase chain reaction (PCR) was used to test whether the organisms possessed the *speA* gene encoding pyrogenic exotoxin A (scarlet fever toxin) (3,13). Most contemporary M1 isolates cultured from patients with invasive disease have this gene (1,3-5), but some lack it because *speA* is bacteriophage encoded (13). Possession of *speA* is therefore a variable trait among M1 organisms. All 30 M1 isolates had the *speA* gene, and sequence analysis of 11 random isolates found that all had allele *speA2* (14). Previous study of the *speA* gene in several hundred contemporary M1 strains showed that all organisms had the *speA2* allele (1,14).

Sequence Analysis of *sic*

Recent molecular genetic studies have documented that *sic* is a uniquely hypervariable gene among M1 GAS strains (7,8). Our *sic* database consists of 252 distinct alleles identified by sequence analysis of ~1,200 M1 isolates from worldwide sources and cultured from patients with a large array of GAS diseases, including pharyngitis and invasive episodes (7;8; unpub. data). *sic* allelic variation has not been identified

Table. Characteristics of serotype M1 Group A *Streptococcus* isolates analyzed

MGAS no.[a]	TDH no.[b]	*sic* allele	*emm1* allele	DR[c] PCR[d] (bp)	DR sequence type	*speA* PCR[e]	IS*1548* type
6151	BE8-776	1.01	1.0	372	4.0	pos	1.0
6168	BE-98-743	1.01	1.0	306	3.0	pos	1.0
6184	BE8-873	1.01	1.0	306	NS[f]	pos	1.0
6199	BE8-917	1.01	1.19	306	NS	pos	1.0
6262	BE8-1085	1.01	1.19	306	NS	pos	1.0
6264	BE8-1087	1.01	1.19	306	3.0	pos	1.0
6181	BE-98-764	1.02	1.0	240	NS	pos	1.0
6293	BE8-1339	1.02	1.0	306	NS	pos	1.0
6294	BE8-1340	1.02	1.0	306	NS	pos	1.4
6140	BE8-629	1.13	1.0	240	NS	pos	1.0
6200	BE8-918	1.13	1.0	240	NS	pos	1.0
6201	BE8-919	1.13	1.0	240	NS	pos	1.0
6281	BE8-1149	1.13	1.24	306	NS	pos	1.3
6137	BE8-563	1.32	1.0	306	3.0	pos	1.0
6148	BE8-773	1.32	1.0	306	NS	pos	1.0
6249	BE8-929	1.32	1.0	306	NS	pos	1.0
6172	BE-98-751	1.34	1.0	306	NS	pos	1.0
5997	BE8-191	1.36	1.0	240	NS	pos	1.0
6135	BE8-548	1.36	1.0	240	2.2	pos	1.0
6254	BE8-1021	1.36	1.24	306	NS	pos	1.0
6189	BE8-88	1.66	1.13	306	NS	pos	1.0
5999	BE8-208	1.99	1.0	306	3.0	pos	1.0
6003	BE8-322	1.100	1.0	240	NS	pos	1.0
6251	BE8-1000	1.100	1.0	240	2.1	pos	1.0
6006	BE8-369	1.101	1.0	306	3.0	pos	1.0
6138	BE8-566	1.118	1.0	240	2.2	pos	1.0
6150	BE8-775	1.119	1.0	306	3.0	pos	1.0
6154	BE8-792	1.120	1.18	240	2.1	pos	1.0
6272	BE8-1111	1.179	1.0	306	NS	pos	1.0
6299	BE8-1380	1.180	1.0	240	2.0	pos	1.0
2221	NA	1.01	1.0	306	NS	pos	1.0
5305	NA	1.01	1.0	306	3.0	pos	1.0
5809	NA	1.01	1.0	305	3.01	pos	1.0
2139	NA	1.02	1.0	306	3.0	pos	1.0
2350	NA	1.09	1.0	306	3.0	pos	1.0
1272	NA	1.35	1.0	306	NS	pos	1.5
5297	NA	1.121	1.0	240	2.0	pos	1.0
279	NA	1.08	1.3	570	7.0	neg	1.6
1632	NA	1.08	1.3	570	7.0	neg	1.6
1653	NA	1.19	1.3	570	7.0	neg	1.6
326	NA	1.20	1.3	570	7.0	neg	1.6
570	NA	1.21	1.3	570	7.0	neg	1.8
1642	NA	1.24	1.3	504	6.1	neg	1.6
6708[g]	NA	1.225	1.6	504	6.0	neg	1.7

[a]MGAS, Musser group A *Streptococcus* strain number. All isolates had no known direct epidemiologic connection except MGAS 6199, 6264, and 6272 (associated household cases); MGAS 6140, 6200, and 6201 (blood and cerebrospinal fluid cultures of same patient); and MGAS 6293 and 6294 (mother-neonate paired isolates).
[b]TDH, Texas Department of Health strain number; NA, not applicable (control isolate).
[c]DR, direct repeat.
[d]PCR, polymerase chain reaction.
[e]pos, PCR-positive for *speA*; neg, PCR-negative for *speA*. The *speA* gene in MGAS 1272, 6135, 6137, 6138, 6150, 6151, 6154, 6168, 6251, 6264, 6272, and 6299 was sequenced and identified as allele *speA2*.
[f]NS, not sequenced.
[g]MGAS 6708 is also known as SF370. The genome of this organism is being sequenced at the University of Oklahoma.

during in vitro laboratory passage, nor has variation been detected among strains that are epidemiologically associated (8). These molecular features suggest that automated sequencing of *sic* may be a convenient method for identifying M1 genetic subtypes and inferring epidemiologic relationships in potential outbreaks.To test this idea, we sequenced the *sic* gene in the 30 M1 isolates and identified 15 *sic* alleles that differed from one another by at least one nucleotide (Figure 2). Seven of the 15 alleles were not found among the ~1,200 M1 isolates previously characterized for *sic* variation. Eight new nucleotide substitutions were identified in eight codons, and one codon had a new dinucleotide change; these changes would result in nine amino acid substitutions in the expressed Sic proteins. As observed in earlier analyses (7,8), the amino-terminal half of the Sic protein had many insertions and deletions, all in frame (Figure 2).

RFLP Analysis with Insertion Sequences IS*1548* and IS*1562*

IS*1548,* a recently described insertion sequence, has been reported to be polymorphic in copy number and location in the chromosome of group A and group B streptococci (9). IS*1562* is an insertion sequence located in the Mga regulon between the *sic* gene and *scpA* gene encoding C5a peptidase in some GAS (10). Relatively few GAS strains have been analyzed by RFLP profiling with these elements, and their ability to differentiate among isolates expressing the same M type has not been assessed. Since insertion sequence profiling has helped elucidate transmission dynamics and evolutionary relationships of *Mycobacterium tuberculosis* (15), *Bordetella pertussis* (16), *Streptococcus pneumoniae* (17), *Escherichia coli* (18), and *Salmonella* Enteritidis (19), we tested the hypothesis that IS*1548* or IS*1562* subtyping would provide additional epidemiologically informative data regarding genetic diversity among M1 isolates.

		SRR	SRR (II)	SRR (II)	SRR (II)	SRR (II)	SRR (II)	SRR (IV)	SRR (IV)	SRR	SRR (II)	SRR (II)	SRR (II)	SRR (II)	SRR	R1	R2	R2	R3	R3								
		113	141	141	141	142	145	220	220	243	261	261	268	271	291	403	474	479	492	506	646	650	659	691	691	691/692	721	806
MGAS strain	*sic* allele	CGC>CAC R - H	15 bp insertion	15 bp deletion	45 bp deletion	CCT> TCT P - S	GAA>GGA E - G	CAA>AAA Q - K	15 bp deletion	48 bp deletion	GAT>GAA D - E	12 bp deletion	GAA>AAA E - K	GAT>TAT D - Y	48 bp insertion	ACT>GCT T - A	GTA>GTG V - V	ATT>ACT I - T	87 bp insertion	GAA>GGA E - G	9 bp insertion	GAA>GGA E - G	TTT> TCT F - S	GCC>ACC A - T	GCC>TCC A - S	GCC>ATC A - I	CCA>ACA P - T	AAC>ACC N - T
5997	1.36							X									X	X		X	X	X	X	X			X	
5999	1.99							X							-	X	X	X		X	X	X	X		X			
6003	1.100					X	X	X									X	X		X		X	X	X			X	X
6006	1.101	X						X								X	X	X		X	X	X	X		X		X	
6137	1.32			X				X									X	X		X	X	X	X	X			X	
6138	1.118		X							X							X	X	X	X	X	X	X	X			X	
6150	1.119		X					X							X		X	X		X	X	X	X	X			X	
6151	1.01		X					X									X	X		X	X	X	X	X			X	
6154	1.120							X									X	X	XX	X	X	X	X	X			X	
6172	1.34		X					X				X					X	X		X		X	X	X			X	
6181	1.02		X					X									X	X		X		X	X	X			X	
6189	1.68		X					X			X						X	X		X	X	X	X	X			X	
6200	1.13							X									X	X		X		X	X	X			X	
6272	1.179		X					X					X	X		X	X	X	X	X		X	X	X			X	
6299	1.180				X				X								X	X		X		X	X			X	X	

Figure 2. Variation in the *sic* gene and Sic protein identified in M1 group A *Streptococcus* isolates characterized in the study. The figure is a compilation of variations found in the 15 distinct *sic* alleles in the sample. The numbers at the top of the figure refer to the nucleotide sequence position of a *sic* allele described in reference 6. Single-letter amino acid abbreviations are used. SRR, amino-terminal short repeat region; Roman numeral, short repeats I-V which recur in SRR; R2 and R3, tandem repeats; MGAS strain, Musser Group A*Streptococcus* strain number; X, presence of polymorphism.

To determine whether the IS*1548* element was present in M1 organisms in our sample, PCR was performed on genomic DNA from 10 random isolates by using the oligonucleotides (forward) 5'-TGCCGTTCATCAACTGATTTCAGTGG-3' and (reverse 5'-CGACGATAACTGAGGTCTTTTTT AGGAAAT-3'(9). A PCR product of the anticipated size of ~1 kb was obtained from all organisms, a result indicating that the isolates had this element or a close relative. The PCR-amplified fragment was subsequently used as a probe for RFLP analysis by Southern blotting after *Eco*NI digestion and electrophoretic separation of chromosomal DNA fragments. The data were analyzed with a Bioimage Analyzer system interfaced with a Sun Sparcstation. Four M1 isolates had the same 6-band IS*1548* RFLP pattern, which was distinct from the 3-band pattern obtained from three random serotype M3 isolates (Figure 3A). Twenty-eight of the 30 M1 isolates studied had the same IS*1548* pattern (Figure 3B and data not shown). The IS*1548* RFLP patterns of the two other isolates were single-band variants of the common M1 pattern, both characterized by the addition of one hybridizing band (Figure 3B). One of the isolates (MGAS 6294) with a variant IS*1548* pattern was recovered from the blood of a neonate born to a woman with GAS sepsis. The isolate (MGAS 6293) from the blood of the infected mother had the common IS*1548* pattern.

To identify other IS*1548* RFLP patterns in M1 GAS organisms, we analyzed 14 non-Texas control isolates. These 14 M1 isolates were selected for analysis because they have been well characterized by several molecular techniques (5). The isolates also have many different *sic* alleles and include representatives of two major genetic subclones of M1 organisms (5). IS*1548* profiling of this group identified the common six-band pattern and also found five organisms with a distinct subtype with four bands (Figure 3C). All organisms with this profile were *speA*-negative. Interestingly, MGAS6708 (SF370), the M1 strain whose genome is being sequenced (20), had a unique five-band IS*1548* fingerprint

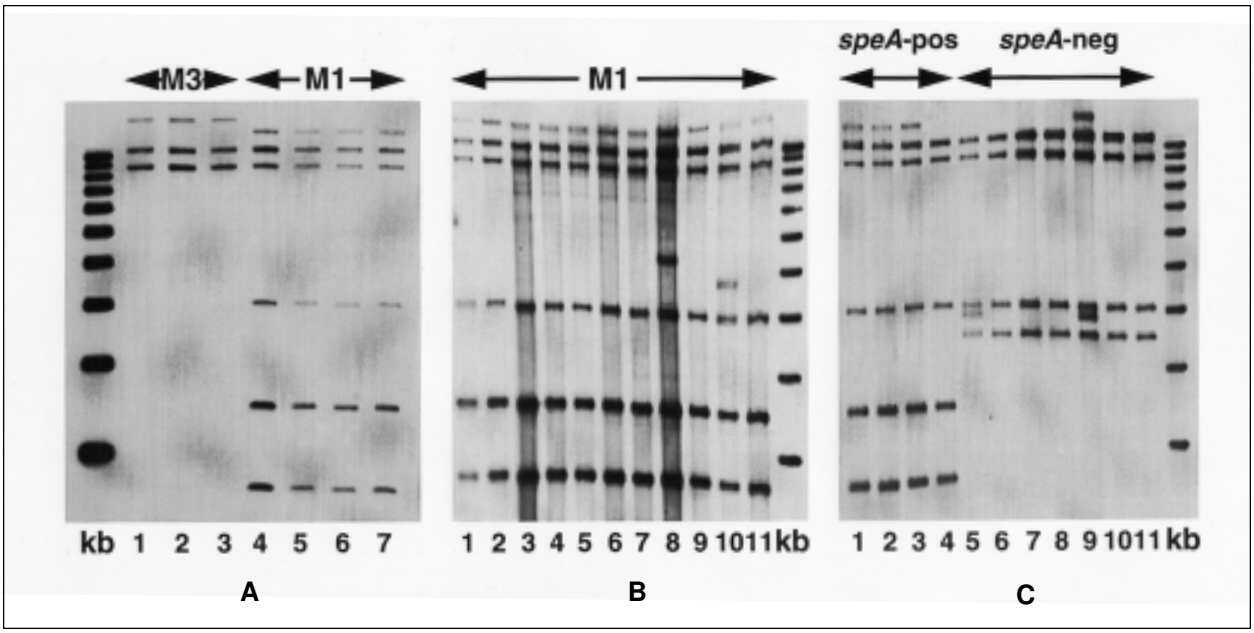

Figure 3. Representative IS*1548* RFLP fingerprint patterns of M1 isolates. Panel A is a lane map showing results from analysis of three serotype M3 control isolates and four M1 isolates with different *sic* alleles. Lane 1, MGAS5892; lane 2, MGAS6004; lane 3, MGAS6005; lane 4, MGAS5997; lane 5, MGAS5999; lane 6, MGAS6003; lane 7, MGAS6006. kb, 1-kb DNA ladder. Panel B is a lane map showing results from analysis of eleven M1 isolates with eight different *sic* alleles. Lane 1, MGAS6201; lane 2, MGAS6249; lane 3, MGAS6251; lane 4, MGAS6254; lane 5, MGAS6262; lane 6, MGAS6264; lane 7, MGAS6272; lane 8, MGAS6281; lane 9, MGAS6293; lane 10, MGAS6294; lane 11, MGAS6299. kb, 1-kb DNA ladder. Panel C is a lane map showing results from analysis of four *speA*-positive and seven *speA*-negative M1 isolates. Lane 1, MGAS2350, lane 2, MGAS2221, lane 3, MGAS2139, lane 4, MGAS1272, lane 5, MGAS6708, lane 6, MGAS1653, lane 7, MGAS1642, lane 8, MGAS1632, lane 9, MGAS570, lane 10, MGAS326, lane 11, MGAS279. kb, 1-kb DNA ladder.

(Figure 3C). The IS*1548* profile for this strain was very similar to the four-copy pattern characteristic of most of the *speA* negative organisms.

We next used PCR to determine whether IS*1562* was present in the 30 M1 organisms from Texas and in 11 of the 14 non-Texas isolates by using oligonucleotide primers 3244 and 3267, as described by Berge et al. (10). A PCR product of the expected size of ~1 kb was obtained from all isolates. The ~1-kb fragment was used to reprobe the nylon membranes used for IS*1548* RFLP analysis. The results showed that all M1 isolates tested had the identical or closely similar RFLP characterized by one copy of IS*1562* (data not shown).

PCR and Sequence Analysis of a Polymorphic Direct Repeat (DR) Chromosomal Region

Several years ago Groenen et al. (21) characterized an unusual region of the *M. tuberculosis* chromosome that contains up to approximately 40 copies of a 36-bp DR sequence interspersed with unique-sequence spacer regions 35 bp to 41 bp in length. Subsequent analysis of this DR region in hundreds of *M. tuberculosis* isolates by a method referred to as spacer oligotyping (spoligotyping) has identified large numbers of distinct subtypes of this pathogen (22), indicating that the DR region is highly polymorphic, even among isolates closely related in overall chromosomal character (23). We examined the M1 GAS genome database maintained by the University of Oklahoma Advanced Center for Genome Technology and identified a region of the GAS chromosome located on contig 208 (database as of February 22, 1999) that consists of seven DR elements separated by six unique 30-bp spacer regions. This area of the M1 chromosome is referred to as a DR region on the basis of its shared structural features with the *M. tuberculosis* DR region.

To test the hypothesis that the DR region is polymorphic among M1 GAS isolates, we analyzed the 14 control isolates by PCR with primers that flank this region (DR003, 5'-GGGCTTTTCAAGACTGAAGTCTAGCTG-3' and DR004, 5'-TCCGACTGCTGGTATTAACCCTC TT-3'). Four sizes of PCR products were identified (data not shown). Six of seven isolates previously identified as RFLP type 1a (*speA*-positive, containing allele *emm1.0*) had an apparently identical size PCR product of ~300 bp. A PCR product of ~240 bp was identified in the remaining isolate. Two sizes of PCR products (~500 bp and ~570 bp) were also identified in the six organisms with RFLP type 1k (*speA*-negative, allele *emm1.3*). Hence, the PCR results indicated that size variation was present in the GAS DR region in M1 organisms and showed that isolates of the RFLP types 1a and 1k categories did not share PCR fragment sizes.

To examine nucleotide variation in this chromosomal region, we sequenced the PCR products obtained from 12 of these control M1 isolates, including 5 with the ~240-bp or ~300-bp PCR product and 7 organisms with either the ~500-bp or ~570-bp PCR product. The one organism with the ~240-bp PCR product, characterized by two identical DR elements and two nonidentical spacer sequences, is arbitrarily designated DR type 2.0 (Figure 4). Three of the four organisms with the ~300-bp PCR product had identical DR-region sequences defined by the presence of three identical DR elements and three nonidentical spacer sequences (Figure 4B). This molecular arrangement was designated DR type 3.0 (Figure 4C). The DR element of the fourth isolate differed from the other three by the absence of 1 base in the second spacer region and is designated DR type 3.01 (Figure 4C). Consistent with the difference in PCR fragment size, the sequences of the DR region in the seven other organisms were distinct from the DR type 3.0 sequence. Five of these seven isolates had an identical DR-region sequence that was characterized by seven spacer regions (designated DR type 7.0). Two organisms lacked one of the spacer regions present in the DR type 7.0 strains; these molecular variants were designated DR types 6.0 and 6.1 (Figure 4C).

We next analyzed the 30 M1 Texas isolates by PCR of the DR region and obtained three PCR fragment sizes: products of ~240 bp (n = 11 isolates), ~300 bp (n = 18 isolates), and ~370 bp (n = 1 isolate). We sequenced the PCR products from 12 organisms selected to represent an array of DR PCR fragment sizes and *emm* and *sic* alleles. Two additional sequences (designated DR types 2.1 and 2.2) were identified among the five isolates with the DR region PCR fragment size of ~240 bp. All six isolates with the ~300-bp PCR product had the identical sequence (DR

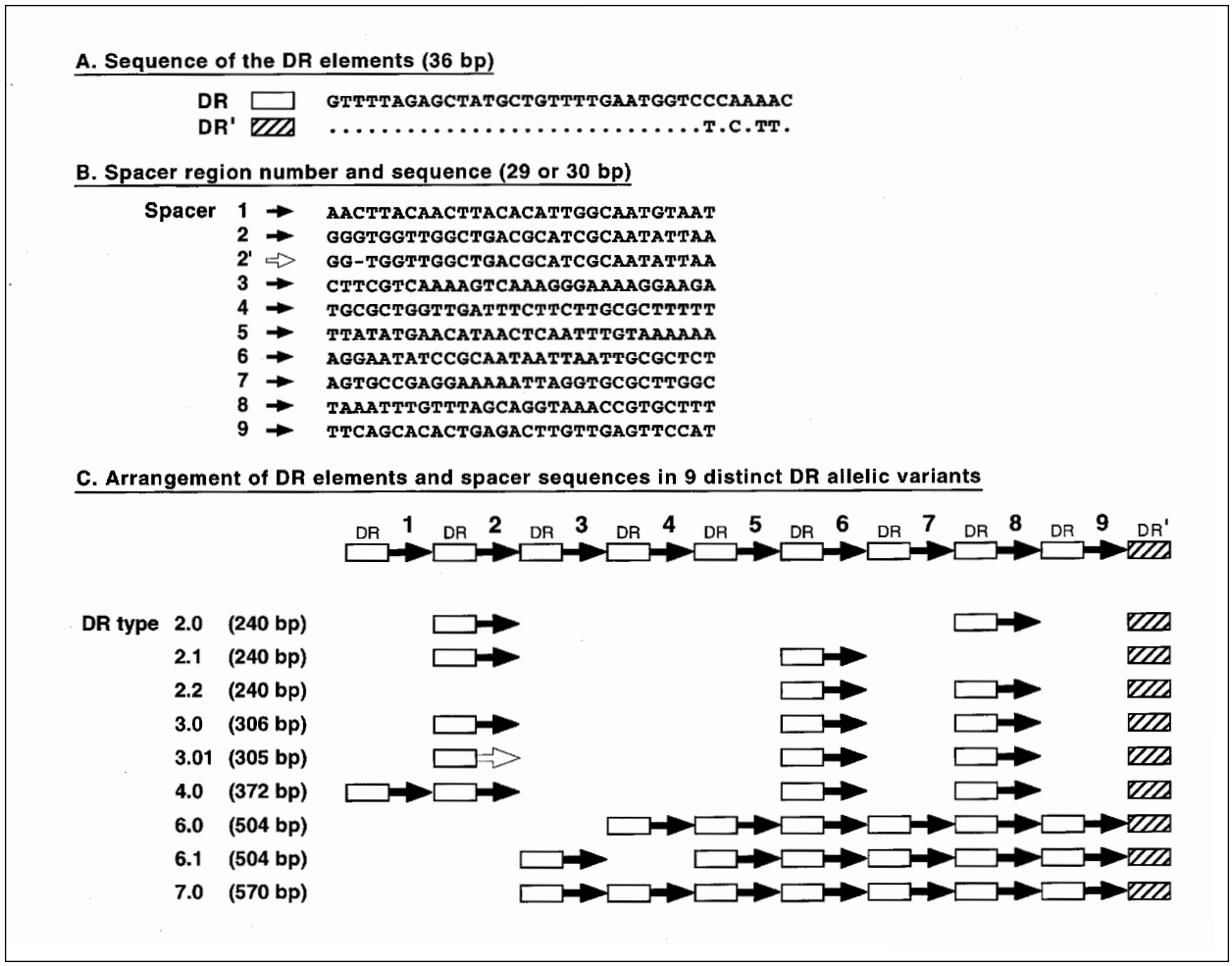

Figure 4. Polymorphism identified in the direct repeat (DR) region of serotype M1 group A *Streptococcus*. The data were generated by automated DNA sequencing of polymerase chain reaction products obtained with the oligonucleotide primers DR003 and DR004 described in the text. (A) The 36-bp sequences of the two related DR and DR' elements. Multiple copies of the DR element present in different M1 isolates all had the identical sequence. (B) The 29-bp or 30-bp sequences of the 10 distinct spacer regions identified in the analysis. (C) Arrangement of the DR elements and spacer sequences in nine distinct DR allelic variants. The DR types were given arbitrary designations based in part on the number of DR elements present. Open or cross-hatched rectangles represent copies of the DR or DR' elements; arrows represent copies of the spacer region sequences connecting the DR elements. The numbers above the spacer region sequences refer to the spacers designated in part B of the figure.

type 3.0). The one isolate with the ~370-bp PCR product had a unique sequence (DR type 4.0) with four spacer regions (Figure 4). The results showed that the DR region had more molecular variation than *emm*. However, the level of allelic variation in *sic* exceeded that found in either *emm* or the DR region.

Conclusions

Our data underscore the importance of molecular typing techniques in rapidly providing information about the epidemiology of GAS infections (24). The *emm* sequence data indicated that a heterogeneous array of GAS M types was present in the sample of 100 GAS isolates; thus, we could rapidly rule out the notion that the invasive cases had been caused by one or a few distinct GAS strains. Moreover, molecular analysis of several other polymorphic loci, including automated DNA sequencing of *sic* and a chromosomal region with multiple DR sequences, showed that M1 organisms, the most

abundant serotype in the sample, had substantial levels of genetic diversity. Of the molecular techniques used in this analysis, sequencing the *sic* gene was the most effective for differentiating among M1 isolates because it identified the most variants. RFLP-based typing with IS*1548* and IS*1562* failed to provide extensive, or even adequate, resolving power among the M1 organisms for epidemiologic purposes. Moreover, the variation in the IS*1548* RFLP profile we detected in two isolates (MGAS 6293 and MGAS 6294) from a woman with puerperal sepsis and the blood of her newborn child suggests that IS*1548* can be mobile in host-pathogen interactions. Instability in insertion sequence profiles has also been reported for IS*6110*, an element commonly used for molecular subtyping of *M. tuberculosis* (25).

Although sequence analysis of *emm* and the DR region provided some useful molecular subtyping data for M1 strains, the level of polymorphism at these loci was less than in *sic*. A rapid PCR-based subtyping system to index polymorphism in the DR region could be formulated for M1 GAS that would be similar to the method available for *M. tuberculosis.* However, this approach would be less useful for M1 GAS than *M. tuberculosis* because in the latter organism 43 distinct spacer regions have been described. Hence, the number of polymorphic markers is considerably greater than in M1 GAS, in which thus far only 13 spacer regions have been found (unpub. data).

Our work, recently reported results (7,8), and unpublished data obtained from ongoing analysis of *sic* polymorphism in large samples obtained from population-based studies demonstrate four emerging themes in the molecular epidemiology and evolutionary biology of M1 organisms. First, several *sic* variants are dispersed over broad geographic areas; some have achieved intercontinental distribution. For example, M1 strains with the *sic1.01* allele have been identified in 14 countries. This allele might be widely disseminated because it is the ancestral condition in M1 organisms or otherwise has had a long-standing association with the M1 serotype. Another plausible hypothesis to explain its widespread dissemination is that expression of Sic1.01 protein bestows greater fitness than do other Sic variants. A third possibility is that the Sic1.01 variant marks an M1 subclone with an unusual propensity to survive and spread. In this regard, we note that virtually all isolates with the *sic1.01* allele are *speA*-positive. GAS isolates with the *speA* gene are statistically overrepresented among organisms recovered from children with pharyngitis who have not been cured by oral antibiotic therapy (26). Bacterial survival despite appropriate antibiotic therapy would likely enhance spread of the organism to new hosts and, hence, assist widespread dispersal. We also note that *speA*-positive M1 isolates are internalized efficiently by human respiratory tract epithelial cells grown in culture (27,28), a process that could provide access to a protective niche that enhances survival capability.

A second important theme is that many *sic* alleles are confined to local geographic areas (e.g., individual countries or communities). For example, seven of the *sic* alleles identified in this study were unique to the Texas M1 isolates. Several unique *sic* alleles also were found among organisms cultured from patients in Mexico (7) and the former East Germany (8). Because many *sic* alleles can be readily linked with one another by a single molecular event such as a nucleotide substitution or one insertion or deletion, some of the variants likely arise rapidly in local areas. Their absence in other regions is explained by lack of sufficient elapsed time required for widespread dispersal. Recent data obtained from study of M1 isolates recovered from population-based surveys in Finland (29), Ontario, Canada (30), and Atlanta, Georgia (31) strongly support this explanation (unpub. data).

The third theme is the remarkable polymorphism in the *sic* gene. Stockbauer et al. (8) reported that virtually all changes in the *sic* gene result in structural changes in the Sic protein and concluded that positive Darwinian selection is mediating Sic variation. Our study confirmed these observations. For example, all 10 new nucleotide changes identified would result in amino acid substitutions in Sic, and all insertions and deletions were in frame. Moreover, most of the amino acid changes were radical replacements, that is, those producing charge changes or polar-nonpolar substitutions. These types of amino acid replacements commonly result in functional differences in the resulting proteins and are a hallmark of positive selection (32).

Last, accumulating data suggest the existence of two genetically divergent M1 subpopulations, which can be thought of as two

evolutionarily distinct lineages. Our study found that organisms with the *speA* gene and chromosomal PFGE type 1a (5) have shorter DR-region sequences and an IS*1548* profile characterized by six hybridizing bands. In contrast, organisms that are *speA*-negative usually have PFGE type 1k (5), longer DR sequences, and an IS*1548* fingerprint with four bands. In addition, we will show elsewhere that the two M1 lineages each have distinct families of *sic* alleles. Together, the data indicate that sufficient time has elapsed since a shared common ancestor for members of the two lineages to have diverged at many chromosomal loci. The data also indicate that transduction of the *speA2* allele between members of the two lineages is apparently rare in natural populations of GAS (5,14). As more comparative analyses are conducted, additional genetic differences will probably be identified between isolates of the two lineages.

In summary, automated sequence analysis of *sic* and a region of the chromosome with DR sequences permitted rapid and unambiguous differentiation among serotype M1 isolates during a period of a significant increase in the number of invasive disease cases. Genetic analysis of these polymorphic markers permitted us to rapidly rule out the idea that a single unusually virulent strain of M1 GAS was responsible. The subtyping methods described in this work will assist other outbreak investigations and studies designed to understand the molecular basis of temporal variation in disease frequency and severity of infections caused by M1 GAS isolates.

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Dr. Hoe is a research associate in the Institute for the Study of Human Bacterial Pathogenesis, Baylor College of Medicine. Her main interests are in the areas of molecular epidemiology and bacterial pathogenesis.

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Gnathostomosis, an Emerging Foodborne Zoonotic Disease in Acapulco, Mexico

Norma Rojas-Molina,* Sigifredo Pedraza-Sanchez,† Balfre Torres-Bibiano,* Hector Meza-Martinez,* and Alejandro Escobar-Gutierrez†

*Hospital Regional "Vicente Guerrero," Instituto Mexicano del Seguro Social, Acapulco, Guerrero, México; and †Secretaría de Salud, México, DF, México

Between 1993 and 1997, 98 gnathostomosis cases were clinically identified in Acapulco, Mexico. Intermittent cutaneous migratory swellings were the commonest manifestation. Larvae were identified in 26 cases, while in 72, final diagnosis was made on the basis of epidemiologic data, food habits, and positive enzyme-linked immunosorbent assay and Western blot results.

Gnathostomosis is a foodborne zoonotic disease caused by several species of nematode *Gnathostoma*. The life cycle of this parasite is as follows: Adult parasites of *G. spinigerum* are found in the stomach of mammals (e.g., dogs and cats). feces containing ova reach the water (i.e., when domestic parasitized animals live at the shore of a lagoon). Free-swimming first-stage larvae are formed, which are ingested by the minute copepod crustacean cyclops, and become second-stage larvae. Freshwater fish eating cyclops are the second intermediate host. Larvae develop to the third state (L3) in the fish muscles. Consumption of this fish by cats, dogs, or other mammals results in development of adults in the gut, closing the cycle. Humans acquire the infection by consuming raw or undercooked freshwater fish. When a larva is ingested by a human host, no further development occurs, but the larva migrates through subcutaneous tissue and internal organs where it produces migratory swelling in the skin and other symptoms depending on the site or organ affected. In most cases, symptoms are not serious; however, if the parasite migrates to vital organs of the host, it can cause severe illness or even death (1,2).

Address for correspondence: Alejandro Escobar-Gutierrez, Departamento de Investigaciones Inmunológicas, Instituto Nacional de Diagnóstico y Referencia Epidemiológicos, SSA, Carpio 470, México, DF, 11340, México; fax 525-341-3264; e-mail: indre@cenids.ssa.gob.mx.

With the highest prevalence in Southeast Asia, gnathostomosis is now an emerging public health problem in Peru, Ecuador and, since 1970, in Mexico (3). In the 1970s and 1980s a few cases were reported in towns around Presidente Miguel Aleman Dam and Papaloapan River basin in the Gulf of Mexico (4). The parasites may have spread when new dams built on rivers of the Pacific Ocean coast were unnoticeably seeded with infected fish. Until now, *G. spinigerum* has been the only species of *Gnathostoma* identified in Mexico (5).

Acapulco (estimated population 890,000), a resort on the southern Pacific coast, is the major Mexican city where gnathostomosis cases have been reported (5). Between December 1, 1993, and July 31, 1997, 98 patients with symptoms compatible with gnathostomosis were identified at the outpatient clinics of the Dermatology and Allergy Services, Hospital Regional "Vicente Guerrero", IMSS. Because the hospital gives medical attention to approximately 45% of the permanent residents of the city, the number of gnathostomosis cases identified can be considered representative prevalence in the local population.

A standardized questionnaire was administered to each patient, requesting demographic data, medical history, clinical features, age, occupation, years of residence at the city, and food habits. Baseline and diagnostic investigations were made after obtaining a written

consent. Gender and age distribution were as follows: 50 were male and 48 female and all but two (5 and 10 years) were 20 to 45 years of age (mean age 36 years). Blood samples were obtained for full blood and eosinophil counts. Serum total immunoglobulin E (IgE) levels were measured by a commercial enzyme-linked immunosorbent assay (ELISA) (Pharmacia Diagnostics, Uppsala, Sweden), and the results were expressed as international units (IU)/mL. Biopsies were taken from the progressing eruption and were examined for worms. Commonest symptoms were intermittent episodes of localized migratory skin swelling, with edema of variable size, slightly reddish, feverish at times, accompanied by stabbing pain, burning sensation and pruritus. Initial edema generally appeared on the abdomen. Recurring edema developed randomly, mainly in the upper and lower extremities, gluteus, thorax, and face. The duration of edema varied from 1 day to approximately 2 weeks. Only two patients reported visceral manifestations, mainly epigastrial pain and nausea, lasting approximately2 days. Medium value for blood eosinophils was 12%, and serum IgE was 302.6 Ul/mL (normal 10 to 180 Ul/mL). Although identification of larvae by a biopsy is recommended for definitive diagnosis, such identification was possible in only 16 cases, including three in which the worms were easily excised after their outward migration to the dermis as a consequence of treatment with albendazole as has been recommended (6).

To assess the effectiveness of serologic tests for diagnosis, a representative sample of patients were selected for antibody screening by ELISA (7) and Western blot confirmation (8). Antigens used were two batches of larval total extract. The first one was prepared with worms recovered from patient's biopsies or from muscles of freshwater fishes (*Dormilatum latrifons*) from laguna Tres Palos (a large freshwater lagoon 20 km southeast Acapulco). The second batch was obtained from *G. spinigerum* freeze-dried specimens donated by Dr. Wichit Rojekittkhum (Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). For ELISA, protein concentration of the antigen was adjusted to 10 g/ml, and sera with OD_{420} values ≥ the OD_{420} average obtained in control sera plus four standard deviations were considered positive. ELISA-positive results were found in four of five parasitologically confirmed cases and 19 of 22 biopsy-negative patients. Immunoblots with sera from ELISA-positive patients showed two strong bands (30 and 38 kDa) and two weak bands (35 and 43 kDa) not observed in sera from the control group. No differences between the two batches of antigens were detected by ELISA or by immunoblotting. However, by ELISA, higher OD_{420} values were seen when *G. spinigerum* lyophilized antigen was used. Lyophilization may make the antigen better for binding to the ELISA pates. No correlation was found with age, time of onset, or parasitologic treatment in patients with negative serologic results.

Although characteristic clinical manifestations could be very indicative of gnathostomosis, up to now confirmation through identification of larvae has been mandatory; nevertheless, larva identification is rare, and a combination of such factors as permanent residence or recent visit to a disease-endemic area, history of eating raw fish, exclusion of other diseases, and results of serologic tests can be taken into account in establishing a definitive diagnosis.

Inability to interrupt the parasite's life cycle and lack of effective medical treatment (2) make preventive measures critical in controlling the disease. Therefore, travelers to disease-endemic areas must be warned of the possibility of acquiring gnathostomosis and be instructed to avoid ingesting any form of raw fish. To protect the general population of disease-endemic areas, public campaigns should be implemented and encouraged against eating or selling raw or poorly cooked freshwater fish, especially in the form of sushimi or "ceviche" (a spicy lime-marinated fish salad of South American origin now very popular in Mexico).

Dr. Rojas-Molina is an allergologist at Service of Allergy, Hospital Regional "Vicente Guerrero," Instituto Mexicano del Seguro Social, Acapulco, Guerrero, México.

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Acute Hemorrhagic Conjunctivitis Due to Enterovirus 70 in India

R.S. Maitreyi, L. Dar, A. Muthukumar, M. Vajpayee, I. Xess, R.B. Vajpayee, P. Seth, and S. Broor
All India Institute of Medical Sciences, New Delhi, India

An outbreak of acute hemorrhagic conjunctivitis occurred in Delhi, India, during August and September 1996. The etiologic agent was confirmed as enterovirus type 70 by a modified centrifugation-enhanced culture method followed by immunofluorescence and neutralization tests. After nearly a decade, this virus is reemerging as a cause of acute hemorrhagic conjunctivitis in India.

Acute conjunctivitis can be caused by viruses including enterovirus 70 (EV-70), coxsackievirus A24, and epidemic adenoviruses. These viruses may also lead to acute hemorrhagic conjunctivitis (AHC), characterized by photophobia, watering, and foreign body sensation, eyelid edema, conjunctival hemorrhages, and superficial punctate keratitis. The disease is self-limiting.

In 1996, an outbreak of AHC occurred in Delhi, north India, during the rainy season (August and September). We conducted a study to identify the etiologic agent by viral culture, immunofluorescence and neutralization tests, and polymerase chain reaction (PCR).

The Study

We enrolled 13 patients with clinically diagnosed bilateral AHC who attended the outpatient clinic of Rajendra Prasad Centre for Ophthalmic Sciences during the outbreak. At the initial visit, all patients had a complete ophthalmic examination, including slit-lamp biomicroscopy. Conjunctival swabs were taken from the right eye of each patient. These swabs were collected in 2 ml of Hanks balanced salt solution with antibiotics and were transported on wet ice to the virology laboratory for processing.

All samples were vortexed thoroughly and treated with antibiotics (1,000 IU/ml penicillin and 1,000 μg/ml streptomycin). Specimens were clarified by centrifugation at 700 x g for 10 minutes at 4°C. Supernatant fluid was separated and used for inoculation in cell culture. The samples were stored at -70°C until they were processed.

For virus culture, Hep-2–cell monolayers were grown in 24-well plates, containing 12-mm cover slips (Bellco Glass Inc., New Jersey, USA). Each plate was seeded with 1 ml of Hep-2–cell suspension, incubated at 37°C in a 5% CO_2 atmosphere, and used for a modification of the centrifugation-enhanced viral culture technique (1).

Cover-slip monolayers were washed twice with phosphate-buffered saline (PBS) before specimen inoculation. Two hundred microliters of each specimen was inoculated in parallel in two 24-well plates containing Hep-2–cell monolayers grown onto 12-mm cover slips. Plates were centrifuged at 700 x g for 60 minutes at room temperature. Inoculum was discarded and washed once with PBS; infected cells were re-fed with 1.0 ml of Eagle's minimum essential medium containing 2% fetal calf serum. The plates were reincubated at 37°C in a 5% CO_2 atmosphere. Cover slips from one plate were removed, washed twice with PBS, and fixed with chilled acetone at 48 hours postinoculation for immunofluorescence. The parallel 24-well plate cultures were observed for viral cytopathic effect, which included rounding and refractility of cells and destruction of the monolayer at 2 to 4 days; the resulting effect suggested enteroviral infection. Because a standardized reverse transcriptase-PCR test for enteroviral RNA was available at the All India Institute of Medical

Address for correspondence: Shobha Broor, Department of Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110029, India; fax: 91-11-686 2663; e-mail: broor@medinst.ernet.in.

Sciences, New Delhi, India, we screened the samples with this test.

Ten of the 13 clinical samples and the positive control showed the amplicon band, while the negative control did not (Figure). The PCR results suggested enteroviral infection, helped narrow the search for the etiologic agent, and provided a rapid preliminary diagnosis.

Viral antigen was detected by indirect immunofluorescence staining (on the fixed cover slips stored earlier) using specific antibodies to EV70, coxsackievirus A24, and adenoviruses (Chemicon International Inc., CA, USA).

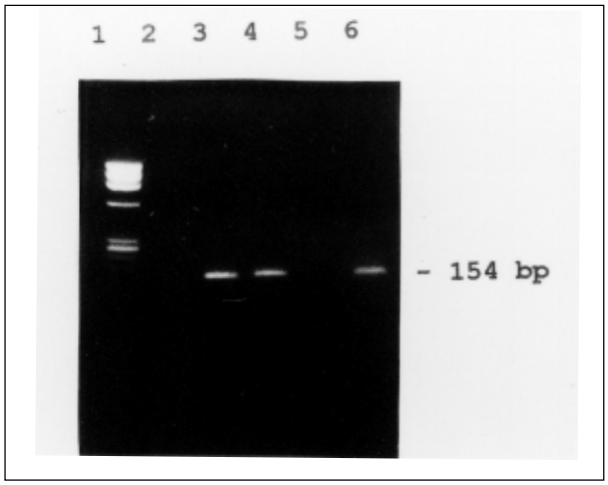

Figure: Reverse transcriptase-polymerase chain reaction (RT-PCR) for enteroviral RNA from acute hemorrhagic conjunctivitis cases. Lane 1: pX174 DNA/Hae III marker; lane 2: negative control; lane 3: positive control (enteroviral RNA); lanes 4-6: clinical samples.

For PCR, viral RNA was extracted by the guanidinium thiocyanate method (2) from 200 µl of clinical specimen. The RNA pellet was resuspended in 10 µl of diethyl pyrocarbonate treated water. A 5-µl volume was used for cDNA synthesis. The primers used for PCR amplification were selected from the highly conserved 5' noncoding region of enterovirus described by Rotbart et al. (3) and, using the same protocol, cDNA for PCR was synthesized by reverse transcription. Briefly, cDNA (10µ Cl) was amplified in a PCR mix volume of 50 µl, containing 100 ng of each primer, 250 µl of each dNTP, 1.5 mM $MgCl_2$, 2.5 units Taq polymerase, and 5 µl of 10X PCR buffer. The tubes were subjected to 30 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes, respectively, followed by a final extension for 7 minutes at 72°C. A 154-bp sized band of the PCR-amplified product was visualized under UV illumination on a 2% agarose gel after ethidium bromide staining.

Infected cover-slip monolayers were stained with 25 µl of monoclonal antibodies, incubated in a moist chamber at 37°C for 45 minutes, and washed twice with PBS for 10 minutes each. Fluorescein-labeled anti-mouse conjugate was added, and the cover slips were reincubated at 37°C for 45 minutes, followed by a PBS washing step, as described above. The cover slips were air-dried, mounted with glycerol buffer, and examined under fluorescence microscopy.

The samples showing a viral cytopathic effect were given a second passage, and if the effect was seen again, the 50% tissue infective dose of the virus isolate was calculated. A virus neutralization test was performed using 100 50% tissue infective doses of the isolate and virus-specific antiserum.

Findings

All 13 patients enrolled in the study (9 male and 4 female, ages 14 to 37 years) had bilateral ocular involvement and described redness and watering of the eyes, mild photophobia, and severe foreign body sensation. Ocular examination showed severe conjunctival congestion, interspersed subconjunctival hemorrhages, and superficial punctate epithelial keratitis. No neurologic manifestations (as reported in an earlier epidemic) were observed (4).

Cover-slip monolayers infected with a known EV-70 prototype (Kono)–like strain from a previous outbreak (5) and 10 of the 13 clinical specimens showed specific cytoplasmic fluorescence with monoclonal antibodies to EV-70. Infected monolayers tested with monoclonal antibodies to adenovirus and coxsackievirus A24 yielded negative results. Neutralization test results confirmed the identification, as all 10 clinical isolates were neutralized by EV-70–specific antisera. Also, the known EV-70 prototype (Kono)–like strain was neutralized by pooled convalescent-phase sera from the patients in this outbreak. PCR was positive for EV-70 in 11 of the 13 cases, including the 10 culture-confirmed cases.

Conclusions

An outbreak of AHC was first reported from Ghana in 1969 and was referred to as Apollo conjunctivitis (6). A new enterovirus (EV-70) was identified as the etiologic agent of AHC (7); subsequently it spread to other parts of Africa and Asia including India.

The first serologic evidence of EV-70 infection in India came from Bombay (western India) in 1971-72 (4), and the first isolation was reported from a single case during a small epidemic in southern India in 1975 (8). During an epidemic in north India in 1981 (also during the rainy season), two isolates of EV-70 prototype (Kono)–like strain were reported from Delhi (5), and antigen-positive cases were found by immunofluorescence in the city of Chandigarh (9). The last reported outbreak of EV-70 from this region (July to September 1986) was also confirmed only by demonstration of antigen in cell scrapings by immunofluorescence (10). AHC due to EV-70 appears to have reemerged in north India after nearly a decade. During the intervening period, a coxsackievirus A24 variant was circulating as a cause of AHC in this region of India (11). However, a prime-type EV-70 isolate was obtained from a case-patient during an outbreak in Pune, western India, nearly 1,200 km from Delhi, in 1991 (12).

In this outbreak of AHC, we achieved an unusually high isolation rate of EV-70 (10 of 13 cases) by using a modification of centrifugation-enhanced culture. The results of neutralization tests indicate that the strains circulating in this part of India continue to resemble the prototype strain (also reported during the 1981 epidemic). PCR was useful because of its rapidity and help in narrowing the search for an etiologic agent to enteroviruses. With the availability of PCR based on EV-70 specific primers (13), this highly sensitive centrifugation-enhanced technique is likely to be used increasingly in the future.

Dr. Maitreyi is a senior research fellow, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India. Her areas of expertise include tissue culture and molecular biology, and her research focuses on respiratory viruses and enteroviruses.

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Mycobacterium sp. as a Possible Cause of Hypersensitivity Pneumonitis in Machine Workers

Brian G. Shelton,* W. Dana Flanders,† and George K. Morris*
*PathCon Laboratories, Norcross, Georgia, USA; and
†Emory University, Atlanta, Georgia, USA

Hypersensitivity pneumonitis (HP) in workers exposed to metal removal fluids (MRFs) is increasing. This study supports the hypothesis that aerosolized mycobacteria colonizing the MRFs likely cause the disease. Three case studies of HP outbreaks among metal workers showed potentially high exposures to a rare and newly proposed *Mycobacterium* species. Retrospective review of samples submitted to our laboratory showed an association between presence of mycobacteria and HP.

Interest in occupational hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis is increasing (1,2). HP, a rare group of diseases characterized by recurrent dyspnea, cough, and systemic signs such as myalgia and fever, is caused by repeated exposure and subsequent sensitization to various antigens. Recognized antigens are often fungal or bacterial. The disease process is thought to involve lymphocyte-sensitization and cell-mediated immune response that ultimately results in alveolitis. Types of occupational HP include farmer's lung, bird fancier's lung, and mushroom worker's lung.

HP has recently been recognized among metal workers, and as evidenced at a recent meeting of the Automotive Manufacturers Association in Detroit (September 1997), controversy exists as to its possible cause(s). Researchers from the National Institute for Occupational Safety and Health recently reported that four of six outbreaks of HP in metal-working facilities yielded unusual flora (e.g., *Mycobacterium chelonae*) in contaminated MRFs (2,3). They discussed acid-fast bacteria, nontuberculous mycobacteria, gram-positive bacteria, and fungi as possible causes (3) but more recently concluded that the specific etiologic agent(s) for HP among workers exposed to (MRF) aerosol remain(s) unknown (2). Advances in metal removal fluid technology have led to the use of synthetic, semisynthetic, and soluble fluids, as opposed to traditional oil coolants. These water-based coolants are typically recycled and often become colonized by microorganisms. We present case reports and observations that further suggest mycobacteria may be a cause of occupational HP.

Address for correspondence: Brian G. Shelton, PathCon Laboratories, 270 Scientific Drive, Suite 3, Norcross, Georgia 30092, USA: fax: 770-446-0610; e-mail: bshelton@pathcon.com.

Case Report 1

We investigated a single case of HP in an employee who worked in an engine production facility in 1997. Other than contaminated fluids, no chemicals or substances known to cause HP were used at the facility. This 45-year-old male nonsmoker, in otherwise good health, worked in a wet machining area of the shop. His symptoms, which manifested themselves while he was at work, included cough, dyspnea, and hoarseness. One episode, which included malaise and fever of 102°F, led to hospitalization. In-hospital evaluation included a chest X-ray, which showed bilateral interstitial infiltrates, a high resolution computed tomography scan consistent with alveolitis, normal spirometry with a decreased diffusing capacity for carbon monoxide, and nonspecific transbronchial biopsy findings. A diagnosis of HP was made, and the patient was treated with oral glucocorticoids and relocated to an office environment at work. His symptoms gradually improved over several weeks, and the steroids were discontinued.

Mycobacteria in the *M. chelonae* complex were identified in high numbers in numerous bulk coolant samples (from nondetectable to $6.6x10^6$ CFU/ml) and counts from 56 to 2,256 CFU/m^3 in positive air samples around the colonized machines. Other organisms identified in the bulk samples included *Pseudomonas pseudoalcaligenes*, *P. alcaligenes*, and yeast with counts ranging from nondetectable to $1.7x10^6$ CFU/ml. The *Mycobacterium* isolates were identical to *M. immunogen* (4), a proposed rare and new species of rapidly growing mycobacteria with similarities to both *M. chelonae* and *M. abscessus*. The case-patient (Case 1) and an unaffected co-worker (Control 1) yielded a significantly elevated antibody immunoglobulin G (IgG) titer against this *Mycobacterium* and other organisms isolated from coolant samples at the facility. Five other unaffected workers (Controls 2-6) who worked in the same area as the case-patient were tested, as were 23 other controls comprising the reference population not known to have exposure to the facility. The standard deviation index used in the Figure was calculated by subtracting the mean IgG/ml level of the reference population from the patient's IgG/ml. This value was then divided by the standard deviation of the reference population to give the standard deviation index. For example, the levels were 5 or more in the case-patient, indicating that his levels were at least 5 standard deviations higher than the corresponding mean antibody levels in the reference population. The serum antigen-specific antibody levels were measured by enzyme-linked immunosorbent assay.

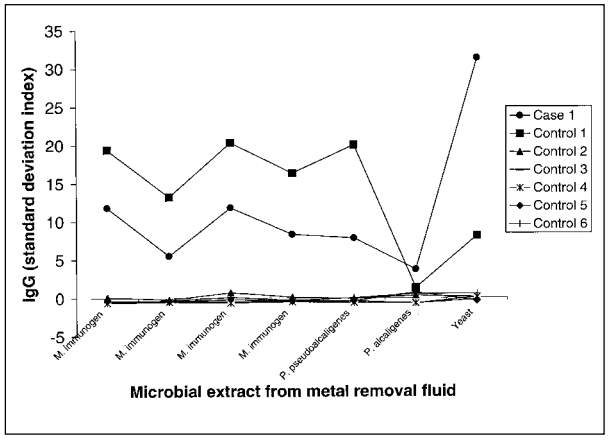

Figure. Immunoglobulin G (IgG) antibody titer vs. microbial extract from metal removal fluid.

Case Report 2

We were also involved in the investigation of HP at an engine manufacturing plant that had numerous machines for cutting and grinding metal. The facility employed approximately 700 machine workers. After HP was diagnosed by a treating pulmonologist in two middle-aged male machinists, the facility was inspected for known causes of HP. No causal exposures were identified other than exposure to cutting fluids, which was common to the two cases. Samples of machine fluid samples yielded *Mycobacterium* colony counts of 10^2 to 10^7 per ml, with mycobacteria being the most predominant organism in some fluids. Air samples near colonized machines yielded high counts of mycobacteria, some of which exceeded the upper limits of the sampler (>9,424 CFU/m^3 of air). Other organisms in air samples included *Corynebacterium*, *Klebsiella*-like organisms, *Bacillus*, *Rhodococcus*, *Hyalodendron*, *Cladosporium*, and nonsporulating fungi at levels of 24 CFU/m^3 to 1,319 CFU/m^3. No mycobacteria were detected immediately outside the facility. The mycobacteria detected were rapid growers identical to the newly proposed species *M. immunogen*. We evaluated a sister plant (in the same state) that had no known cases of HP; no mycobacteria were detected from samples of cutting fluids.

Case Report 3

We are currently investigating a cluster of five cases of physician-diagnosed HP at a large metal working factory in the midwestern part of the United States. Other than contaminated fluids, no chemicals or other substances known to cause HP are used at the facility. Samples of MRFs showed counts of mycobacteria ranging from nondetectable to >10^6 CFU/ml of sample. Again, the *Mycobacterium* species was identical to the newly proposed species, *M. immunogen*. Other organisms identified in bulk samples included *Bacillus*, *Pseudomonas*, *Cladosporium*, *Trichoderma*, and nonsporulating fungi at concentrations of <10 CFU/ml to 6,000 CFU/ml.

Conclusions

All the cases we report occurred in men exposed to metal removal fluids heavily colonized with mycobacteria. Moreover, we have documented high airborne concentrations of mycobacteria in the workplace. At least five

other clusters of HP in workers exposed to mycobacteria have been reported (2,3,5-7). In one outbreak of HP in metal workers (6,7), a sputum culture sample from one of the six patients in the outbreak yielded *M. chelonae*—a possibly rare event in a person without pulmonary infection attributed to that organism. By documenting further clusters of HP among workers exposed to MRF contaminated with mycobacteria, our findings provide an additional link suggesting a possible association between mycobacteria and HP.

It is possible that mycobacteria commonly contaminate MRFs even in the absence of HP. To assess this possibility, we identified all samples of MRF that had been submitted for culture to our laboratory since 1993 when we routinely started testing these samples for mycobacteria. Of the seven facilities from which we received samples as part of an investigation of known HP, six had mycobacteria. Of the eight facilities without known HP, only one had mycobacteria (odds ratio = 42, 95% confidence interval = 1.5-2194.47, p = 0.01 by Fisher's exact test). Thus, in facilities without HP, mycobacteria were not commonly found in MRFs. Even though they represent a review of existing records and not a prospectively designed study, these data suggest that mycobacterial contamination of MRFs may increase the risk for HP.

The hypothesis that mycobacterial contamination in cutting fluids is a cause of HP has high biologic plausibility supported by several observations: Cutting fluids and their contaminants can become easily aerosolized thus providing a high opportunity for inhalation exposure; mycobacteria have an acid-fast cell wall that is highly antigenic; mycobacterium adjuvants, often used by immunologists to elicit a stronger immunologic reaction, may lead to granuloma formation if used more than once; elevated IgG antibody levels directed at mycobacteria were found in patients with HP—the case-patient in case report 1 had elevated antibody titers to several antigens including mycobacteria in the MRFs (although not diagnostic of HP, this elevated titer documents exposure and immunologic response to mycobacteria in this patient); outbreaks of HP in hot tubs have been reported with mycobacteria (*M. avium*) as the suspect cause (8); and animal models used to study HP have been produced by using antigenic extracts from mycobacteria (9).

This hypothesis—mycobacteria as an antigen, or as an adjuvant that enhances reactions to other antigens found in the environment—is biologically plausible. Mycobacteria may be an important cause of HP in occupational settings or other settings in which exposure to contaminated aerosolized aqueous solutions is possible. These data and observations alone neither prove nor disprove a causal link. However, given the available data from the case studies presented here and elsewhere (2,3,5-7), the ability of *Mycobacterium* to proliferate to high levels in certain water-based MRFs, biologic plausibility, and the lack of use of other substances known to cause HP at these facilities, exposure to mycobacteria should be considered as a possible cause.

Dr. Shelton is vice president of PathCon Laboratories, a private laboratory specializing in the investigation of airborne environmental microorganisms. His research interests are in the epidemiology of environmental microorganisms in building water systems and indoor air. He has conducted numerous outbreak investigations and worked on the prevention and control of Legionnaires' disease. He was the first to use environmental concentrations of Legionella to predict disease risk.

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Evaluating Diagnosis and Treatment of Oral and Esophageal Candidiasis in Ugandan AIDS Patients

Maurizio Ravera, Alberto Reggiori, Anna Maria Agliata, and Roberto Pidoto Rocco
Regional Teaching Hospital, Hoima, Uganda

A randomized cross-over clinical and endoscopic evaluation of 85 Ugandan patients showed that esophageal candidiasis in AIDS patients with oral candidiasis could be managed without endoscopy and biopsies. Oral lesions, especially when accompanied by esophageal symptoms, were sufficient for diagnosis. Miconazole was more effective than nystatin in treating esophageal candidiasis and could be a valid alternative to more expensive azolic drugs in developing countries.

Candidiasis, a well-known opportunistic infection of AIDS patients, is the leading cause of infectious esophagitis (1,2). Studies show similar prevalence of *Candida* esophagitis in AIDS patients in the West (9.1% to 31%) (3-5) and in Africa (7.3% to 27%) (6-7). In most treated patients (80% to 100%) *Candida* esophagitis recurs after 3 months (8,9). Nevertheless, in patients with AIDS, candidiasis generally does not become systemic, and thus, clinical cure is important (9). Defining the most effective diagnostic and therapeutic approach to curing *Candida* esophagitis in AIDS patients is especially important in developing countries, which often have limited resources.

Diagnosis of esophageal candidiasis is usually based on the endoscopic appearance of the typical mucosal lesions and on histopathologic studies (10-12). Several western studies have shown that the diagnosis of this disease in AIDS patients can be made on clinical findings alone because the positive predictive value of esophageal symptoms as indexes of esophageal infection is 71% to 100% (10,13,14). Such an evaluation has not yet been made in African AIDS patients.

Several therapeutic regimens have been effective in treating oral and esophageal candidiasis (8,15-23). For the past decade, oral nystatin therapy has been considered effective in controlling *Candida* esophagitis (11). In tropical countries, the efficacy of nystatin in treating this disease is not well known, although a recent study in Zaire reported a cure rate of less than 10% (24). We evaluated the diagnostic accuracy of esophageal symptoms in predicting *Candida* esophagitis in Ugandan AIDS patients with oral candidiasis and compared the effectiveness of miconazole and nystatin in treating oral and endoscopically proven esophageal candidiasis in these patients.

The Study

From September 1994 to December 1995, 320 consecutive AIDS patients were observed at the Gastroenterology Department of Hoima Hospital in Uganda. Among them, 85 (45 women, 40 men, mean age 27.1, standard deviation [SD] 5.3 years) fulfilled admission criteria: positive HIV test or clinical diagnosis of AIDS and presence of oral patchy white plaques as markers of oral candidiasis. The district medical officer and the hospital medical superintendent granted approval for the study, and informed consent was obtained from each patient. Patients were considered symptomatic if they had any of the following symptoms: odinophagia, dysphagia, or retrosternal burning pain.

All patients were hospitalized, and the upper digestive tract was examined endoscopically. The diagnosis of esophageal candidiasis was made at the examination. All patients had the

Address for correspondence: Maurizio Ravera, c/o AVSI P.O. Box 6785, Kampala, Uganda; fax: 256-41-266967; e-mail: petra@swiftuganda.com.

same spectrum of lesions: patchy white plaques, confluent pseudomembrane, and friable mucosa. Routine histopathologic assessment was not performed, mainly because of cost.

Patients were randomly assigned to the nystatin or miconazole regimen; a stratified randomization method was used to balance treatment groups by esophageal symptoms, age, and sex (Table). Nystatin tablets were given at a dose of 1,000,000 I.U. every 8 hours for 7 days (according to Uganda Ministry of Health 1993 National Standard Treatment Guidelines), while miconazole tablets were administered at a dose of 250 mg every 6 hours for 7 days (25). At a mean follow-up of 7.6 days (SD 0.9), the patients' symptoms were reassessed, and the upper digestive tract was reexamined endoscopically. The endoscopist was blind to the treatment used. Patients given nystatin who still had candidiasis were placed on the miconazole regimen and tested for candidiasis 1 week later.

Table. Characteristics of patients participating in the study

	No.	Mean age (yr) (S.D.)	Sex M/F
Oral candidiasis	85	24.0 (6.7)	32 / 53
Esophageal symptoms[a]	40		17 / 23
Nystatin group	20	23.9 (6.1)	7 / 13
Miconazole group	20	23.7 (6.6)	8 / 12
Esophageal candidiasis	77	23.7 (6.4)	27 / 50
Nystatin group	37	24.2 (6.5)	12 / 22
Miconazole group	40	23.4 (6.1)	15 / 28

[a]Esophageal symptoms are any of the following: odinophagia, dysphagia, retrosternal burning pain.

Findings

Most (90.8%) (42 female, 35 male, mean age 28.0 ± 5.8 years) of the study participants had both oral and esophageal candidiasis. Forty (47.1%) had esophageal symptoms, and all had esophageal candidiasis at endoscopy. Sensitivity, specificity, and the positive and negative predictive values of esophageal symptoms as markers of esophageal infection were 83.3% (confidence interval [CI] 69.2 to 92.0), 100% (CI 88.3 to 100), 100% (CI 89.1 to 100), and 82.2% (CI 67.4 to 91.5), respectively.

Esophageal symptoms disappeared in 10 (27.0%) of the 37 patients in the nystatin group and in 38 (95.0%) of 40 patients in the miconazole group (Yates chi-square = 34.99, p <0.001). Oral candidiasis was cured in all patients in both groups; esophageal candidiasis was cured in 8 (21.6%) patients in the nystatin group and in 37 (92.5%) patients in the miconazole group (Yates chi-square = 36.89, p <0.001). Of the 29 patients who did not respond to nystatin, 27 (93.1%) were cured with miconazole (Figure). No adverse effects were observed in either group.

More than 90% of AIDS patients with oral candidiasis in this study also had esophageal candidiasis, thus confirming that such an association is also very strong in Uganda (14,26). A little more than half (51.9%) of 77 patients with esophageal candidiasis also had esophageal symptoms. Our findings and those of other studies support the observation that esophageal candidiasis could be suspected if oral thrush is present, especially when esophageal symptoms are associated (10,14,26,27). Thus, in tropical countries, endoscopic assessment and biopsies might best be reserved for patients who have esophageal symptoms after receiving prolonged antifungal treatment to confirm diagnosis of candidiasis or to determine other infectious causes of this symptoms (e.g., herpes simplex virus infection, cytomegalovirus infection, cryptosporidiosis) (2,23).

Although our study was not designed to detect recurrence of candidiasis, esophageal candidiasis is likely to recur in AIDS patients within 12 months from any antifungal treat-

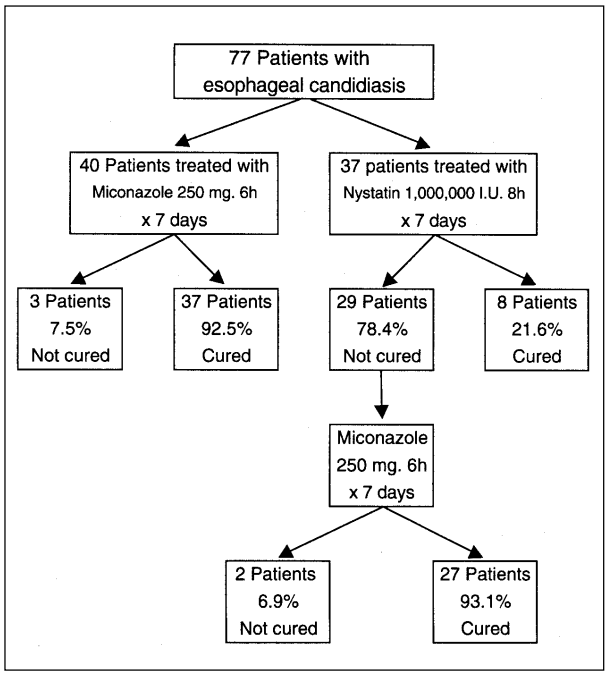

Figure. Esophageal candidiasis treatment flow.

ment; if it does, response to therapy is worse than response to initial therapy (28). For this reason, as well as the possibility of resistance to miconazole (sporadic cases have been reported [29]), more expensive azolic drugs (e.g., fluconazole) should be reserved for recurrences of the disease; the disease should be treated initially with less expensive, but more effective drugs (e.g., miconazole).

In sum, AIDS patients with oral candidiasis in countries similar to Uganda can be managed without endoscopic and bioptic assessments since oral lesions are typical and a high prevalence of esophageal involvement is expected (with or without symptoms) (>90% of cases in our study). In our patients, nystatin had a very low cure rate in the treatment of esophageal candidiasis in AIDS patients; however, it could still play a role in the treatment of oral candidiasis, especially in nonimmunocompromised patients, in whom concomitant esophageal involvement is less common. On the other hand, miconazole, a medium-priced azolic drug, was very effective and could be a valid alternative to more expensive azolic drugs in developing countries.

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Dr. Ravera is a specialist in gastroenterology and digestive endoscopy. A researcher at the Italian National Health Institute, he serves as site coordinator/monitor of the UNAIDS PETRA study at Nsambya Hospital, Kampala, Uganda. His research interests include gastroenterology, endoscopy, infectious diseases, and HIV/AIDS.

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Neospora caninum Infection and Repeated Abortions in Humans

Eskild Petersen,* Morten Lebech,* Lene Jensen,† Peter Lind,† Martin Rask,† Peter Bagger,‡ Camilla Björkman,§ and Arvid Uggla§
*Statens Serum Institut, Copenhagen, Denmark; †State Veterinary Laboratory, Copenhagen, Denmark; ‡Rigshospitalet, Copenhagen, Denmark; and §Swedish University of Agricultural Sciences, Uppsala, Sweden

To determine whether *Neospora caninum*, a parasite known to cause repeated abortions and stillbirths in cattle, also causes repeated abortions in humans, we retrospectively examined serum samples of 76 women with a history of abortions for evidence of *N. caninum* infection. No antibodies to the parasite were detected by enzyme-linked immunosorbent assay, immunofluorescence assay, or Western blot.

Neospora caninum, an intracellular protozoan parasite closely related to *Toxoplasma gondii* (1,2), was first described in dogs in Norway in 1984 and later in a wide range of other mammals including cattle, goats, horses, and sheep. The life cycle of *N. caninum* is only partially known, but the dog has recently been established as its definitive host (3). The pathogen's only known natural route of transmission (which can occur during sequential pregnancies in cattle) is transplacental (4).

N. caninum is now recognized as the most common cause of repeated abortions and stillbirths in cattle, and infected herds have been reported in most parts of the world, including Scandinavia (4-6). Infected, live-borne offspring may have neurologic symptoms including progressive paralysis. When experimentally transferred to pregnant nonhuman primates, *N. caninum* has caused fetal infection. The fetal lesions closely resembled those in congenital toxoplasmosis (7). *N. caninum* organisms are morphologically very similar to *T. gondii*, the pathogen responsible for toxoplasmosis; however, the two species have distinct antigenic characteristics and can be distinguished by serologic and immunohistochemical methods (4).

No case of *N. caninum* infection has been described in humans. However, because of the organism's close phylogenetic relationship to *T. gondii* and its wide range of potential hosts, the possibility of human *N. caninum* infection cannot be excluded. We investigated serologically the possible presence of *N. caninum* infection in Danish women who had repeated abortions of unknown cause.

Address for correspondence: Eskild Petersen, Laboratory of Parasitology, Statens Serum Institut, DK-2300 Copenhagen S. Denmark; fax:45-3268-3033; e-mail: ep@ssi.dk.

The Study

The study included 76 women (mean age 30.8 years, range 19 to 41 years) who had had repeated abortions or intrauterine death of the fetus. Blood samples were obtained at the time of abortion or within 3 months of fetal death. The study participants had been referred to the Department of Gynecology and Obstetrics, Rigshospitalet, Copenhagen, Denmark, between 1 September 1991 and 31 October 1992 as part of a larger study of pregnant women with repeated primary or secondary abortions or repeated intrauterine fetal deaths. Serum specimens were tested for antibodies to *N. caninum* and *T. gondii* as described below.

Findings

The absorbence values for the human serum samples were 0.10 to 1.24 absorbence units, whereas the mean value for the presumed *N. caninum*-negative human control serum was 0.26 (0.13 to 0.56). The mean absorbence values for the high-positive and low-positive control pig sera were 1.73 (1.54 to 1.93) and 0.87 (0.85 to 1.07), respectively. As no true *N. caninum*-negative or -positive human sera were available, serum specimens with absorbencies 0.50 (n = 12) were selected for further investigation (Table).

Table. Results of enzyme-linked immunosorbent assay (ELISA)[a] tests for *Neospora caninum* and of the Sabin-Feldman dye test[b] for *Toxoplasma gondii*

Sample no.	ELISA mean OD	Dye test
90	0.720	0
107	0.627	0
262	0.578	1:50
264	1.238	0
276	1.043	1:50
279	1.032	0
282	0.647	1:10
285	0.656	0
287	1.143	1:6250
289	0.818	0
295	0.583	0
297	0.541	0

[a]The ELISA was modified from Björkman et al. (8,9). A *T. gondii*-negative human serum specimen was used as a presumed *N. caninum*-negative control, and serum specimens from experimentally *N. caninum*-infected pigs were used as positive controls. A low-positive serum was collected from a pig infected 11 days before sampling and a high-positive control serum was pooled from pigs infected for at least 3 weeks (10). No reaction in human sera was definitely positive.

[b]The dye test was used to demonstrate antibodies to *T. gondii* as described (11) but using in vitro cultured *T. gondii*.

None of the 12 specimens showed specific fluorescence in the indirect fluorescence antibody test (IFAT) at dilution 1:640 with *N. caninum* tachyzoites that had been cultivated in vitro (6). (Sera had been diluted in twofold serial dilutions from 1:20 in phosphate-buffered saline.) Of the 12, only 3 had *T. gondii* antibodies. The reactivities in the *N. caninum* enzyme-linked immunosorbent assay (ELISA) were not associated with the presence of *T. gondii* antibodies (Table). Only 1 of the 12 human serum specimens tested showed reactivity against the *N. caninum* antigen by Western blot analysis. This specimen, number 279, recognized an antigen with apparent molecular weight of 60 kDa (Figure, lane 11 and 12). This antigen was not recognized by the *N. caninum*-positive pig sera, and serum 279 did not recognize any of the low-molecular weight antigens recognized by the *N. caninum*-positive pig sera. Three serum specimens reacted with the *T. gondii* antigen; all were *T. gondii*-positive in the dye test.

Because of the biologic similarities between *N. caninum* and the human pathogen *T. gondii*,

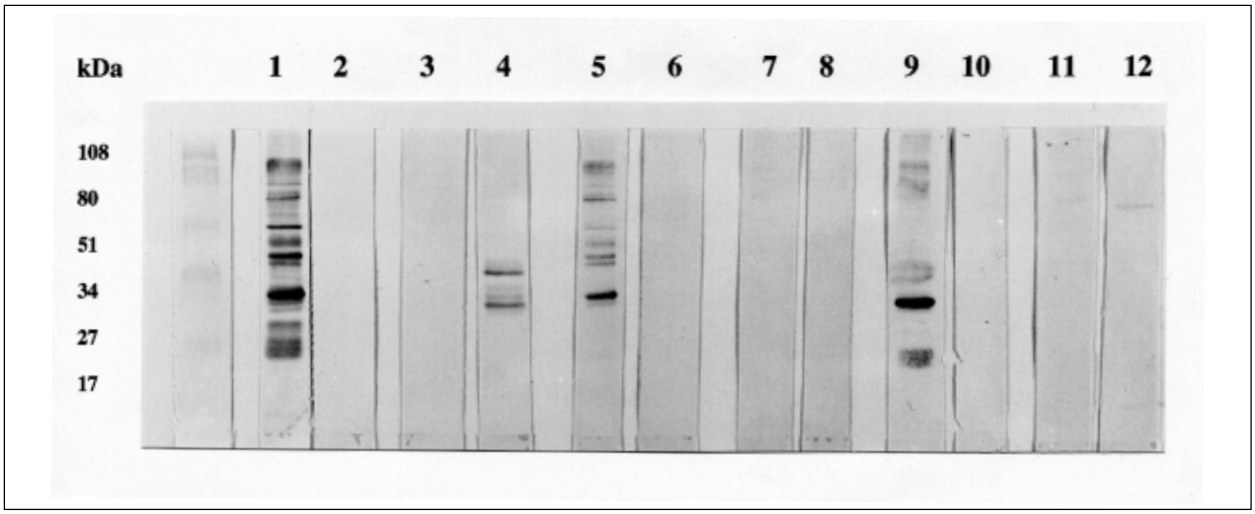

Figure. Western blot of *Toxoplasma gondii* or *Neospora caninum* antigen. Analysis was performed essentially as described by Sharma et al. (12) by using tachyzoites from in vitro culture of the *N. caninum* NC-1 isolate (13) and the *T. gondii* RH strain (10). Lanes 1-4 were probed with control sera and lanes 5-12 with human sera with high absorbencies in the *N. caninum* enzyme-linked immunosorbent assay.
Lane 1: *T. gondii*-positive human serum and *T. gondii* antigen; Lane 2: *N. caninum*-positive pig serum and *T. gondii* antigen; Lane 3: *T. gondii*-positive human serum and *N. caninum* antigen; Lane 4: *N. caninum*-positive pig serum and *N. caninum* antigen; Lane 5 and 6: Serum 262: *T. gondii* and *N. caninum* antigens, respectively; Lane 7 and 8: Serum 264: *T. gondii* and *N. caninum* antigens, respectively; Lane 9 and 10: Serum 276: *T. gondii* and *N. caninum* antigens, respectively. Lane 11 and 12: Serum 279: *T. gondii* and *N. caninum* antigens, respectively.

it has been speculated that *N. caninum* could be transmissible to humans. Since repeated abortions and stillbirths are common manifestations of neosporosis in cattle (4), women with a history of repeated abortions seemed an obvious category to investigate for human *N. caninum* infection. However, in this study of serum samples from women with repeated abortions, no evidence of *N. caninum* infection was detected.

The assays we used were based on methods used for *T. gondii* analyses; we used the same conjugates and serum dilutions found optimal in these analyses. The *N. caninum* immunostimulating complex antigen has a high specificity (14) and has been used for serologic investigations in different animal species (8,9,15). It was therefore anticipated that it would be applicable in a human system as well. However, because we could not define a proper cut-off for the assay, we further investigated the serum samples with the highest ELISA absorbence values by IFAT, regarded as the reference test for *N. caninum* antibodies in different species (4), and Western blot. None of the human sera investigated showed any reactivity in IFAT. Only one of the specimens reacted with the *N. caninum* antigen in the Western blot. However, because it only reacted with a band not recognized by sera from the infected pigs, the reaction was considered unspecific, and cross-reactivity between *T. gondii* and *N. caninum* was not found.

That we found no evidence of *N. caninum* infection in women who had repeated spontaneous abortions does not rule out the possibility that the infection might occur in humans. The predominant effects of neosporosis in dogs are primarily progressive neurologic signs including paralysis. It might, therefore, be worthwhile to examine human patients with clinical symptoms other than abortions, e.g., neurologic disorders of unknown etiology. Furthermore, the possible presence of *N. caninum* in patients with weakened immune systems should be considered. Researchers might continue the search for *N. caninum* by using serologic tests, as we did, or, alternatively, by using material collected at biopsy or autopsy for polymerase chain reaction or immunohistochemical analysis.

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Dr. Petersen is a specialist in infectious diseases and tropical medicine at the Laboratory of Parasitology, Statens Serum Institut, Denmark's national reference center for diagnosis and research of human parasitic infections. His areas of expertise include immunology and epidemiology, primarily applied to malaria and congenital toxoplasmosis.

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Lack of Association between First Myocardial Infarction and Past Use of Erythromycin, Tetracycline, or Doxycycline

Lisa A. Jackson, Nicholas L. Smith, Susan R. Heckbert, J. Thomas Grayston, David S. Siscovick, and Bruce M. Psaty
University of Washington, Seattle, Washington, USA

To evaluate the association of prior treatment with antibiotics active against *Chlamydia pneumoniae* with the risk for incident myocardial infarction, we conducted a population-based case-control study. We found that use of erythromycin, tetracycline, or doxycycline during the previous 5 years was not associated with risk for first myocardial infarction. These results suggest little or no association between the use of these antibiotics and the risk for first myocardial infarction in the primary prevention setting.

Chlamydia pneumoniae has been associated with atherosclerotic cardiovascular disease in seroepidemiologic studies, by detection of the organism in atherosclerotic plaque, and in animal model studies (1-4). Two small clinical trials to assess the effect of treatment with antibiotics active against *C. pneumoniae* on cardiovascular disease outcomes have indicated a possible effect of azithromycin (5) or roxithromycin (6) in the secondary prevention of coronary heart disease. To evaluate whether past use of antibiotics active against *C. pneumoniae* is associated with decreased risk for first myocardial infarction (MI), we conducted a retrospective, population-based case-control study of patients enrolled at Group Health Cooperative of Puget Sound (GH), Seattle, WA, USA.

The Study

Case-patients were GH enrollees, ages 30 to 79 years, in whom an incident fatal or nonfatal MI was diagnosed during July 1986 through December 1995. Controls were a stratified random sample of GH enrollees frequency-matched with the case-patients by age (within decade), sex, calendar year, presence of treated hypertension, and menopausal status (post versus peri and premenopausal). The case-patients and controls had been identified for two previous cardiovascular studies, one of persons with pharmacologically treated hypertension (7) and one of postmenopausal women (8,9), by using methods previously reported. Therefore, men and women with treated hypertension and women without hypertension were included as both case-patients and controls, but men without hypertension were not included in the study population. Medical records were reviewed for all study participants to confirm the diagnosis of incident MI (case-patients) and obtain information on other cardiovascular risk factors.

All study participants had an index date. For the hospitalized case-patients, it was the date of admission for the first MI; for out-of-hospital case-patients who died, it was the date of death; and for the controls, it was a computer-generated random date within the calendar year for which they had been sampled as controls. We excluded persons who had been enrolled for fewer than 5 years or had fewer than four visits to a GH provider before their index date.

The GH computerized pharmacy database, which contains records of all prescriptions dispensed at GH pharmacies, was used to assess prescriptions for antibiotics. A survey conducted

Address for correspondence: Lisa Jackson, 1730 Minor Avenue, Suite 1600, Seattle, WA 98101-1448; USA; fax: 206-287-4677; e-mail: lajack@u.washington.edu.

in conjunction with the previous study of postmenopausal women determined that 95% of study participants filled all their prescriptions at a GH pharmacy (9). During the period of interest to this study, newer macrolidelike antibiotics such as azithromycin and clarithromycin were not routinely available at GH; thus, we selected erythromycin, tetracycline, and doxycycline, antibiotics available for routine use during this period, for evaluation because of their in vitro activity against *C. pneumoniae* and their indication for treatment of *C. pneumoniae* respiratory infections. To determine duration of use for each agent, we defined 1 day of use as equivalent to 2 g erythromycin, 2 g tetracycline, or 100 mg doxycycline. The total duration of therapy with these drugs was calculated by using the sum of the quantities dispensed during the 5 years before the index date.

The Findings

We identified 1,796 eligible case-patients with an incident fatal or nonfatal MI and 4,882 eligible controls during the study period (Table 1). At least one prescription for erythromycin, tetracycline, or doxycycline was recorded for 775 (43%) of the case-patients and 2,061 (42%) of the controls. In multivariate logistic regression models controlling for the matching variables (age, sex, hypertension status, menopausal status, and index year) or the matching variables and known cardiac risk factors (smoking, diabetes, cardiovascular disease), risk for incident MI was not associated with the cumulative duration of prescribed treatment with erythromycin, tetracycline, doxycycline, or the three agents combined (Table 2). In addition, risk for incident MI was not associated with increasing cumulative duration of therapy across these categories for any of the agents individually or for the three agents combined. Further, no association was detected when assessment of the use of antibiotics was restricted to 1 year before the index date (data not shown).

Table 1. Characteristics of case-patients with myocardial infarction and controls

Variable	Case-patients n=1,796	Controls n=4,882
Mean age (yr)	67.0	66.7
Male	38%	41%
Mean duration of enrollment in GH[a] (yr)	16.8	17.4
Angina	26%	10%
Hypertension	75%	70%
Diabetes mellitus	27%	12%
Current smoker	28%	15%

[a]Group Health Cooperative of Puget Sound, Seattle, WA.

There are several possible explanations for these findings. Treatment with antibiotics active against *C. pneumoniae* may not affect the risk for heart disease, either because *C. pneumoniae* does not play a causal role in the atherosclerotic process or because its effect on that process cannot be modified by antibiotics. Although two published clinical trials have suggested a protective effect of antibiotics on the secondary prevention of coronary outcomes, these findings are not conclusive. The study of azithromycin (5), which enrolled 80 men with a history of MI who had serologic evidence of *C. pneumoniae*, was not a randomized controlled trial and so was subject to bias. The results reported from the roxithromycin study (6), which randomized patients hospitalized for unstable angina or non-Q wave infarctions to 1 month of treatment with roxithromycin or placebo, were preliminary findings from the first 31 days of the 6-month follow-up. Even if the protective effect indicated by the preliminary analysis persists in the final analysis, further studies will be needed to replicate and confirm those findings. Increasing evidence supports a causal association of *C. pneumoniae* and atherosclerotic disease; however, additional data are needed to validate this hypothesis.

Alternatively, treatment with antichlamydial antibiotics may be associated with a protective effect, but this effect may vary depending on the specific agent; the dose, duration, or timing of treatment; or the patient's clinical status. Azithromycin and roxithromycin (both highly active against *C. pneumoniae* in vitro) achieve much higher intracellular levels, and in particular much higher levels in macrophages, than do erythromycin, doxycycline, or tetracycline; they also have longer half-lives than those agents (10,11). The beneficial effect of roxithromycin in the published clinical trial may have been due to a nonspecific antiinflammatory effect (12,13) rather than to a direct antimicrobial effect. A true cardiovascular protective effect associated with azithromycin or roxithromycin treatment may not, therefore, be seen after treatment with erythromycin, tetracycline, or

Table 2. Cumulative duration of prescribed treatment with erythromycin, tetracycline, doxycycline, and the three agents combined, and the risk for incident myocardial infarction

Drug	Cumulative duration (day)[a]	Case-patients (n=1,796) (%)	Controls (n=4,882) (%)	OR[b] (95% CI)	OR[c] (95% CI)
Erythromycin	0	1,266 (70)	3,493 (72)	1.0 reference	1.0 reference
	1-14	401 (22)	1,124 (23)	0.99 (0.87-1.13)	0.91 (0.79-1.05)
	15-28	92 (5)	182 (4)	1.41 (1.08-1.82)	1.18 (0.89-1.54)
	29+	37 (2)	83 (2)	1.23 (0.83-1.83)	1.05 (0.69-1.59)
Tetracycline	0	1,507 (84)	4,149 (85)	1.0 reference	1.0 reference
	1-14	223 (12)	564 (12)	1.11 (0.94-1.31)	1.02 (0.86-1.22)
	15-28	39 (2)	88 (2)	1.24 (0.84-1.81)	1.04 (0.69-1.55)
	29+	27 (2)	81 (2)	0.95 (0.61-1.47)	0.98 (0.62-1.54)
Doxycycline	0	1,597 (89)	4,365 (89)	1.0 reference	1.0 reference
	1-14	37 (2)	86 (2)	1.18 (0.79-1.74)	1.06 (0.71-1.59)
	15-28	85 (5)	244 (5)	0.94 (0.73-1.22)	0.90 (0.69-1.18)
	29+	77 (4)	187 (4)	1.10 (0.84-1.45)	1.17 (0.88-1.56)
Erythromycin, tetracycline, or doxycycline	0	1,021 (57)	2,821 (58)	1.0 reference	1.0 reference
	1-14	422 (24)	1,178 (24)	0.99 (0.87-1.14)	0.93 (0.81-1.07)
	15-28	185 (10)	454 (9)	1.13 (0.94-1.35)	0.99 (0.81-1.20)
	29+	168 (9)	421 (9)	1.11 (0.91-1.34)	1.03 (0.84-1.26)

[a]For duration of each agent, 1 day is equivalent to 2 g erythromycin, 2 g tetracycline, or 100 mg doxycycline.
[b]Adjusted for sex, age, hypertension status, menopausal status, and index year.
[c]Adjusted for sex, age, hypertension status, menopausal status, index year, smoking status, diabetes, and cardiovascular disease.
OR = odds ratio; CI = confidence interval.

doxycycline because of differences in pharmacodynamics or in the mechanisms of action of those agents. Additionally, in our study population, the cumulative duration of treatment with the antibiotics assessed was relatively limited; only 9% of all participants had been prescribed more than a total of 28 days of treatment with the three antibiotics combined during the 5-year study period. Thus, the exposure to antibiotics in routine clinical care may be insufficient to reduce risk. A protective effect of antibiotic treatment may also be limited to the secondary, but not the primary, prevention setting; to patients in the high-risk period after an acute event; or to subsets of patients defined by factors that we could not evaluate, such as seropositivity to *C. pneumoniae*. Further, if the organism plays a role during the initiation or early progression of atherosclerotic lesions, but not in later stages, treatment of older adults may not be effective.

Lastly, while it is possible that our study may have failed to detect a true beneficial effect of past antibiotic treatment, our sample size was relatively large. Assuming a prevalence of exposure among the control group of 42%, this sample size had 90% power (at a 95% confidence level) to detect a 20% reduction in risk associated with antibiotic use.

In summary, even though the results of two small clinical trials have suggested that newer macrolidelike antibiotics active against *C. pneumoniae* may provide effective secondary prevention of coronary artery disease, their effectiveness in the primary prevention setting has not been evaluated prospectively. Our results suggest that treatment with erythromycin, tetracycline, and doxycycline in doses commonly prescribed in routine clinical practice is not associated with a reduction in the risk for incident MI among our study population. Further clinical trials of the newer agents for secondary prevention and further observational studies of these agents for the primary prevention of heart disease are indicated.

Dr. Jackson is an assistant professor in the Department of Epidemiology, University of Washington, and an assistant investigator in the Center for Health Studies, Group Health Cooperative in Seattle. She is an infectious diseases epidemiologist; her research focuses on *Chlamydia pneumoniae*, vaccine evaluations, and the epidemiology of vaccine-preventable diseases.

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An Epidemic of Bloody Diarrhea: *Escherichia coli* O157 Emerging in Cameroon?

Patrick Cunin,* Etienne Tedjouka,† Yves Germani,‡ Chouaïbou Ncharre,§ Raymond Bercion,* Jacques Morvan,‡ and Paul M.V. Martin*

*Centre Pasteur du Cameroun, Yaoundé, Cameroon; †Lomié Health District, Lomié, Cameroon; ‡Institut Pasteur, Bangui, Central African Republic; and §World Health Organization Representation, Yaoundé, Cameroon

Between November 1997 and April 20, 1998, bloody diarrhea sickened 298 persons in Cameroon. Laboratory investigation of the epidemic (case-fatality rate, 16.4%) documented amoebiasis in one of three patients and three types of pathogens: multidrug-resistant *Shigella dysenteriae* type 1, *S. boydii,* and enterohemorrhagic *Escherichia coli.* We report the first isolation of *E. coli* O157:H7 in Cameroon and the second series of cases in the Central African region.

In December 1997, an epidemic of bloody diarrhea was reported in Ngoïla, a village of approximately 500 inhabitants, approximately 400 km from Yaoundé. Canoes and motorbikes are necessary to reach Ngoïla, which is linked to Lomié by a difficult road across the Dja River. The remote zone to the south of the river is inhabited by 2,610 persons, who belong to two ethnic groups (Bantus and Baka Pygmies) and live in 22 villages (Figure 1). The population of the outbreak area is 20,600. The sanitation system is weak, latrines do not exist, and human feces are used as fertilizer. No village has running water; drinking water, which is neither chlorinated nor filtered, comes from wells or small streams.

Between December 1997 and March 1998, teams from Lomié Hospital and the East Provincial Delegation of Health went to Ngoïla to treat patients and make an inventory of the cases. After a March 19, 1998, wire service report of a possible viral hemorrhagic fever epidemic in Ngoïla, a joint mission of Centre Pasteur du Cameroun and the World Health Organization visited the area March 25-28, 1998.

Address for correspondence: Paul Martin, Centre Pasteur du Cameroun, BP 1274 Yaounde, Cameroun; fax: 237-231-564; e-mail: C.pasteur@camnet.cm.

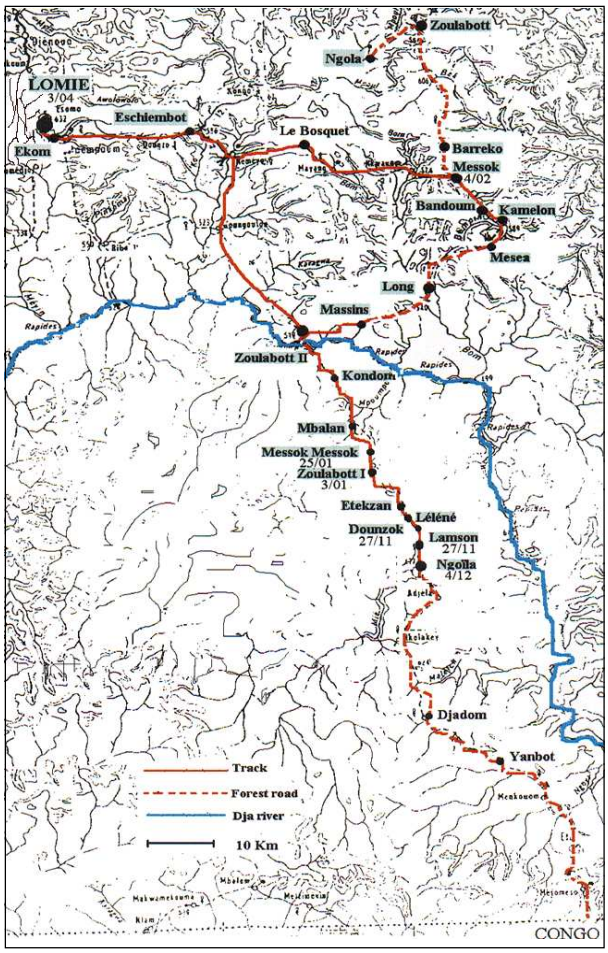

Figure 1. Progression of the bloody diarrhea epidemic, Cameroon.

The Investigation

Investigators defined a case as bloody diarrhea. Data on patients' age, sex, village of origin, onset of disease, drugs received, and disease diagnosis were obtained from the observation record book of the Lomié medical officer. Standardized observation forms were filled out for 34 patients (from nine villages) seen during the mission. Serum specimens were obtained from 21 patients (both current and former) in three different villages, and stool specimens were obtained from 22 patients. Serum specimens were immediately frozen in liquid nitrogen. Stool specimens were cultured immediately in a field laboratory set up in each of the three villages. Later, in the laboratories of Centre Pasteur in Yaoundé and the Institut Pasteur of Bangui, the cultures were identified, their susceptibility to antibiotics was determined (according to the diffusion method on agar plates), and genetic studies were conducted. Hektoen agar plates, purple bromocresol agar plates, MacConkey Sorbitol medium and thiosulfate-citrate-bile- saccharose medium (Sanofi, Marnes la Coquette, France) were used.

Isolates that agglutinated in O157 antiserum were confirmed biochemically as *Escherichia coli* and screened for the lack of enzyme ß glucuronidase, using the substrate 4-methylumbelliferyl-ß-D-glucuronide. Specific anti-O157 and anti-H7 were obtained from Difco, USA. Toxin production was assayed as follows. We used the Gb3 enzyme-linked immunosorbent assay described by Ashkenazi and Cleary (1) for detection of Verotoxin. Cytotoxicity assays and seroneutralization tests were performed according to procedures (2) with Vero cells. The antisera to purified Verotoxin and Shiga toxin had been used in previous studies (3,4) and were prepared in New Zealand white rabbits to perform seroneutralization. The Verotoxin 1 preparations we used were purified from *E. coli* E40705 (O157:H7) provided by the Public Health Laboratory Service, London, United Kingdom, by the procedure described by O'Brien and LaVeck (5). Sera to Shiga toxin from *Shigella dysenteriae* type 1 (provided by the Institut Pasteur, Paris, France) were prepared according to a previously described procedure (4). Virulence genes of *E. coli* were investigated by molecular biology techniques; polymerase chain reaction was used to detect enterohemorrhagic Shigalike toxins 1 and 2 genes and the attaching and effacing gene *eae* (6-8).

Part of each stool specimen was conserved in a medium containing Merthiolate-formalized iodine for further parasitologic analyses. Viral hemorrhagic fever agents were sought by gene amplification and immunoglobulin M detection.

The Outbreak

The first cases were reported at the end of November 1997 in two small villages, Dounzok and Lamson, near Ngoïla. An additional two cases were found retrospectively, at the end of November, one each in Djadom and Yanebot, villages south of Ngoïla. The epidemic began to affect Ngoïla at the beginning of December 1997 and then, traveling north along the roads, spread to all villages in the area. It crossed the Dja River and reached Messok on February 4 and Lomié, divisional headquarters of the district, on April 3 (Figure 1). By April 20, 298 cases had been reported from 28 villages.

In the first villages affected, each with fewer than 100 inhabitants, the epidemic lasted an average 3 months (57 days to 112 days), with an attack rate of more than 50%. Villages south of the Dja had an attack rate of 9.7% (253 of 2,610). In Ngoïla, the village with the most cases, more than a fourth of the inhabitants were sick. The graph of the epidemic for the 271 patients with a known date of illness onset suggests person-to-person transmission, with a slow increasing gradient consisting of successive waves (Figure 2).

Of 10 patients examined at Messok, 7 belonged to the same family (of 12); 2 carried *S. dysenteriae* type 1, and 3 carried enterohemorrhagic *E. coli* (EHEC). Disease onset spread out over 4 weeks, with the index

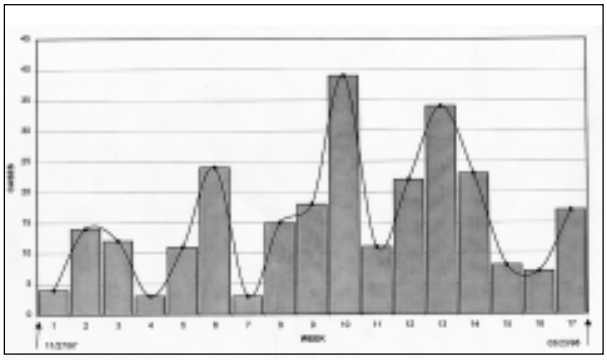

Figure 2. Cases of bloody diarrhea, by week, Cameroon, Nov. 27, 1997–March 23, 1998.

case in week 1, two cases in week 2, one case in week 3, and three cases in week 4. The median delay between illness onset was 6 days (range 3 to 8 days). A relative who came to visit family members in the Messok Health Center was the last case in this family. He became ill 7 days later; he had *Shigella* and *Entamoeba* infections. The many Pygmies in the region (who live in separate villages) were not affected by the epidemic. Both sexes in all age groups became sick. The attack rate for the villages south of the Dja (with known population sizes) was higher among female (11.4%) than among male residents (8.1%) ($p < 0.02$) and increased with age (Table 1). The case-fatality rate was highest for women ages 60 years and older (39.7%).

Table 1. Bloody diarrhea epidemic in 22 villages south of the Dja River, East Cameroon, 1997–1998

Age group (yr)	Female			Male			
	Cases	No.[a]	Rate (%)	Cases	No.[a]	Rate (%)	p
0-4	16	221	(7.2)	14	218	(6.4)	NS[b]
5-9	16	191	(8.4)	11	191	(5.8)	NS
10-19	25	321	(7.8)	18	313	(5.8)	NS
20-29	22	211	(10.4)	25	207	(12.1)	NS
30-39	15	127	(11.8)	14	154	(9.1)	NS
40-49	16	89	(18.0)	14	101	(13.9)	NS
50-59	11	63	(17.5)	4	70	(5.7)	<0.05
60 +	25	63	(39.7)	7	69	(10.1)	$<10^{-4}$
Total	146	1,286	(11.4)	107	1,324	(8.1)	<0.01
p			$<10^{-8}$			<0.05	

[a]Number of village inhabitants.
[b]NS, not significant.

Illness, Treatment, and Deaths

The most frequent signs of illness in the 34 investigated patients were abdominal pain (97%) and mucus in stools (91%), followed by fever (53%), vomiting (50%), and dehydration (41%). The average number of stools per day was 10; mucus and blood were observed in stools starting on the third day. The quantity of blood in the stools varied; occasionally only blood was excreted.

Stool specimens were obtained from 12 female and 10 male patients; their median age was 25 (2 years to 60 years), and the median length of illness was 7 days (3 days to 58 days). African hemorrhagic fever was not detected. Parasitologic examination of stool specimens showed heavy infestation with numerous parasites. Hematophagous trophozoites of *Entamoeba histolytica histolytica* were observed in 7 (31.8%) of 22 of the cases. Bacteriologic studies isolated at least 1 enteropathogen in 20 (90.9%). EHEC were isolated in 12 (54.5%), *S. dysenteriae* type 1 in 9 (40.9%), and *S. boydii* in 2 (9.1%). Potentially dangerous associations were common. Of the 22 patients with stool specimens, 7 (31.8%) had both *E. histolytica histolytica* and one enteropathogenic bacterium; 3 (13.6%) had both *E. histolytica histolytica* and *S. dysenteriae* type 1; and 2 (9.1%) had both *S. dysenteriae* type 1 and EHEC. Simultaneous infection with all three pathogens—*S. dysenteriae*, EHEC, and *E. histolytica*—were found in patients from all three villages, while dual infections with *S. dysenteriae* and EHEC were observed in patients from two villages.

Drug susceptibility testing found that *S. dysenteriae* type 1 was resistant to amoxicillin, amoxicillin in combination with clavulanic acid, tetracycline, chloramphenicol, cefsulodin, and cotrimoxazole; it was sensitive to nalidixic acid, piperacillin, cefalotin, ceftazidin, gentamicin, and ofloxacin. *E. coli* O157:H7 was resistant to amoxicillin, chloramphenicol, and cefsulodine, and sensitive to amoxicillin in combination with clavulanic acid, tetracycline, cotrimoxazole, nalidixic acid, and cefalotin. DNA amplification showed that *E. coli* O157:H7 strains had genes that coded for Shigalike toxins 1 and 2 and the attaching and effacing gene *eaeA*.

By April 1998, five different medications, depending on availability, had been used, sometimes in combination. Of the 292 patients with known drug regimens, 182 took cotrimoxazole (62.3%), 190 took metronidazole (65.1%), 34 took chloramphenicol (11.6%), and 19 took tetracycline (6.5%). Of these, 67 (22.9%) were given oral rehydration salts, while 71 (24.3%) received no treatment or received traditional medicine. More female than male patients were not treated, 51 (31.1%) of 164 versus 20 (15.6%) of 128 ($p < 0.01$), respectively. The groups that received the least or no drug treatment were children younger than 5 years of age (12 [34.3%] of 35) and those ages 50 years and older (26 [46.4%] of 56). The proportion of patients treated with drugs increased during the epidemic: 24 (54.5%) of 44 of the patients were treated in December 1997, while 41 (68.3%) of 60, 85 (76.6%) of 111, and 44 (88%) of 50 were treated in January, February, and March, 1998, respectively.

Of the 275 patients for whom disease outcome was known, 45 died (case-fatality rate

16.4%). The rate was higher in female (21.3% [33 of 155]) than in male patients, (10% [12 of 120]) (p <0.02) (Table 2). Case-fatality rates were 23.5% (8 of 34) among those younger than 5 years old, 40% (22 of 55) among those 50 years of age and older, and 53.5% (38 of 71) among patients who received no treatment.

Table 2. Bloody diarrhea epidemic in East Cameroon, case-fatality rate of by age and sex

Age group	Female			Male			
(yr)	Cases	Deaths	(%)	Cases	Deaths	(%)	p
0-4	18	5	(27.8)	16	3	(18.8)	NS[a]
5-9	16	2	(12.5)	13	1	(7.7)	NS
10-19	25	1	(4.0)	20	1	(5.0)	NS
20-29	24	1	(4.2)	27	1	(3.7)	NS
30-39	16	3	(18.8)	18	1	(5.6)	NS
40-49	17	3	(17.6)	12	1	(8.3)	NS
50-59	13	6	(46.2)	8	2	(25.0)	NS
60 +	26	12	(46.2)	6	2	(33.3)	NS
Total	155	33	(21.3)	120	12	(10.0)	<0.02

[a]NS, not significant.

Conclusions

During the first half of 1997, an epidemic of bloody diarrhea with a case-fatality rate of 19% was reported in villages near Mintom, a district headquarters approximately 100 km west of Ngoïla. Then, the region of Ngoïla in the East Province of Cameroon was hit by an extremely severe and deadly epidemic of bloody diarrhea, which continued until July 1998. In April 1998, 34 (11.4%) of 298 (the affected population) were examined, and stool specimens were obtained from 22 patients with an active infection, which represented 7.4% of the total epidemic population (298). Although the sample was relatively small, three bacterial enteropathogens (*S. dysenteriae* type 1, *S. boydii,* and EHEC) associated with numerous parasites were isolated from the stool specimens. Thus, which of these pathogens were responsible for the epidemic and its severity is not known with certainty. The isolation of *E. coli* O157, in contrast to *Shigella,* is quite recent in Africa. We believe that this is the first case of *E. coli* O157 described in Cameroon and the second (after the 1996 epidemic in the Republic of Central Africa) in the Central African region (9).

A few persons may have contracted the disease through food or drink. However, the epidemic curve with a slow rise of successive waves does not depict a common source of contamination; it suggests person-to-person transmission. The spread of the disease followed roads, and women were the most likely infected, probably because they live together in groups and take care of the sick. Because of difficulties in communication and the necessity to return to the laboratory to process the specimens, we could not investigate in detail with a case-control study. The protracted course of the illness, poor sanitation, and length of time patients were infected with pathogens favored transmission. The bloody diarrhea was long-lasting for some patients. In one patient with *S. dysenteriae* type I, the onset of disease had begun 58 days before, while in another with EHEC, it had begun 39 days before.

In contrast to shigellosis, which is most often transmitted from person to person (10) (often through the hands) (11) and affects mostly women (12), *E. coli* O157 infection has been described in the northern hemisphere as food poisoning (13). For example, in Japan an epidemic involving more than 5,000 cases was described in school children contaminated by food prepared in the school canteen in 1996 (14). Sources of contamination most commonly incriminated are meat in hamburgers (15) or sandwiches (16), milk (17), drinking water (18), water absorbed during baths (19), nonpasteurized apple juice (20), and salads (21). The main reservoir of the germ is cows and other ruminants (22), but the association between *E. coli* O157 and cows is not absolute (23). Its transmission from person to person has been observed in a small number of cases, most often intrafamilially (24). In the Republic of Central Africa during the first isolation of *E. coli* O157, food was the suspected vehicle (9). The rain forest zone in South and East Cameroon, like Ngoïla, has no cattle; breeding is limited to pigs, goats, and chickens; and meat is not imported.

Deaths caused by the disease were quite high, with a case-fatality rate of 16.4%. In comparison, the case-fatality rate for cholera was globally estimated at 4.7% in 1996 (25) and rose to 48% in the worst outbreak (the beginning of the epidemic in Rwanda refugee camps in Goma in 1994) (26). Shigellosis is one of the main causes of severe diarrhea in Africa, accounting for 12% of all deaths in Kibue Sector in Burundi in 1992 (12) and 19% of pediatric hospital deaths in KwaZulu-Natal in 1995 (27). The portion of

deaths due to shigellosis varies—from 13% in hospital statistics in children in KwaZulu-Natal to 3.8% in a Burundan refugee camp in Rwanda in 1993 (28) and 0.25% during the first *Shigella* epidemic in Mozambique in the same year (29). The severity of *E. coli* O157 is associated with its complication, hemolytic uremic syndrome; an estimated 200,000 cases of this syndrome per year occur in the United States, with 250 deaths and a case-fatality rate of 0.1% (30). In the epidemic in the Republic of Central Africa, the case-fatality rate was 3.7% (9).

The causes of death were unknown. The case-fatality rate diminished during the course of the epidemic: 14 (31.8%) of 44 in patients in December 1997, 9 (15%) of 60 in January, 19 (18.3%) of 104 in February, and 2 (5%) of 40 in March 1998 ($p < 0.02$), suggesting progressively better disease management during the epidemic. The case-fatality rate was 3.4% in patients who received either cotrimoxazole (6/174), metronidazole (6/176), or chloramphenicol (1/29); 5.5% in those who were given tetracycline (1/18); 0% in patients who also received oral rehydration salts; and 53.5% (38/71) in those who received no treatment. In Ngoïla, health facilities did not have the resources to perform the classic hematologic and chemical investigations. However, the case-fatality rate in patients who received oral rehydration salts was zero, suggesting that fluid loss was a major part of the disease. Other cofactors (e.g., anemia) could have played a role in lethality. To determine the risk for death from the disease, we constructed a logistic regression model that included age, sex, onset of illness, and drug regimen received. Oral rehydration salts were not included in the model (all rehydrated patients survived). The only factor strongly associated with death was age >50 years (odds ratio [OR] = 5 ; 95% confidence interval [CI]: 2.2-11.5); having received cotrimoxazole was a protective factor (OR = 0.05, 95% CI: 0.02 - 0.13, $p < 10^{-5}$). Because the treatment groups were not randomized, the decline in case-fatality rates cannot be attributed to this drug. The use of other drugs, however, as well as sex and month of onset of the illness, were not associated with death. *S. dysenteriae* type 1 was resistant to cotrimoxazole, while *E. coli* O157 was susceptible.

The multiple resistance to antibiotics of *S. dysenteriae* type 1 strains isolated from Ngoïla could also explain the high case-fatality rate. Studies from Central and East Africa confirm that *S. dysenteriae* type 1 is resistant to multiple drugs (31-34). There may, therefore, be a new emerging disease due to the association of enterohemorrhagic *E. coli* O157:H7 and *S. dysenteriae* type 1 in this region of Africa, an association that could be as deadly as that of shigellosis and cholera. Finally, the management of cases will be rendered difficult by the resistance of the pathogens implicated to multiple drugs. After our microbiologic investigations, the health authorities distributed nalidixic tablets and ciprofloxacin. The case-fatality rate fell to 3.7% (8/213).

Dr. Cunin, an epidemiologist and specialist in tropical diseases, is affiliated with Coopération française and has worked in Africa (Morocco, Côte d'Ivoire, Mauritania, Cameroon) since 1975. He now works at the Pasteur Center in Cameroon. His research focuses on epidemic investigation, infectious diseases (tuberculosis, arbovirus, schistosomiasis), mother-to-child transmission of HIV, and maternal and child health.

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Genospecies Diversity of Lyme Disease Spirochetes in Rodent Reservoirs

Dania Richter,*§ Stefan Endepols,† Andreas Ohlenbusch,‡ Helmut Eiffert,‡ Andrew Spielman,§ and Franz-Rainer Matuschka*§
*Medizinische Fakultät der Humboldt-Universität zu Berlin, Berlin, Germany; †Geschäftsbereich Tiergesundheit, Bayer AG, Leverkusen, Germany; ‡Universitätskliniken der Georg-August-Universität Göttingen, Göttingen, Germany; and §Harvard School of Public Health, Boston, Massachusetts, USA

To determine whether particular *Borrelia burgdorferi* s.l. genospecies associate solely with rodent reservoir hosts, we compared the genospecies prevalence in questing nymphal *Ixodes* ticks with that in xenodiagnostic ticks that had fed as larvae on rodents captured in the same site. No genospecies was more prevalent in rodent-fed ticks than in questing ticks. The three main spirochete genospecies, therefore, share common rodent hosts.

The several genospecies of the Lyme disease spirochete, *Borrelia burgdorferi* sensu lato, that infect people in Eurasia produce a broad spectrum of human disease. Particular genospecies have been associated with characteristic symptoms; chronic skin disease, for example, results from infection by *Borrelia afzelii* (1). Rodents (e.g., various *Apodemus* mice [2], Norway rats, *Rattus norvegicus* [3,4], edible dormice, *Glis glis* [5]) serve unambiguously as reservoir hosts for Lyme disease spirochetes. Although each major European genospecies has been associated with birds (6), *B. afzelii* is thought to perpetuate in rodents and *B. garinii* is thought to perpetuate in avian reservoir hosts (7,8). The European vector of Lyme disease, *Ixodes ricinus*, maintains an unusual diversity of pathogens in an extraordinarily broad array of hosts.

The vertebrates that are infested most frequently by arthropods generally also serve as reservoir hosts for any pathogens transmitted by these vectors. The transmission cycle would likely be broken if some vector acquired a pathogen from one host but injected it into an ill-adapted host. If *B. afzelii* thrived mainly in rodents (7), transmission might not be sustained if a larval tick acquired these spirochetes from a mouse and attached subsequently as a nymph to a bird. If *B. garinii*, on the other hand, thrived mainly in birds, a corresponding diversion of its vector to a rodent would similarly result in transmission failure. Efficient perpetuation of rodent-borne *B. afzelii* as well as bird-borne *B. garinii* by the same subadult *I. ricinus* vector ticks, therefore, would seem paradoxical.

Because rodents may serve as reservoir hosts for both *B. afzelii* and *B. garinii*, we determined whether the genospecies distribution of spirochetes naturally infecting rodents corresponds to that in questing vector ticks. We compared the genospecies diversity of spirochetes infecting questing nymphal *I. ricinus* ticks with that of spirochetes infecting nymphs that had fed as larvae on Norway rats or on yellow-necked mice, *Apodemus flavicollis*, captured in the same site.

The Study

Norway rats and yellow-necked mice were captured in an urban park in Magdeburg, Germany, from June through September 1994, by using apple- and bread-baited Tomahawk traps (Tomahawk Live Trap Company, Tomahawk, WI) and apple- and rodent chow–baited Longworth traps (Longworth Scientific Instruments, Abingdon, UK) (4). Each captured rodent was caged over water for 1 week to permit detachment of all ticks that had attached to these rodents in the field. Subsequently, rodents were

Address for correspondence: Dania Richter, Institut für Pathologie, Charité, Medizinische Fakultät der Humboldt-Universität zu Berlin, Malteserstraße 74-100, 12249 Berlin, Germany; fax: 49-30-776-2085; e-mail: dania.richter@charite.de.

infested with noninfected laboratory-bred *I. ricinus* larvae for xenodiagnosis. To confirm that larvae used for xenodiagnosis were free of spirochetes, a sample of each batch was routinely fed on a laboratory-bred mouse; no spirochetes were found when midguts of 20 of the resulting nymphs were examined by dark-field microscopy, and subsequent xenodiagnosis of the mouse showed no evidence of spirochetes. The water was changed and inspected at least twice a day and detached larvae were removed promptly. Engorged larvae were enclosed in screened vials and kept at 20±2°C in sealed desiccator jars over supersaturated $MgSO_4$ under a light-dark regimen (16:8) until they molted.

Questing *I. ricinus* ticks were collected from the vegetation in the study site by using flannel flags. Collected ticks were confined in screened vials and stored at 15±1°C in sealed desiccator jars containing supersaturated $MgSO_4$ until they were examined microscopically, identified to stage, and prepared for polymerase chain reaction (PCR) analysis.

DNA Extraction, Amplification, and Sequencing

Total DNA from the ear pinnae of field-caught rodents was prepared with a QiAamp Tissue kit (Qiagen GmbH, Hilden, Germany). Engorged *I. ricinus* larvae that served for xenodiagnosis were permitted to molt to nymphs. The opisthosoma was then opened, andTthe contained mass of soft tissue was dissected out into 400 µl ice-cold Tris-EDTA buffer (pH 7.4, 10mM Tris, 1mM EDTA). Suspensions of tick tissue were adjusted to 0.5% sodium dodecyl sulfate, 0.2M NaCl, 10mM Tris, and 5mM EDTA at pH 8.0; proteinase K (Boehringer Mannheim, Mannheim, Germany) was added (0.2mg/ml) and incubated at 56°C for 3 hours. DNA was extracted with phenol-chloroform. Ethanol-precipitated DNA was resuspended in 50 µl distilled water.

Borrelia genospecies were characterized by amplifying and sequencing a 400-nucleotide segment of the gene encoding the outer surface protein A (OspA) (9-11). To increase the sensitivity for detection of spirochetal DNA in ticks, we used nested PCR (10). Aliquots of DNA suspensions (20 µl) were diluted to 50 µl by using 200 µM each of deoxynucleoside triphosphate, 4mM $MgCl_2$, 10mM Tris at pH 8.3, 50mM KCl, 0.01% Tween-20, 0.01% gelatin, and 0.8 units Taq polymerase (Amersham, Braunschweig, Germany), as well as 10pmol of the outer primer pair or 20pmol of the inner primer pair. We used the following primer sequences (5'-3') of the *ospA* gene (9): outer primers GGTCTAATATTAGCCTT AATAGGCATG (positions 169-194) and TCAG CAGCTAGAGTTCCTTCAAG (positions 665-643); inner primers CATGTAAGCAAAATGTTAG CAGCC (positions 191-214) and CTGTGTATTCA AGTCTGGTTCC (positions 589-568). The mixture was overlaid with mineral oil (Sigma, Deisenhofen, Germany), placed in a thermocycler (Omnitech, Heidelberg, Germany), heated for 2 min at 95°C, and subjected to 40 cycles of 20 sec denaturation at 95°C, 20 sec each for the first annealing reaction at 59°C, for the second at 61°C and with a 20-sec extension at 72°C. After the first amplification with the outer set of primers, 5 µl of product was transferred to a fresh tube containing 45 µl of reaction mixture as described for the inner set of primers. PCR products were detected by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. DNA was extracted, reaction vials were prepared for amplification, and products were electrophoresed in separate rooms. For comparison, each series of PCR amplification included two laboratory-reared nymphs that had fed as larvae on *B. afzelii*-infected jirds (*Meriones unguiculatus*) and two that had fed on noninfected jirds.

Each PCR amplification product was purified by using a QIAquick-Spin PCR column (Qiagen). Amplified DNA fragments were directly sequenced in both directions using the inner primers by the dideoxynucleotide chain-termination method on an ABI 373 DNA-sequencer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Each resulting sequence was compared with sequences of the same fragment representing *B. burgdorferi* sensu stricto, *B. afzelii,* and five serotypes of *B. garinii* (9-11); an exact fit was required. With this technique, each of these genospecies can be detected with equal sensitivity; the technique detects and identifies two different coinfecting spirochete genospecies, even when one is five times as numerous as the other (10).

The Findings

We first described the frequency of spirochetal infection and the distribution of

spirochete genospecies infecting questing *I. ricinus* ticks collected from vegetation. Although all three spirochete genospecies were present in these ticks, *B. garinii* was somewhat more prevalent than *B. afzelii*; *B. burgdorferi* s.s. was infrequent (Table 1). A similar distribution of genospecies was found in questing adult ticks (data not shown). Spirochetes of each of the major pathogenic European genospecies infect questing vector ticks at our study site.

We then identified the spirochete genospecies that naturally infected rodents transmitted to xenodiagnostic larvae. Spirochetes were present in virtually all nymphal ticks that had fed as larvae on rats captured at the study site and in approximately half of those that had fed on mice (Table 1). Each of the three main genospecies was present. The 1:2:1 ratio of *B. afzelii* to *B. garinii* to *B. burgdorferi* s.s. in ticks that fed on rats did not differ from the 3:3:1 ratio in nymphal ticks that fed on mice (Chi-square, p=0.7). The 2:3:1 overall ratio of genospecies in xenodiagnostic ticks did not differ from the 4:5:1 ratio in questing ticks (Chi-square, p=0.9). In contrast, only *B. afzelii* DNA was amplified from the ear pinnae of these hosts (data not shown). The array of spirochete genospecies acquired by ticks feeding on field-derived rodents is similar to that in questing ticks but differs sharply from that present in skin samples of these rodents.

To examine the transmissibility of the three genospecies of Lyme disease spirochetes, we compared genospecies diversity in *I. ricinus* ticks that had fed on individual rodents. At least two such infected ticks were available for each of these rats and for all but one mouse; that mouse was excluded from this analysis (Table 2). Although more than one spirochete genospecies infected the cohort of ticks that fed on each rodent, no individual tick was infected with more than one genospecies. The *B. garinii* genospecies infected somewhat more ticks than did *B. afzelii*, and *B. afzelii* infected somewhat more than did *B. burgdorferi* s.s. We found that no particular spirochete genospecies is transmitted more frequently than any other.

Table 1. *Borrelia* genospecies diversity in questing nymphal *Ixodes ricinus* ticks and in nymphal ticks that had fed as larvae on Norway rats, *Rattus norvegicus*, or yellow-necked mice, *Apodemus flavicollis*

	Borrelia prevalence		Distribution of genospecies			
			No.			
Tick source	No. tested	% infected	ticks tested	% with *Borrelia* *afz*[a]	*gar*	*bur*
Questing	112	9	10	40	50	10
Fed on rat[b]	50	86	25	28	52	20
Fed on mouse[c]	36	42	15	40	47	13

[a]*afz*,*afzelii*; *gar, garinii*; *bur*, *burgdorferi* sensu stricto.
[b]Ticks fed on five infected rats.
[c]Ticks fed on four infected mice.

Table 2. Natural infectivity for xenodiagnostic *Ixodes ricinus* ticks of various genospecies of Lyme disease spirochetes

		No. ticks infected by *Borrelia*		
Infected rodent	Rodent no.	*afzelii*	*garinii*	*burgdorferi* sensu stricto
Rattus norvegicus (Norway rats)	1	2	2	1
	2	0	3	2
	3	1	4	0
	4	3	2	0
	5	1	2	2
	Total	7	13	5
Apodemus flavicollis (Yellow-necked mice)	1	3	3	1
	2	3	2	0
	3	0	1	1
	Total	6	6	2

Conclusions

Although *B. garinii* spirochetes most frequently infect questing ticks in our German study site, the other two major genospecies predominate elsewhere in Europe. *B. afzelii* predominates in questing ticks in four European sites (12-14) and *B. burgdorferi* s.s. in four others (Table 3) (7,15-17). In three sites, different combinations of two genospecies predominate (7,15,18). No regional pattern of genospecies diversity in questing ticks seems evident, nor does the sampling method influence the genospecies ratio. If *B. garinii* were to perpetuate in an avian reservoir (8,19) and *B. afzelii* in rodents (7), the relative availability of these hosts to vector ticks would determine genospecies distribution in a site. Alternatively, the founder's principle (i.e., in the absence of some selective force, the present ratio randomly reflects that of the past) may influence genospecies distribution. No available longitudinal study, however, permits such a temporal

Table 3. *Borrelia* genospecies diversity in questing *Ixodes ricinus* ticks from various European sites

Site[a]		No. infected ticks	*Borrelia* genospecies Relative ratio *afz*[b]	*gar*	*bur*	% mixed	Sampling method	Ref.
CH	1	7	1	2	2	8	Culture	7
	2	6	0	1	5	0	Culture	7
	v	50	1	6	9	0	Culture	17
CR	v	56	19	3	1	20	Direct	13
D	1	52	5	11	1	2	Direct	1
	2	10	4	5	1	0	Direct	TA[c]
F	1	25	10	5	1	24	Direct	12
GB	1	16	0	10	1	0	Direct	8
IR	1	11	1	4	5	9	Direct	15
	2	10	0	2	1	0	Direct	15
	3	20	4	1	4	15	Direct	15
NL	1	15	1	1	0	14	Direct	18
	v	63	1	9	14	0	Culture	16
SL	1	47	3	2	1	5	Culture	14
	v	13	12	1	0	0	Culture	14

[a]CH, Switzerland; CR, Croatia; D, Germany; F, France; GB, Great Britain; IR, Ireland; NL, Netherlands; SL, Slovenia; v, sum of various sites in a country.
[b]*afz, Borrelia afzelii*; *gar, Borrelia garinii*; *bur*, *Borrelia burgdorferi* sensu stricto.
[c]This article.

interpretation. The distribution of European *B. burgdorferi* s.l. genospecies appears to be site-specific and may be random. For this reason, genospecies comparisons designed to differentiate reservoirs of these spirochetes should be based on diversity within a single site.

The method used to sample spirochetes from a mammal host may bias the results in favor of one or another genospecies. When spirochetes are isolated from European mice by culturing segments of their ear pinnae, the *B. afzelii* genospecies seems to predominate (7). In direct identification by PCR and sequence analysis, we confirmed the sole presence of this genospecies in ear pinnae from Norway rats and yellow-necked mice captured at our study site. A more diverse array of genospecies, however, infects ticks questing at this site. Although this finding would suggest that *B. afzelii* is the sole spirochete genospecies infecting these rodents, xenodiagnostic observations indicate that samples based on ear biopsies do not reflect the total diversity of spirochetes infecting such a rodent. Interestingly, *B. burgdorferi* s.s. is readily detected in earpunch samples taken from American mice (20). The apparent association of *B. afzelii* with European rodent hosts may derive from a sampling artifact.

In contrast to the reported association of *B. afzelii* with rodents, *B. garinii* is reported to depend on avian reservoir hosts (7,8,21). Evidence for such a genospecies-specific avian reservoir derives originally from observations on an isolated island site where numerous seabirds nested and where no other spirochetes were detected (19). DNA characteristic of *B. garinii* was detected in questing and seabird-feeding *I. uriae* ticks and in the footweb of a razorbill. This genospecies also predominates in pheasants in a British site in which no *B. afzelii* spirochetes are evident (8). Although *B. garinii* infects bird-feeding *I. persulcatus* ticks in a Japanese site and *B. afzelii* infects those feeding on rodents (21), another Japanese study detected both genospecies in voles (22). Experimental studies, however, suggest that birds are relatively incompetent as hosts of Lyme disease spirochetes. Domestic chickens, for example, become only transiently competent (23), and European blackbirds, *Turdus merula*, and Canary finches, *Serinus canarius*, appear not to become infected (3,24). Spirochetes (probably *B. burgdorferi* s.s.), however, have been detected in larval *I. dammini* taken from particular North American birds (25). Evidence of nonspecificity in avian hosts is provided by observations in Scandinavia, where spirochetes of the three major genospecies infect larval ticks that had fed on various passerine birds (6). Indeed, our finding that vector ticks ingest *B. garinii* spirochetes from rodents at least as frequently as *B. afzelii* argues against the concept of genospecies-reservoir specificity.

Surprisingly, few ticks questing in Europe appear to contain more than one spirochete genospecies (Table 3). No more than one in four ticks is multiply infected (12) (generally far fewer). None of the questing ticks collected in our European study site appear to contain more than one kind of spirochete, nor did any laboratory-reared ticks permitted to feed on rodents captured in this site. These rodents, however, were multiply infected: each rodent infected some of the ticks that fed on them with two or more kinds of spirochetes. Although our diagnostic procedure may more reliably detect DNA of the more abundant of two genospecies coinfecting a tick, our findings may have a biologic basis. We suggest that a single genospecies becomes established in a tick far more frequently than do multiple genospecies.

Host specificity of a vector contributes powerfully to the intensity of transmission of a tick-borne pathogen. Such a pathogen would fail to perpetuate unless the host-range of its vector corresponds closely to that of its reservoir. It would seem paradoxical if the same *I. ricinus* population maintained spirochetes of one genospecies in birds, while maintaining another in rodents. This "one-vector-one-reservoir" principle is consistent with our discovery of a similar genospecies distribution in rodents and in ticks questing at our study site. If the *B. garinii* genospecies were to predominate there in birds (7), such spirochetes would have been more prevalent in questing ticks than in ticks that had engorged on sympatric rodents; however, this is not the case. Our site-specific observation that the genospecies distribution in rodent-fed ticks reflects that in questing ticks argues that all three pathogenic spirochete genospecies share common reservoir hosts.

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Dr. Richter is a postdoctoral fellow conducting joint research in the laboratories of Dr. Matuschka, 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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Preparing for Pandemic Influenza: The Need for Enhanced Surveillance

Epidemic influenza, an age-old infectious disease, kills approximately 20,000 men and women in the United States every year. The emergence of influenza viruses bearing novel surface antigens in 1918 (A/H1N1), 1957 (A/H2N2), and 1968 (A/H3N2) led to three worldwide pandemics of disease and more than 600,000 deaths in the United States. During the influenza pandemic of 1918-19, 500,000 deaths were reported, with persons younger than 65 years of age accounting for 99% of all influenza-related deaths (1).

Because it establishes the scientific foundation for a public health response, surveillance is the single most important tool for identifying new or reemerging infectious diseases with potential to cause serious public health problems. Surveillance can be useful in rapidly identifying and monitoring persons at highest risk, changes in disease rates, modes of transmission, and groups at risk. Surveillance can help in planning and evaluating disease prevention and control programs and can improve capacity to control annual epidemics as well as the next influenza pandemic. The outbreak of human infection with H5N1 (avian) influenza in Hong Kong in 1997 highlighted the potential of new and lethal pathogens to emerge unexpectedly and questioned the capacity of local and state health agencies to expand surveillance activities in response to a possible pandemic influenza strain.

In 1993, a Working Group on Influenza Pandemic Preparedness and Emergency Response (GrIPPE), which included influenza experts from the public and private sectors, began to develop an updated, comprehensive blueprint for an action plan for pandemic influenza for the United States (2). GrIPPE identified surveillance as a key component of the pandemic plan. The group has also recognized that to effectively address the threat of an influenza pandemic, measures to reduce the impact of influenza must be in place and operational at the state and local level now, during the prepandemic period. Because more influenza-related illness and death occur in the aggregate during regularly recurring influenza epidemics than during the pandemics themselves, GrIPPE has attempted to link its plan to other relevant public health initiatives such as those related to emerging infections and adult immunization.

In 1994, the Council of State and Territorial Epidemiologists (CSTE) was asked by GrIPPE to participate in the national pandemic influenza planning process. As part of this effort, CSTE conducted a survey of state epidemiologists in March 1995 to assess current influenza surveillance systems; all 50 states and the District of Columbia responded. Questions sought to determine the source and type of illness reports received, the type of virologic surveillance activities performed, the state's perceived preparedness for a pandemic, obstacles in detecting a new pandemic strain, the need for a pandemic plan specific to the state's jurisdiction, whether more influenza surveillance would be conducted if additional resources were made available, and how much increased surveillance would cost.

All 51 respondents reported at least one source of influenza surveillance information, and 39 (77%) identified sentinel physicians as the primary source of disease reports. Forty-eight (94%) states had the capability, through some private or public health laboratory, to identify influenza viruses isolated in tissue culture. Of 47 laboratories that indicated to what degree they could characterize influenza viruses, 37 (79%) could subtype the viruses, while 10 (21%) could identify viruses only as influenza A or B. Another 1995 survey by the Association of State and Territorial Public Health Laboratory Directors of its membership was more specific in defining influenza virologic resources available at each state's public health laboratory, with 10 (20%) states indicating no state public health laboratory capacity to isolate viruses and 13 (25%) state public laboratories reporting no ability to subtype influenza isolates (A. Di Salvo, pers. comm.).

In the CSTE survey, 34 (67%) states responded that their laboratory surveillance system would be adequate to detect a new pandemic virus, with 29 (57%) states indicating that their disease surveillance system would be adequate. Among the reasons given for the difficulty in detecting a new pandemic strain were inadequate financial resources (20 [83%]), inadequate personnel (19 [79%]), and low disease priority (10 [42%]).

Only 29 (59%) states reported that an influenza pandemic plan specific for their jurisdiction was needed. Reasons for not developing a state pandemic plan included lack of resources 4 (31%), insufficient interest 4 (31%), and the perception that pandemic influenza did not pose an immediate threat 3 (23%). However, if targeted resources were made available, 44 (86%) respondents indicated that they would increase laboratory surveillance for influenza, and 39 (76%) indicated they would increase disease surveillance activities. The estimates provided for increasing surveillance activities were $2,000 to $100,000 (mean $37,602) for laboratory surveillance and $2,000 to $100,000 (mean $40,914) for disease surveillance.

In the United States, public health services (including surveillance) are provided most directly by municipal, county, and state health departments, or a combination of all three. The 1995 CSTE survey found that many states lacked surveillance activities dedicated to influenza; however, many states would expand virologic and disease-based surveillance systems if nominal resources were made available, despite the lack of urgency given to any pandemic planning effort at the state level.

Several efforts have been undertaken at the national level to respond to the states' needs and to promote enhanced preparedness for pandemic influenza. A Pandemic Influenza Planning Guide for State and Local Health Officials was developed as a result of these efforts. The guide provides a checklist or set of guidelines for states to develop their own pandemic plan. A draft guide was pilot-tested in six sites during February-March 1998.

The surveillance component of the planning guide calls for enhancements in virologic and disease-based surveillance and improvements in surveillance information systems. Specifically, the planning guide recommends that during the prepandemic period 1) virologic surveillance capability be improved by ensuring that at least one laboratory in each state or major metropolitan area can isolate and subtype influenza viruses; 2) disease-based surveillance capability be improved by expanding the existing sentinel physician network, with the aim of establishing a population-based system of approximately one sentinel physician per 250,000 population; 3) contingency plans be developed for enhancing state and local virologic and disease-based surveillance systems in the event of a novel virus alert or pandemic alert; and 4) electronic and telecommunications capability with neighboring jurisdictions and with the Centers for Disease Control and Prevention (CDC) be enhanced.

Because the antigenic properties of influenza viruses change constantly, surveillance to monitor the changes and their impact is necessary. One type of surveillance information without the other is of limited value.

Although a disease-based surveillance system for influenza is in place in the United States, the system is in jeopardy because of the misperception that influenza is no longer an important public health problem and because of continued erosion of resources supporting the public health infrastructure at the state and local levels. Moreover, influenza surveillance is compromised because influenza is not a nationally notifiable disease.

One component of the existing surveillance system is weekly reports to CDC's national notifiable disease system from each state epidemiologist designating the level of influenza activity during the preceding week. Levels of estimated activity are reported as widespread, regional, sporadic, or nonexistent. The validity of these estimates has long been questioned, since they may primarily reflect local interest or availability of resources. Data on associated disease incidence are only rarely collected.

Many states lack the financial resources for influenza virologic surveillance, which is critical for monitoring antigenic drift and shift of influenza viruses circulating among humans and is the basis for each year's vaccine formulation. State public health laboratories that perform virologic surveillance have a continuing need to culture and characterize isolates on a timely basis. Submissions of specimens for virus isolation are expected to decrease as rapid antigen test kits are improved and become more widely available. Having fewer isolates for characterization is a potential public health problem.

The demands of pandemic planning have prompted CDC and CSTE to begin changing influenza surveillance. The premise of changes is that a solid surveillance infrastructure must be in existence during the prepandemic period. Disease- and laboratory-based surveillance is being reinforced and other databases are

explored as potential sources of additional qualitative or quantitative data. Efforts are under way to upgrade the sentinel physician network by enlisting and retraining more participants, integrating influenza reporting with other state-based systems, standardizing reporting procedures, and developing a semiautomated data management system to provide rapid feedback. Twenty-eight states and the District of Columbia pilot-tested steps to revise the existing sentinel physician surveillance system during the 1997-98 influenza season. One benefit of such efforts may be to increase the public's, medical providers', and public health practitioners' understanding of influenza as a potentially preventable disease.

A national plan is an important first step in highlighting the public health problem posed by influenza and the need to identify ways in which the federal, state, and local public health community can combine efforts to address the problem.

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K. F. Gensheimer,* Keiji Fukuda,† Lynnette Brammer,† Nancy Cox,† Peter A. Patriarca,‡ and Raymond A. Strikas†

*Maine Bureau of Health, Augusta, Maine, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and ‡Food and Drug Administration, Rockville, Maryland, USA

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An Outbreak of Gastroenteritis in Japan due to *Escherichia coli* O166

To the Editor: Enteroaggregative *Escherichia coli* (EAggEC) heat-stable enterotoxin 1 (EAST1) was originally found as an enterotoxin of EaggEC (1). Recently, Yamamoto et al. (2) reported that the *EAST1* gene, or its variants, were present not only in EAggEC but in other diarrheagenic *E. coli,* including some enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC). Hedberg et al. (3) found that an outbreak of gastrointestinal illness in 1991 had been caused by EAST1-producing *E. coli* that possessed the EPEC gene locus for enterocyte effacement. We propose that *E. coli* producing EAST1 but possessing no other identifiable pathogenic properties may compose either a new group of diarrhea-associated *E. coli* or a new subgroup of ETEC.

In an outbreak of gastroenteritis on July 23, 1996, in Osaka, Japan, 54 of 91 persons attending a meeting held in an office building on July 22, 1996, became ill. The patients did not eat any common foods except the lunch served at the office. Symptoms were diarrhea in 52 (96%); abdominal pain in 32 (59%); nausea in 8 (15%); fever in 8 (15%); and vomiting in 5 (10%). The mean incubation period was 17 hours.

Stool specimens of 33 patients were examined, and *E. coli* O166 with an unidentifiable H antigen were isolated from 29 specimens. Laboratory tests for other bacterial pathogens and viruses were negative. The isolates showed the same DNA banding pattern in pulsed-field gel electrophoresis after treatment with the restriction enzymes *Xba* I or *Not* I.

The *E. coli* O166 organisms did not adhere to HEp-2 cells in a localized, diffuse, or enteroaggregative manner and did not give mannose-resistant hemagglutination of human or bovine red blood cells. Although the organisms were further analyzed for expression of known ETEC colonization factors by a dot-blot assay using specific monoclonal antibodies, they did not express CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, PCFO159, PCFO166, or CFA/III. In polymerase chain reaction (PCR) tests, the bacteria did not have coding genes for verocytotoxin of enterohemorrhagic *E. coli,* heat-labile, or heat-stable enterotoxin of ETEC, attachment and effacement (*eae*A) of EPEC, or invasion (*inv*E) of enteroinvasive *E. coli.* Consequently, they are not assigned to any of the recognized diarrheagenic groups of *E. coli*: EPEC, ETEC, enterohemorrhagic *E. coli,* enteroinvasive *E. coli*, EAggEC, and diffusely adhering *E. coli*. According to the PCR method of Yamamoto et al. (2), however, the organisms possessed the *EAST1* gene.

To our knowledge, this is the first report of an outbreak caused by EAST1-producing *E. coli* that did not have other well-characterized virulence genes. We believe that these strains should be assigned to a new subgroup of ETEC. Such strains would not be detected in most current surveys for diarrheagenic *E. coli*, as tests for EAST1 are rarely included. The role of EAST1 in pathogenicity has been controversial. We propose that diarrheal specimens be examined for EAST1-producing *E. coli* so that the distribution of these organisms worldwide can be determined.

Yoshikazu Nishikawa,* Jun Ogasawara,* Anna Helander, † and Kosuke Haruki*
*Osaka City Institute of Public Health and Environmental Sciences, Osaka, Japan;
†Göteborg University, Sweden

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Vibrio cholerae Outbreak in Italy

To the Editor: On 16 June, the microbiology unit of the Hospital of Lodi communicated to the local public health unit that *Vibrio cholerae* had been isolated and identified by standard biochemical tests in stool samples of an outpatient whose

clinical data were unknown. On the same day, we contacted and interviewed the patient to investigate risk factors for cholera. The patient reported abdominal pain starting on 6 June and then severe diarrhea (10 to 12 stools per day) until 13 June; on that day the patient went to his general practitioner, who gave him loperamide and suggested a coproculture. The patient never traveled to cholera-endemic areas; did not eat raw mussels, uncooked fish, or vegetables of uncertain origin or from cholera-infected areas; and did not swim in rivers or lakes. The patient reported that he ate a seafood salad in the canteen of his work place on 5 June and that three out of the four persons who ate the same kind of salad also had abdominal symptoms. Subsequently, the Istituto Superiore di Sanità (ISS) in Rome confirmed isolation of toxinogenic *V. cholerae* O1, biotype El Tor, serotype Ogawa, from stools from the index patient.

The canteen where the index patient had eaten the seafood salad was 1 of 17 supplied by a single cooking center that used a precooked, frozen, ready-to-eat product including shrimps, scallops, mussels, hen clams, cuttlefishes, and squid. Each product was cooked and frozen in the country of origin and mixed in Italy by an importer who packaged the seafood salad. Tracking the products around the world was difficult, but we learned that at least some had come from Far East countries where cholera is endemic. Approximately 125 servings of the same food were distributed within our local public health area (Azienda Sanitaria Locale) and more than 400 in other areas.

We performed an epidemiologic case-control investigation beginning 18 June involving 454 persons (94 who had eaten the seafood salad and 360 controls who had eaten in the same canteen any food except seafood salad); 37 (39%) of the persons who had eaten the seafood salad had had at least one episode of diarrhea or other relevant gastrointestinal symptoms, as compared to one (0.3%) of those who had not eaten it. We did not find symptomatic patients. The corresponding odds ratio was 233 (95% confidence interval, 97 to 560). No symptomatic person had to be hospitalized because of symptoms or required intravenous treatment; three or more loose or watery stools during a 24-hour period were reported in 24 cases. We performed coprocultures (using TCBS medium) between 23 June and 3 July of all 94 persons who had eaten the seafood salad. One positive coproculture for *V. cholerae* O1 Ogawa (same strain) was identified on 25 June; the isolation was subsequently confirmed by the ISS. This second patient with a positive culture worked in a factory in a different town. She had severe diarrhea on 6, 7, and 8 June. Her family doctor gave her rifaximin but did not ask for a coproculture, but a specimen was obtained on 23 June. She did not report risk factors for cholera infection, except having eaten seafood salad on June 5 in the canteen of her work place. The delay between exposure to *V. cholerae* and the coprocultures was longer than 1 week (median delay 26 days, range 19 to 31), and it is therefore not surprising that others who had eaten the seafood salad did not have positive results. Both the culture-positive index case-patient and the woman were recultured three more times; negative results were obtained.

The identification of this cholera outbreak is a sentinel episode confirming (1,2) that, if not adequately monitored, food preparation and distribution can cause serious infectious diseases in industrialized countries.

Luca Cavalieri d'Oro,*† Elisabetta Merlo,† Eugenio Ariano,† Maria Grazia Silvestri,† Antonio Ceraminiello,† Eva Negri,* and Carlo La Vecchia*‡

*Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy; †Azienda Sanitaria Locale della Provincia di Lodi, Lodi, Italy; and ‡Università degli Studi di Milano, Milan, Italy

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Shiga Toxin–Producing *Escherichia coli* O157:H7 in Japan

To the Editor: Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 infection, which can cause hemolytic uremic syndrome and death, is a global public health concern. Patients younger than 5 years of age are at high risk for hemolytic uremic syndrome (1) and shed the organism longer than adults (2). The public health

importance of this symptomatic shedding in transmission among preschool children is well established (3); however, that of symptom-free shedding in adults is unknown. We report here that the rate of symptom-free STEC O157:H7 shedding is higher in adults 30 to 49 years of age than in others.

STEC infections have been notifiable in Japan since August 1996. When STEC is found in the feces of patients in schools, families, and hospitals, local health centers and public health institutes must test (generally using MacConkey sorbitol agar with cefixime-potassium tellurite medium) for the pathogen in stool specimens of contacts of the patients. The pathogen is also sought twice a month in the stool specimens of food-handlers. All isolates from culture-positive patients are collected by Japan's National Institute of Infectious Diseases.

In 1997, 1,412 STEC O157:H7 human isolates were examined for subtyping of Shiga toxin genes *stx1* and *stx2* by polymerase chain reaction, for genotyping by *Xba*I-digested pulsed-field gel electrophoresis (PFGE) (4,5), and for their relationship with symptoms; 1,381 isolates (from culture-positive persons with well-characterized clinical status) were further analyzed. The rates by age group among STEC O157:H7-shedding persons reporting one or more symptoms (vs. culture-positive persons without symptoms) were as follows: 82% (475 of 576) younger than 10 years old; 81% (145 of 178), 10 to 19 years; 63% (98 of 156), 20 to 29 years; 25% (32 of 128), 30 to 39 years; 34% (34 of 100), 40 to 49 years; 54% (57 of 106), 50 to 59 years; 56% (38 of 68), 60 to 69 years; 68% (47 of 69), older than 70 years. Culture-positive persons under 20 years of age, especially children under 10 years of age, were more likely to have symptoms than other age groups. Intermediate rates of symptom-free persons with positive stool cultures occurred in young adults (20 to 29 years of age) and the elderly (70 years of age), while the highest rates of stool-positive, symptom-free persons were adults, especially those between 30 and 49 years of age. In terms of pathogen virulence, we did not find significant differences in the distribution of *stx* subtypes and PFGE genotypes between strains shed by symptomatic and asymptomatic persons. These results suggest that the rate of symptom-free STEC O157:H7 shedding may be associated with age rather than organism-related factors. Possible age-related host factors that could influence the presence of STEC O157:H7 in the stools of symptom-free persons include qualitative and quantitative differences in intestinal cross-reactive antibodies against STEC O157:H7, intestinal bacterial flora, or the sensitivity to Stx toxins between children and adults. Further investigations will be required to determine the relative importance of these and other host factors. Our finding of a high rate of asymptomatic shedding in adults may suggest the potential for secondary transmission of the bacteria from symptom-free STEC O157:H7-shedding adults to healthy children.

Acknowledgments

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Jun Terajima, Hidemasa Izumiya, Akihito Wada, Kazumichi Tamura, and Haruo Watanabe
National Institute of Infectious Diseases, Tokyo, Japan

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Streptococcus pyogenes Erythromycin Resistance in Italy

To the Editor: *Streptococcus pyogenes* resistance to erythromycin began to emerge as a serious problem worldwide in the early 1990s. In some areas in Italy, 30% to 40% of strains have beome

resistant (1-3). Throughout Italy, the use of macrolides, particularly the newest ones (azithromycin and clarithromycin), has increased in the treatment of infections caused by Group A streptococci. This therapeutic approach is contrary to current guidelines, which recommend using betalactam antibiotics as first-choice therapy and reserving macrolides only for patients allergic to betalactams.

In 1997 in Finland, a decrease was observed in the use of macrolide antibiotics in ambulatory patients from 2.40 defined daily doses per 1,000 inhabitants in 1991 to 1.38 in 1992. Subsequently, the maintenance of doses at 1.28 to 1.74 defined daily doses resulted in a substantial decrease in the percentage of group A streptococcal resistance to erythromycin, reported as 16.5% in 1992, 19% in 1993, 15.6% in 1994, 10% in 1995, and 8.6% in 1996 (4). These data prompted us to evaluate such phenomena in our geographic area, the urban area of Genoa, Italy (approximately 120,000 inhabitants).

From January 1991 to June 1998, 311 (6.1%) of 5,117 strains of *S. pyogenes* throat swabs from patients with pharyngotonsillitis were isolated. We observed a higher number of group A streptococci isolates from throat swabs starting in 1996 than we had in 1991 to 1995 (chi-square = 35.653, $p < 0.0001$). All isolates were tested for susceptibility to penicillin and erythromycin by standard susceptibility tests (broth microdilution) as recommended by the National Committee for Clinical Laboratory Standards. All isolates were susceptible to penicillin. From 1991 to 1996, the percentage of *S. pyogenes* resistant or with intermediate resistance to erythromycin increased from 0% to 50% (1992, 6%; 1993, 13%; 1994, 14%; 1995, 24%; 1996, 50%). In 1997 and the first half of 1998, resistance to erythromycin decreased to 39% and 34%, respectively. The number of resistant strains before 1996 was significantly lower than from 1996 to 1998 (chi-square = 50.386, $p < 0.0001$). Analysis of antibiotic consumption in our district showed an increase in the use of macrolides (erythromycin and the new compounds clarithromycin and azithromycin) from 0.445 defined daily dose per 1,000 inhabitants in 1994 to 1.140 in 1996. In 1997 and in the first half of 1998, consumption decreased to 0.9 and 0.8, respectively; we observed a correlation between the number of resistant isolates and the defined daily dose increase (correlation $[R^2] = 0.795$, $p = 0.0153$).

S. pyogenes resistance to erythromycin rose from 6% to 50% in only 4 years and then rapidly decreased from 50% to 34% in an 18-month period, corresponding to a 57% decrease in defined daily dose (from 1.41 in 1996 to 0.8 in the first half of 1998). Our data suggest that *S. pyogenes* resistance to erythromycin is associated with frequency of macrolide use.

Matteo Bassetti, Enrico Mantero, Giorgio Gatti, Antonio Di Biagio, and Dante Bassetti
University of Genoa, G. Gaslini Children's Hospital, Genoa, Italy

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Estimated Incidence of *Clostridium difficile* Infection

To the Editor: Since the publication of our article Increasing hospitalization and death, possibly due to *Clostridium difficile* diarrheal disease (1), we have received several requests to estimate the incidence of *C. difficile* infection. Our original study included only hospitalized patients treated at the Lovelace Medical Center from 1993 to 1996, and no information on the incidence of *C. difficile* infection. In response to these requests, we used inpatient and outpatient medical claims for the Lovelace managed care population to calculate incidence rates. We searched medical claims for the Lovelace Health Plan/Senior Plan (LHP) to identify patients who had a *C. difficile* diagnosis between January 1, 1993, and December 31, 1997. LHP members are residents of New Mexico, most residing in or near Albuquerque.

LHP had approximately 713,000 person-years of enrollment from 1993 to 1997. We identified 104 members with a *C. difficile* diagnosis on a claims record during this period. This group includes most of the patients in our original study. Most patients (62.5%) were identified exclusively from inpatient records; another 15.4% had both an inpatient and an outpatient record with a *C. difficile* diagnosis; and 22.1% had only an outpatient *C. difficile* diagnosis. We calculated an age-adjusted rate of infection (adjusted to the 1990 U.S. population), for each year and for the 5-year period. The incidence of *C. difficile* infection for all members during 1993 to 1997 was 14.8 cases per 100,000 person-years of enrollment. The patients rates for male and female were essentially the same (14.4 vs. 15.5, respectively). The rates increased dramatically with age. For persons ages 0-4, the age-adjusted rate per 100,000 person-years of enrollment (number of cases) was 5.3 (2); for 5-14, 2.7 (3), for 15-24, 2.2 (2); for 24-34, 6.4 (6); for 35-44, 9.2 (12); for 45-54, 15.7 (17); 55-64, 16.8 (10); 65-74, 38.5 (19); and 75+, 98.9 (33). The overall average rate of infection was 15.4; there were 104 cases.

The rate of infection may have declined since 1993 in this population. The 1993 rate was 24.5 per 100,000 person-years of enrollment, declining to 11.1 in 1997 (1993, 24.4; 1994, 19.1; 1995, 9.9; 1996, 12.3; and 1997, 11.1).

Our method for estimating rates has some limitations. We did not examine laboratory records to confirm the diagnosis. In addition, some laboratory-confirmed infections may not have resulted in a claims record with a *C. difficile* diagnosis. The Lovelace managed-care population is an insured, generally healthy population that may not have the characteristics of patients in other health care delivery settings or, because of its geographic restriction, the characteristics of the general U.S. population. Nevertheless, these estimates provide a basis for determining the magnitude of the public health problem of *C. difficile* infection. Additional surveillance studies are needed to better estimate the incidence of infection and to determine whether the incidence has declined during recent years.

Floyd Frost,* Judith S. Hurley,*
Hans V. Petersen,* and Roman N. Casciano†
*Southwest Center for Managed Case Research, Albuquerque, New Mexico, USA; and †Analytica Group, Ltd., New York, New York, USA

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Diphtheria in Eastern Nepal

To the Editor: Diphtheria, caused by *Corynebacterium diphtheriae*, was a major childhood killer until the advent of the Expanded Program on Immunization when diphtheria, pertussis, and tetanus (DPT) vaccination was greatly increased; diphtheria gradually declined in many countries. We report two cases of diphtheria identified at the B.P. Koirala Institute of Health Sciences Hospital, Dharan, Nepal.

During April 1996, a 6-year-old patient had fever (for 5 days), difficulty in swallowing and breathing, and change of voice (for 4 days). Throat examination showed a grayish-white membrane over the right tonsil, uvula, and adjacent soft palate. The membrane could not be removed, and the larynx could not be examined. Swabs were taken from the membrane area and sent to the laboratory, where smears were made and stained by Gram and Albert stains. Gram-stained smears showed a large number of gram-positive bacilli with the appearance of Chinese letters, and Albert stain showed bacilli with numerous metachromatic granules. A diagnosis of faucial diphtheria, with a strong possibility of laryngeal diphtheria, was made. The patient was treated with parenteral penicillin and diphtheria antitoxin. His condition improved after 6 days of therapy.

In December 1996, a 9-year-old patient sought treatment for chronic pain and discharge in the left ear. On examination, he had mucopurulent discharge, antral perforation, and mastoid tenderness. Throat examination showed bilateral tonsilitis. A provisional diagnosis of acute mastoiditis associated with acute septic tonsillitis was made. Throat swabs were collected and sent to the laboratory; smear findings showed typical organisms morphologically resembling *C. diphtheriae.* Culture done on 10% defibrinated sheep blood agar and Loefflers serum slope grew colonies consistent with *C. diphtheriae.* In addition to local antibiotic to the ear, the patient was given parenteral penicillin, gentamicin, and metronidazole. Because the patient had no features of systemic

toxicity, no antidiphtheria serum was administered. The patient became well and was discharged on day 4.

In the first case, a throat culture could not be done because the patient had already received local antiseptic paint. However, the diagnosis was clinically consistent with classic diphtheria with features of toxicity. In the second case, diphtheria was suspected only after bacteriologic examination. Unlike patient 1, patient 2 had no evident features of systemic toxicity. Hence the isolate could be nontoxigenic. Localized diphtheria due to nontoxigenic *C. diphtheriae* is known to occur (1).

The two patients did not give a complete history of immunization and may not have been vaccinated (or may have been partially vaccinated) with DPT. On the Indian subcontinent, DPT vaccination coverage is reported to be 80%. However, it may not be so in all areas, and immunization may have decreased to approximately 50% in certain areas of Southeast Asia (2). This may also be true in certain areas of eastern Nepal. An immunization status survey done in midwestern Nepal from 1989 to 1990 showed that DPT coverage was unsatisfactory (3). Lack of sustained immunization may even result in outbreaks. The recent epidemics of diphtheria in the Ukraine, Russian Federation, and other countries of the former Soviet Union are examples of resurgence due to ineffectively maintained immunization programs (4,5).

Diphtheria, still occasionally seen in many Southeast Asian countries including India and Nepal, is thought to be declining in these areas. However, accurate data have not been recently available, particularly from Nepal, because reporting is infrequent, laboratory confirmation is not available, and the extent of carriers is not clearly known (2).

These two cases show the persistence of diphtheria in a population in Nepal immunized with DPT and underscore the need for careful surveillance, laboratory documentation of clinical diphtheria, and increased immunization of children in this area.

H. Srinivasa, S.C. Parija, and M.P. Upadhyaya
B.P. Koirala Institute of Health Sciences,
Dharan, Nepal

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Commercial Use of *Burkholderia cepacia*

To the Editor: In their review of the potential threat to human health by the commercial use of *Burkholderia cepacia*, Holmes et al. (1) focus on the biopesticidal uses of this bacterium in agriculture. By virtue of its ability to antagonize a number of soilborne plant pathogens, *B. cepacia* is an attractive natural alternative to currently used chemical pesticides, such as captan, mancozeb, and metalaxyl. The replacement of these highly toxic agents, which are among the mainstays of crop protection chemicals, by safer products is a laudable goal. However, despite being nonpathogenic to healthy humans (and thus classified as a Biosafety Level 1 species), *B. cepacia* can cause life-threatening pulmonary infection in persons with cystic fibrosis. Holmes et al. call for a moratorium on the use of *B. cepacia* in agriculture until more is known about risks from such use.

Perhaps of greater concern than agricultural use is *B. cepacia*'s use as a bioremedial agent. Holmes et al. only briefly refer to the capacity of this species to degrade chlorinated aromatic substrates such as those found in certain pesticides and herbicides. By virtue of its extraordinary metabolic versatility, *B. cepacia* can use such compounds as nutrient carbon energy sources. In addition, some strains produce enzymes capable of degrading nonnutritive substrates, such as trichloroethyl-

ene (TCE), a major ground water contaminant used in the dry cleaning industry and in degreasing solvents.

The degree to which *B. cepacia* is being used in bioremediation products is unknown; however, the species has been used extensively to degrade ground water TCE contamination in at least one large U.S. city. A number of environment-friendly bioremediation products containing only naturally occurring, nonpathogenic bacteria are being marketed for use in drain opening and grease eradication systems. Because their formulations are proprietary, it is not known if these products contain *B. cepacia*; however, franchises that distribute such totally natural, noncorrosive, nontoxic products specifically target fast-food restaurants, photo processing facilities, and hospital radiology departments.

In the United States, the biopesticidal use of microorganisms such as *B. cepacia* is regulated by the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act; however, the use of naturally occurring, nonpathogenic bacteria as bioremedial agents is essentially unregulated. Only new microorganisms (i.e., intergeneric or formed by combining genetic material from organisms in different genera) are regulated by EPA under the Toxic Substances Control Act (TSCA) (2). Ironically, TSCA regulations provide a strong disincentive to the development of safer microbiologic alternatives for use in bioremediation. For example, although the genetic elements responsible for TCE degradation by *B. cepacia* have been cloned, their recombination into another nonpathogenic bacterial host (e.g., *Escherichia coli*) would constitute a new microorganism, the licensure of which would be considered prohibitively time-consuming and expensive by many companies.

In Canada, biopesticidal uses of microorganisms are regulated by the Pest Management Regulatory Agency of Health Canada, under the Pest Control Products Act (PCPA); bioremedial uses are regulated by Environment Canada under the Canadian Environmental Protection Act (CEPA) (3). Both naturally occurring and genetically engineered microorganisms are strictly controlled under these acts. However, accurate species identification is the cornerstone of all notification of products under the Canadian regulations. This presents a further dilemma. At least five genomovars (discrete species) constitute what has recently been designated the "*B. cepacia* complex" (4). Insofar as the taxonomy of this group is poorly defined, there are no conventional taxonomic designations to distinguish pathogenic from nonpathogenic species. At present, it appears that all five *B. cepacia* genomovars are capable of causing infections in vulnerable persons (4).

Because the epidemiology of *B. cepacia* complex infection in humans is incompletely understood, the threat posed by the inclusion of this species in biopesticides and bioremedial products is difficult to quantify. However, we agree with Holmes et al. that such use should be approached with considerable caution. In a broader context, the commercial use of *B. cepacia* illustrates our incomplete understanding of nonpathogenic bacteria and their potential to cause human disease. Regulations governing the use of microorganisms in industry must constantly adapt to keep pace with the emergence of infections due to nonpathogens and limit risk to human health.

John J. LiPuma* and Eshwar Mahenthiralingam†
*MCP Hahnemann University, St Christopher's Hospital for Children, Philadelphia, Pennsylvania, USA; and †University of British Columbia, Vancouver, British Columbia, Canada

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Human Rabies in Israel

To the Editor: Rabies, a major zoonotic disease in the Middle East, has two main epidemiologic forms: urban and sylvatic. The last case of

human rabies in Israel was in the Golan Heights in 1971 (1). Twenty-five years later, in 1996, rabies was reported in a 20-year-old soldier, and then two cases were documented in 1997.

The first case-patient, a soldier in the Golan Heights, was bitten on the lip by an unidentified animal while sleeping. The wound was cleansed and sutured; clinical signs started 39 days later with high fever and headache. The patient was admitted to an emergency room with hallucinations, difficulty in swallowing, and generalized weakness, and rabies was considered in the differential diagnosis; 3 days later the patient became comatose. Samples of saliva, serum, and cerebrospinal fluid; skin biopsy tissue; and corneal impressions were sent to the Pasteur Institute, Paris, France. Eight days after clinical signs developed, rabies was diagnosed by the Kimron Institute by heminested reverse transcription-polymerase chain reaction (hnRT-PCR) on the patient's saliva (2). The RT step was performed with the specific primer 113 (5'-GTAGGATGATATATGGG-'3 at 1013-1030), followed by PCR with the 509 (5'-GAGAAAGA ACTTCAAGA-'3 at 1156-1173) and 304 (5'-GAGT CACTCGAATATGTC-'3 at 1513-1533) primers. The hn-PCR was performed with the 509 and 105 (5 ' - T T C T T A T G A G T C A C T C G A A T A TGTCTTGTTTAG-'3 at 1393-1426) primers (3). The PCR results were confirmed by the Pasteur Institute 3 days later, and the patient died 35 days after clinical symptoms appeared.

The second case-patient was a 7-year-old girl admitted to the hospital unconscious. Two months before admission, she had been scratched while sleeping by an unidentified animal. On the second hospital day, generalized convulsions and gasping occurred. During the following days, brain stem function progressively deteriorated. Rabies was diagnosed by hnRT-PCR on the saliva sample, and the diagnosis was confirmed by the Centers for Disease Control and Prevention (CDC). The patient died despite supportive care.

The third case-patient, a 58-year-old man with fever, headache, and sore throat, was diagnosed as having pharyngitis and received an oral antibiotic. The patient had been bitten 3 months earlier while sleeping. On admission, the lumbar puncture, computerized tomography scan, and electroencephalogram were normal. On the third hospital day, he had respiratory arrest; during orotracheal intubation, acute laryngospasm with copious amounts of salivation occurred. Rabies was suspected, and viral RNA in the saliva was detected by hnRT-PCR. One day later the patient died.

We injected antemortem saliva and postmortem brain tissue from these patients into suckling mice intracerebrally. Virus was isolated from saliva samples of case-patients 1 and 3 but not from the sample of case-patient 2. Rabies virus antigen in the brain tissue was confirmed by direct immunofluorescence assay, and viral RNA was detected by RT-PCR.

For genetic analysis, we used brain samples from the three case-patients and from animals that died of rabies near the location of the case-patients to amplify and sequence a 328-bp (264 bp from the 3' of the N gene and 64 bp of the 3' NS-N region) fragment. On the basis of homologic results of nucleotide sequences in the three case-patients and in virus isolates from animals in the same regions, we concluded that a reservoir for rabies in foxes is responsible for infection of all three humans.

The three human isolates were tested with a panel of 19 anti-N protein monoclonal antibodies (CDC, Atlanta, GA, USA) and compared with those of rabies isolates from the geographic vicinity of the human cases. Isolates from case-patients 1 and 3 belonged to variant 1 (MAb C18 negative) and were similar to virus isolates from 10 foxes, one jackal, and four cattle in the same regions. Isolates from case-patient 2 belonged to antigenic variant 2 (MAbs C2, C7, C12, C13, C18 negative) and were similar to isolates from four foxes, one dog, and one cow in the vicinity of the second case-patient.

Early antemortem diagnosis of virus in an infected human is very important. Checking for virus in saliva eliminates the difficulty of tissue sampling from humans with suspected cases of rabies, and the sensitivity of hnRT-PCR makes it the technique of choice for detecting limited amounts of virus. Previous work showed that a 200-bp region of the N gene had only one nucleotide difference between them (4). Moreover, two samples from a region in western Mexico, isolated 30 years apart, were identical in sequence (4). Incorporation of the reference strains Pasteur and SAD B19 into our phylogenetic tree indicated that the three human viruses we isolated belong to lyssavirus genotype 1.

Dan David,* Charles E. Rupprecht,† Jean Smith,† Itzhak Samina,* Shmuel Perl,* and Yehuda Stram*
*Kimron Veterinary Institute, Bet Dagan, Israel; and †Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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Emerging Infections and Disease Emergence

To the Editor: Emerging infections have been defined as diseases whose incidence in humans has increased within the last 2 decades or threatens to increase in the near future (l). This definition, with minor variations, has continued to be used, although occasional debate erupts over whether one disease or another is truly emerging. Use of the term "emerging" has facilitated communication about the changed pattern of infectious diseases in recent years. While the study of infectious organisms and clinical training about emerging infections are manifestly necessary, they are not a sufficient foundation for understanding the process of disease emergence. I would propose, specifically, that we distinguish between emerging diseases—the study of specific infections that are changing—and the study of disease emergence.

Studies of emerging infections typically rely on disease, organismic, or syndromic approaches. Meetings on emerging infections typically cover newly recognized or characterized organisms or diseases and update information about the recognition, diagnosis, treatment, prevention, and control of these infections. This ongoing education is essential for practicing clinicians, who finished their formal training before AIDS, Lyme disease, ehrlichiosis, *Helicobacter pylori* infection, cryptosporidiosis, cyclosporiasis, and many other infections were described. These meetings also help clinicians learn how to fit new information into their existing knowledge base: What is the probability that a person with rash and fever has ehrlichiosis and that a person with fever and pulmonary infiltrates has hantavirus pulmonary syndrome?

By contrast, understanding the process of disease emergence involves studying the origins and ecology of emerging infections. Many disciplines relevant to disease emergence lie outside traditional infectious disease training and research and include evolutionary biology, demography, population dynamics, ecology, vector biology, climatology, epidemiology, genetics, veterinary medicine, and behavioral sciences (2). Infectious diseases of animals and plants have both a direct and indirect impact on human health. The study of infectious diseases in other species may provide important insights into understanding the process of disease emergence in humans. The study is also relevant to understanding the species-to-species spread of organisms.

Tools used to study and understand disease emergence include mathematical modeling, geographic information systems, remote sensing, molecular methods to study the genetic relatedness of organisms, and molecular phylogeny. Paleobiology, paleoecology, and studies that allow the reconstruction of past events may help inform future research and policy.

A major challenge is to reach people with relevant skills, knowledge, and experience and develop a coherent framework to advance the understanding of the process of disease emergence. No one institution, organization, or country can itself prevent or manage emerging infectious diseases.

In the study of emerging infections we focus on the organism, the patient, and the human population. The study of disease emergence must be at the systems level and must look at ecosystems, evolutionary biology, and populations of parasites and hosts, whatever their species. A primary goal should be to identify conditions or combinations or sequences of events that herald a changed pattern of infections so that preventive strategies can be used.

Mary E. Wilson
Mount Auburn Hospital, Cambridge, Massachusetts, USA; Harvard Medical School and Harvard School of Public Health, Boston, Massachusetts, USA

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Malaria Control in South America

To the Editor: The article by Roberts et al. regarding DDT use and malaria in South America (1) correctly observes that health policy makers have shifted the emphasis of malaria control programs from vector control to case detection and treatment and that malaria control has been woefully underfunded in recent years. However, their conclusions that increased malaria is due to decreased spraying of homes with DDT and that DDT is still needed for malaria control do not withstand close scrutiny.

The authors did not mention several factors influencing malaria increase in recent decades, including growing antimalarial-drug resistance, the deterioration of public health systems responsible for malaria control, and large-scale migration to areas at high risk for malaria (e.g., almost all Brazilian malaria cases occur in the Amazon region) (2,3). Extradomiciliary malaria transmission, poor housing conditions, and human behavior in frontier areas such as the Amazon region limit the usefulness of insecticides. Thus, the deduction of causality between less house spraying with DDT and increased malaria incidence under those circumstances is questionable.

Roberts et al. have not actually linked increased malaria with eliminating DDT use but rather with eliminating house spraying altogether, without implementing effective alternatives. Malaria's recent decline in Brazil is due to a strategy that combines health education, aggressive case detection and treatment, and environmental management to eliminate *Anopheles* breeding sites (C. Catão Prates, unpub. data). A similar strategy has sharply reduced malaria incidence and deaths in Colombia (W. Rojas, unpub. data). In Mexico, use of two synthetic pyrethroid insecticides (deltamethrin and lambda cyhalothrin) for bed-net treatment and house spraying is controlling malaria at a much lower cost than the use of the alternative insecticides tried earlier and mentioned by Roberts et al. (4). Far from being pursued "without meaningful debate," the reduction and phaseout of DDT and other persistent organic pollutants is the subject of a 3-year United Nations–facilitated global negotiation process begun in June 1998.

Roberts et al. assert that DDT applied indoors does not move easily from the application site; however, a mass balance model indicates that 60% to 80% of the DDT ends up outdoors within 6 months (K. Feltmate, A model and assessment of the fate and exposure of DDT following indoor application [bachelor's thesis]. Ontario: Trent University; 1998). From there, DDT can be transported long distances in air, waterborne sediments, and biota, accumulating in humans and other nontarget species (5). Meanwhile, residents of sprayed houses accumulate high, persistent body levels of DDT through skin contact and food contaminated with DDT from air and dust (6).

Long considered a probable human carcinogen, DDT also is associated with reduced lactation, premature births, absorbed fetuses, and lower birth weights (7-9). In addition, recent animal research has raised the possibility that exposure of human fetuses or infants to DDT may cause permanent behavioral changes and impairment of body systems (10-12).

Synthetic pyrethroid insecticides used on bed nets or for house spraying against malaria-infected mosquitoes seem safer for human health than DDT because humans and other mammals possess the ability to hydrolyze the pyrethroids rapidly and excrete them from the body (13-14). Nevertheless, DDT and pyrethroids share known health risks, notably endocrine disruption, and the possible transgenerational consequences of chronic human exposure to pyrethroids have not yet been studied (10,15-16). Optimal protection of human health requires the development of integrated malaria control strategies that eliminate or reduce routine insecticide use by taking maximum advantage of environmental management, biological controls, and other nonchemical vector control measures (17).

Patricia C. Matteson
U.N. Food and Agriculture Organization
Programme for Community Integrated Pest
Management in Asia, Hanoi, Vietnam

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Malaria Control in South America—Response to P.C. Matteson

To the Editor: Dr. Matteson, whose letter relies heavily on unpublished information and nonrefereed publications, states that growing drug resistance has contributed to increasing malaria. While drug resistance is important, when DDT use declined below effective levels (1), the proportion of *Plasmodium falciparum* infections (including infections with resistant strains) compared with *P. vivax* infections (no resistance) did not progressively increase (2). Moreover, malaria has increased in Central America, where drug resistance is unknown (3-6). As for attributing increasing malaria to deteriorating public health systems, the changes imposed on developing countries (in organizational structures of malaria control programs and prohibiting DDT [1,7]) correlate with increasing malaria rates (1).

Dr. Matteson states that large-scale migration explains why almost all Brazilian malaria cases occur in the Amazon Basin. However, DDT cleared malaria from the more populated and temperate southern regions of the country (8, unpublished report: U.S. Agency for International Development review in 1973-74 of Brazil's malaria eradication program). When DDT was in full use (pre-1980), large increases in malaria did not accompany population movement (1). With the 1970s' colonization program of the Basin came malaria problems, but not large population-based malaria increases. DDT prevented that (1,9-11). However, since DDT has been eliminated, persistent urban malaria is again becoming a problem (12-16).

Other factors (biting behavior, housing conditions, and human behavior), which Dr. Matteson attributes to increasing malaria, have always thwarted interdiction of malaria transmission in the Amazon Basin (17;18; an unpublished report: U.S. Agency for International Development review in 1973-74 of the malaria eradication program in Brazil) and are no more important today than they were before.

A UN-facilitated global negotiation process cited as a meaningful debate for malaria control is an effort to provide a legally binding agreement for global elimination of DDT and other persistent organic pollutants, not an open forum for debate of DDT use for malaria control.

Dr. Matteson claims that DDT is associated with reduced lactation. In the United States, where DDT has been banned for 26 years, mothers who stay home breast-feed for an average of 25.1 weeks—mothers who work parttime, for 22.5 weeks (19). In Belize, mothers in urban areas, where DDT is not used for malaria control, breast-feed less than 38.4 weeks—mothers in rural areas with lifetime exposures to DDT breast-feed more than 57.2 weeks (20).

The World Wildlife Fund's mass balance model of DDT sprayed in houses used to refute our assessment that DDT does not readily move away from sprayed houses also mentions that "There are few...data against which to validate the results of this...model, although actual data...should not be difficult to obtain." (21). Studies of DDT use in agriculture show that most DDT settles where it is applied (22).

Studies have shown no meaningful population-based adverse health effects from DDT use, despite more than 50 years' exposure, and evidence argues forcefully that DDT does not cause breast cancer (23).

Donald R. Roberts and Larry L. Laughlin
The Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

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On the Etiology of Tropical Epidemic Neuropathies

To the Editor: In a recent report of an epidemic of optic neuropathy in Dar es Salaam, Tanzania (1), Dolin et al. state that the disease is clinically identical to one of the forms of epidemic neuropathy found in Cuba between 1991 and

1993 (2). Cases of peripheral neuropathy have been part of both epidemics (1,2). Both epidemics occurred in nutritionally deficient populations (1,3).

Dolin et al. state that the cause of the Tanzanian epidemic is unknown and probably difficult to establish; however, we believe findings from the Cuban epidemic could be used to study the etiology of this and other tropical epidemic neuropathies.

In Cuba, several research groups isolated and characterized an enterovirus in the cerebrospinal fluid (CSF) of epidemic neuropathy patients (4,5). Enterovirus sequences were found in CSF of 40 (36%) of 111 epidemic neuropathy patients versus 1 (8%) of 12 control surgical patients ($p < 0.01$, chi-square test with 2 x 2 contingency tables) (5). Recently, this enterovirus has been shown to form quasispecies, which could account for altered biologic properties (de la Fuente et al., submitted for pub.). We thus propose that epidemic neuropathy has a nutroviral etiology: Nutritional deficits and stress make the population more likely to become ill after infection with enterovirus quasispecies with altered biologic properties.

The relationship between the host's nutritional status and virus evolution could be key in understanding the cause of epidemic neuropathy, the Tanzanian epidemic of optic neuropathy, and other tropical epidemic neuropathies. Etiologic factors must be identified before appropriate intervention and treatment strategies can be implemented.

José de la Fuente and María P. Rodríguez
Centro de Ingeniería Genética y Biotecnología,
Havana, Cuba

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Risk for Ebola Virus Infection in Côte d'Ivoire

To the Editor: In Taï National Park, Côte d'Ivoire, where a new strain of Ebola virus was isolated (1), the World Health Organization is conducting a project to identify the reservoir of the virus and evaluate the risk for its emergence in local populations. In March 1998, we conducted qualitative and quantitative surveys of the villagers' awareness of and risk for Ebola infection. In four villages close to Taï National Park (4 km to 10 km), we carried out structured interviews with 150 villagers and in-depth interviews with 17 villagers and three traditional healers.

Of the 150 villagers participating in the structured interviews, 18.0% had heard of Ebola (90.7% had heard of yellow fever). Of those aware of Ebola, 96.3% thought it life-threatening; 65.4% of them thought it preventable. When ill, 81.2% of the respondents generally relied on traditional healers or herbal medicine. During in-depth interviews traditional healers discussed their treatment practices. In one treatment, an incision is made on the skin and medicinal herbs are applied to the incision. Such traditional practices were implicated in the spread of Ebola virus in Gabon, where a traditional healer and his assistant (who were infected with Ebola virus) were suspected of spreading the virus to their patients through an unsterilized blade (1). The same practices would seem to pose a risk for virus transmission in Côte d'Ivoire.

Even though officially Taï National Park is protected from human activities to preserve its natural ecology, 84.0% of the 150 respondents to our survey often hunted or farmed in the park, 62.2% had encountered chimpanzees, and 53.3% had eaten chimpanzee meat. According to the in-depth interviews, chimpanzee meat is available at bush meat markets and is thought safe for eating, even though primates infected with Ebola virus have been linked with human cases (2,3).

Our survey results show that, even though no large-scale Ebola outbreaks have occurred in this area, villagers living near the park are at particularly high risk for infection because they are not aware of Ebola and do not know that their local customs and behavior may be putting them at risk. To prevent future Ebola epidemics in Africa, information, education, and communication (IEC) programs should be established (3). Moreover, further sociocultural studies on perceptions and behavior should be conducted in addition to exploring the nature of the virus and its cycle in the wild (2,4,5).

Osamu Kunii,* Pierre Formenty,† Jeanne Diarra-Nama,‡ and Noël Nahounou‡

*International Medical Center of Japan, Tokyo, Japan; † WHO Taï Forest Project, Abidjan, Côte d'Ivoire; ‡Ministère de la Santé Publique et des Affaires Sociales, Abidjan, Côte d'Ivoire

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Automation in Threat Reduction and Infectious Disease Research: Needs and New Directions

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A scientific colloquium entitled Automation in Threat Reduction and Infectious Disease Research: Needs and New Directions will be held in Washington, D.C., from April 29 to 30, 1999. Sponsors are the Association for Laboratory Automation; Centers for Disease Control and Prevention; Department of Energy; Department of Health and Human Services; Los Alamos National Laboratory; National Academy of Engineering; and University of California, Los Angeles.

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Emerging Pathogens Initiative: An Automated Surveillance System

The Veterans Health Administration, Department of Veterans Affairs (VA), debuted its nationwide computer-based Emerging Pathogens Initiative on October 1, 1998. The initiative is an automated surveillance system that collects data from all 171 VA medical centers (from 146 reporting sites) on 14 specific pathogens or diseases: vancomycin-resistant enterococcus, penicillin-resistant pneumococcus, *Escherichia coli* O157, *Candida* bloodstream infections, *Clostridium difficile*, *Cryptosporidium*, dengue, antibody-positive hepatitis C, Legionella, leishmaniasis, malaria, tuberculosis, group A streptococcus, and Creutzfeldt-Jakob disease. Other information (e.g., patient demographics, antimicrobial susceptibility where appropriate, co-morbidities, and number of patients by facility) is also collected and downloaded into a central database on a monthly basis. After aggregate reports are compiled, the 22 VA patient-care networks will receive network-specific data, along with national VA quartile rankings. For further information, contact Gary A. Roselle, M.D., program director for infectious diseases, VA Headquarters, at 513-475-6398.

Erratum Vol. 5, No. 1
In the article, "Genetic Diversity and Distribution of *Peromyscus*-Borne Hantaviruses in North America," by Martha Monroe et al., on page 85, reference 8 should read Mills JN, Ksiazek TG, Ellis BA, Rollin PE, Nichol ST, Yates TL, et al. Patterns of association with host and habitat: Antibody reactive with Sin Nombre virus in small mammals in the major biotic communities of the southwestern United States. Am J Trop Med Hyg 1997;56:273-84. We apologize to our readers for this error.

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

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

Manuscript Submission

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

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS C-12, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

Types of Articles

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

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

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

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

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

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

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

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

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