EMERGING INFECTIOUS DISEASES

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

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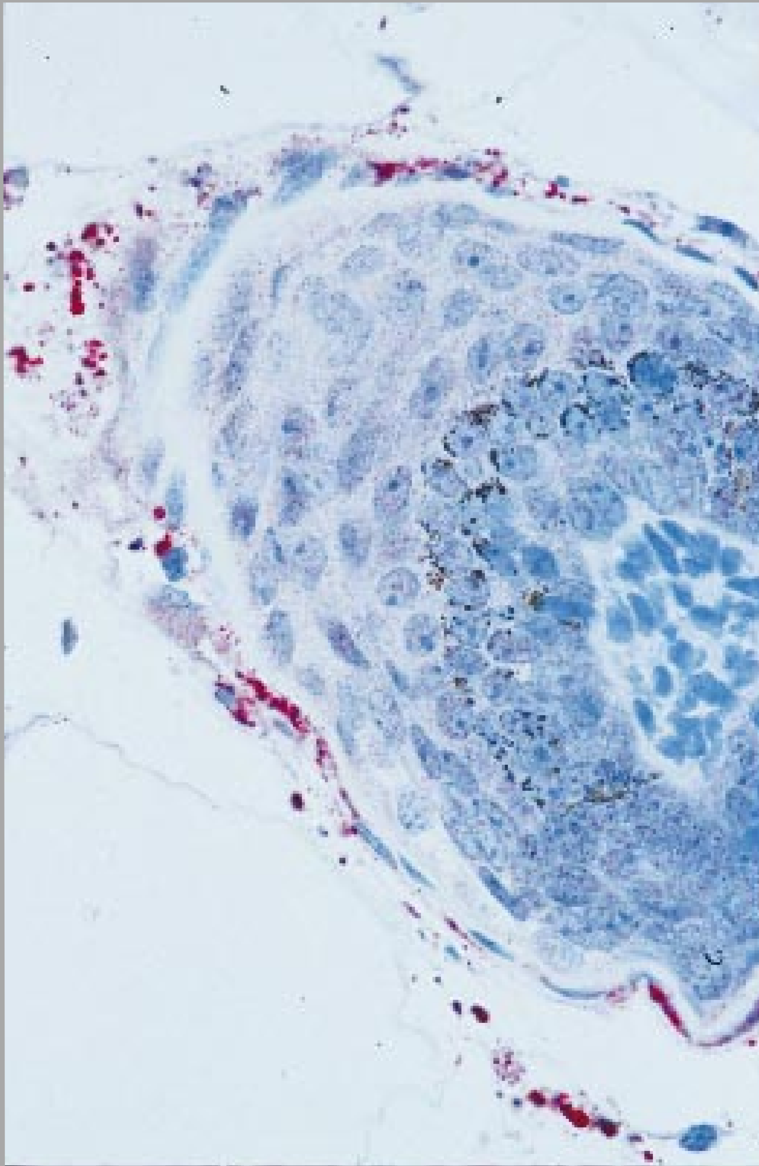

International Regulations

Syphilis Spirochetes

Mycoplasmas

Fluoroquinolone Resistance

Ebola Virus

CJD in Canada

C. pseudodiphtheriticum

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service

CDC
CENTERS FOR DISEASE CONTROL AND PREVENTION

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Emerging Infectious Diseases

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Cover photo: Immunostaining of Ebola virus antigens within subepithelial vascular endothelial cells in a skin biopsy from a chimpanzee that died during the 1996 Gabon outbreak; Dr. Sherif R. Zaki, Centers for Disease Control and Prevention, Atlanta, GA.

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Designing an International Policy and Legal Framework for the Control of Emerging Infectious Diseases: First Steps

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As the pace of emergence and reemergence of infectious diseases quickens, the International Health Regulations, which have served as the legal and policy framework of epidemic control for 45 years, are being revised by the World Health Organization (WHO). In this article, we review the recent history, legal construction, and application of these regulations and related international treaty-based sanitary measures, especially the General Agreement on Tariffs and Trade and the Agreement on the Application of Sanitary and Phytosanitary Measures, and the history of applying the regulations in the maritime and aviation industries. This review indicates that revision efforts should address 1) the limited scope of disease syndromes (and reporters of these syndromes) now in the regulations and 2) the mismatch between multisectoral factors causing disease emergence and the single agency (WHO) administering the regulations. The revised regulations should expand the scope of reporting and simultaneously broaden international agency coordination.

The Division of Emerging and Other Communicable Disease Surveillance and Control, World Health Organization (WHO), is revising the often criticized International Health Regulations (referred to as regulations hereon), the central legal framework for addressing the international spread and control of infectious disease (1,2). This process is under way as emerging and reemerging infectious diseases are the objects of broadening efforts and analysis by the national (3) and international public health communities (4). At least 30 new diseases have emerged in the last 20 years, and still others, including tuberculosis, malaria, cholera, dysentery, and pneumonia, have developed varying degrees of resistance to antimicrobial drugs (4).

The Intersectoral Nature of the Factors Involved in Disease Emergence

Infectious diseases are emerging in the increasingly global context of commercial and demographic activities. The journey of microbial agents from one country to another, often shorter than the incubation period of the disease, is rendering border controls futile. The response to emerging diseases is increasingly global as well: national agencies, international organizations, and other groups coordinate efforts to monitor, prevent, and control the spread of these diseases (5). While no substitute for adequate national health services and infrastructure, international efforts against emerging diseases have increased in importance as national programs have been compromised by complacency, economic recession, international debt, civil turmoil, or natural disasters (3,5,6). At the same time, detecting new agents often requires up-to-date diagnostic facilities unavailable in many parts of the world. International agencies have improved access to such facilities to help define the causes of disease outbreaks.

The Current Legal Framework: International Health Regulations

The regulations, which play a central, albeit limited, role in addressing global disease outbreaks (Table 1), have served as the primary legally binding framework for preventing the international spread of infectious disease (Table 2). In addition to its other legal and policy options for promoting international public health, WHO's

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Table 1. Selected international health regulatory provisions pertinent to emergent infectious disease

Provision	Source/Lead Agency
International Health Regulations	World Health Organization
Agreement on the Application of Sanitary and Phytosanitary Measures	World Trade Organization
Codex Alimentarius	FAO/WHO Codex Alimentarius Commission
Annex 9: Facilitation to the Convention on International Civil Aviation	International Civil Aviation Organization

World Health Assembly is specifically authorized to adopt regulations concerning "sanitary and quarantine requirements and other procedures designed to prevent the international spread of disease" (7, art. 21[a]). The twin objectives of the regulations balance "maximum security against the international spread of diseases with a minimum interference with world traffic" (1, Foreword).

The regulations evolved from efforts to deal with epidemics. The use of quarantine dates back at least to the Middle Ages (3). Steps to regulate countries' actions to protect themselves against the introduction of communicable diseases and to report disease outbreaks developed before the International Sanitary Regulations were adopted in 1951 (8). Inadequacies of the current regulations have been associated with outdated quarantine and frontier-based practices.

The current regulations outline procedures for limiting disease transmission in international traffic, including the disinsectization, disinfection, and deratting of ships and aircraft and the provision of sanitary conditions and health facilities at sea and airports. In addition, the regulations focus on two core obligations of member states relating to disease incidence reporting and response; these obligations apply primarily to the three diseases subject to the regulations: cholera, plague, and yellow fever (1, art. 1). First, states must report to WHO, within specific periods, cases of these three diseases within their territories (1, arts. 3-7,9,11-12). Second, to facilitate reporting and deter unnecessary interference with international travel and trade, members must limit their responsive health measures (applied to international traffic for the protection of their territories against these diseases) to maximum measures permitted by these regulations (1, art. 23). States must also report to WHO any health measures they have applied to international traffic (1, art. 8). Most of such permitted health measures (and related provisions in the regulations limiting or prohibiting health measures) focus on cholera, plague, and yellow fever; some more general provision, however, limit measures directed at other diseases (1, arts. 28, 29, 31, 81; 9).

Like most international agreements, the regulations are to be implemented through national laws and policies that incorporate or otherwise accommodate the regulations' various provisions, minimum requirements, and limitations. The regulations have served as international reference standards for some states creating their own national quarantine provisions; this relationship magnifies the effect of outdated and problematic aspects of the regulations (and increases the urgency of their timely revision).

Even though the regulations pose a legal obligation for WHO members who have not officially "opted out" from participating, lack of compliance has been an ongoing problem (8,9). The regulations have weaknesses: 1) the limited scope of reported information vis-a-vis the burgeoning scope of new infections, and 2) the mismatch between the narrow institutional, political, and legal bases of the regulations issued under the circumscribed authority of a single specialized United Nations (U.N.) agency, WHO, and the varied factors affecting the international emergence and control of infectious disease. The factors include international trade and travel, economic development and land use, changes in human demographics and behavior, and the breakdown of public health infrastructure (3). WHO's broad mandate within the U.N. system (10, art. 57) and under the WHO Constitution (7, preamble, arts. 1-2), to address these factors is ultimately rooted in the one agency. While WHO's natural institutional allies tend to be

Table 2. Development of the International Health Regulations

Year(s)	Action
1951	WHO Int'l Sanitary Regulations and additional regulations in 1955, 1956, 1960, 1963, 1965)
1969	Revised and renamed Int'l Health Regulations (regulations)
1973	Regulations revised
1981	Regulations revised (current version)
1995	WHO resolves to revise regulations

national health ministries, the factors affecting disease emergence are multisectoral and can be more directly addressed by a constituency of international organizations and agencies. WHO members' violations of regulations during epidemics (e.g., the implementation of unjustified and illegal health-based trade barriers) are critical symptoms of the institutional mismatch. Such barriers are rarely the product of sole actions by the Ministry of Health, and WHO has little influence over other national agencies such as Ministries of Trade, Commerce, or Planning.

These core weaknesses are exacerbated by the administration of regulations. In spite of the regulations' strong legal basis, WHO has frequently preferred nonmandatory urging or mediation to a legally binding approach to members' obligations (8,11). In the area of trade, for example, a Pan American Health Organization epidemiologic bulletin on the 1991 cholera outbreak in Latin America referred to unjustified trade restrictions imposed on Peruvian marine exports as "not in accordance with the recommendations of WHO" (12). More substantive is the ambiguous, indeterminate, or otherwise vague nature of many of the regulations' articles, which create further difficulties in application (1, arts. 46[1] and 36 [3]). WHO is unlikely to use formal mandatory enforcement such as sanctions against member states who do not comply with the regulations (13). Under the WHO Constitution, typical of such treaties (13), there are no formal punitive sanctions. While under article 7 of the Constitution, the World Health Assembly is authorized in "exceptional circumstances" to withdraw membership privileges, under treaties, such provisions are rarely invoked and then usually on political grounds against otherwise marginalized states (13). These sanctions would also hinder the WHO objectives of tracking, controlling, and preventing incidence and transmission of disease.

The regulations contain a dispute resolution provision that authorizes member states to refer "any question or dispute" concerning the regulations to the Director General or a WHO committee to "settle," rather than to enforce (1, art. 93[1]). If this referral process fails, a member state is authorized to bring the dispute to the International Court of Justice in the Hague for decision (1, art. 93[3]). The International Court of Justice has, however, never determined a case under the regulations and is a relatively rare choice for disposition of international disputes (13). WHO's right to request advisory opinions from the International Court of Justice has yielded court rulings in only two cases. Thus, disputes appear to be usually handled informally through the WHO bureaucracy (8).

In this article, we propose two steps for improving the effectiveness of the regulations. Some of the following issues are addressed in the December 1995 Report of WHO Informal Consultation of Experts on Revision of the Regulations (14,15).

Step One: Expand the Information Base

The Limited Scope of Reporting Under the Regulations

The current narrow scope of disease reporting undercuts the relevance of the regulations and has been criticized (16). In contrast to the long list of known emerging and other infectious diseases that threaten world communities and the threats posed by as yet unknown or unrecognized diseases or syndromes, only three diseases (cholera, plague, and yellow fever) are expressly covered by the regulations' reporting requirements.

Additional obstacles to effectiveness and compliance regarding both outbreak reporting and minimizing of health restrictions arise from limiting the range of information sources that can report on these issues to WHO. WHO has been criticized for relying solely on information reported officially by member states regarding outbreaks within their borders or health measures applied to traffic from the country involved in an outbreak (9). To avoid self-incrimination, members do not report on a timely basis, if at all, as required (8,9,16). A related problem arises from the right of the affected country's health administration to determine the "infected area" of an outbreak (1, art. 1), although that decision can influence health measures other countries may apply to persons, vehicles, and cargo from the outbreak area (1, arts. 46[1], 59, 64[2], and 66). WHO appears to limit its sources for listing infected areas in the Weekly Epidemiological Review "only [to] official governmental information" (17).

Consequences of an Inadequate Information Base

Driven by political and economic pressures and other concerns (8,18), neighbors or trading partners of countries affected by epidemics often overreact by setting up border or other restrictions in excess of those permitted under the

regulations. From the beginning of the seventh cholera pandemic into Latin America in 1991, for example, trading partners of affected countries—particularly Peru—at times rejected food imports (19) or even nonfood manufactured goods (20), restricted travel (19), or closed their borders. Peru lost an estimated $770 million because of the epidemic (4). In response to the 1994 plague outbreak in India, some countries severed air and shipping links with India; the country sustained a reported $1.3 billion in export losses in 2 months (21). Concerns about potential trade and travel restrictions have caused countries not to report outbreaks within their borders (8,12,22).

Proposed Solutions to Information Weaknesses in Regulations

The regulations' disease coverage, reporting of outbreaks, and disease incidence (and permitted responsive health measures) should be based upon an up-to-date schedule of the most relevant clinical syndromes in conjunction with a broadened list of relevant serious diseases. Syndromes should be reportable until the underlying disease is identified. Descriptions of syndromes and appropriate and inappropriate health responses should be stated as clearly and specifically as possible in the regulations. This approach would align the coverage of the binding regulations with the most appropriate and dangerous diseases; it would also facilitate more direct and coherent risk evaluation and present a framework for addressing unknown or unrecognized diseases and syndromes. It is also likely to expedite reporting as there would be no need for disease identification. It may also stimulate participation by member states that have ceased complying with what they regard as an outdated system (16). An operations research effort by WHO and its member states could facilitate the adoption of these suggested improvements by testing the sensitivity and specificity of syndromic outbreak reporting.

Similarly, to enhance the effectiveness of the regulations, the acceptable sources of information on disease outbreaks and health measures put in place by member states under the regulations should be broadened to include other reliable sources. WHO's use and acceptance of reliable outside information for such determinations will provide more accurate information and may also prompt more timely reporting by the affected countries faced with preemption by WHO and others.

The complex issues arising in international relations are discussed elsewhere (14,23-26), but the history of noncooperation with the regulations does not preclude potential for improved compliance in the future. If the regulations are substantially and meaningfully revised (14,15) and key countries are sufficiently concerned about the dangers of emerging diseases to press for compliance with the revised scheme, compliance should improve (14).

Step Two: Expand Policy and Programmatic Collaboration

International organizations other than WHO deal more directly with the underlying issues affecting the transmission of disease. The expansion of international regulatory provisions in all areas has increased the potential for overlapping policies and even regulation.

Interorganizational and Interagency Consultations

Revising the regulations presents an opportunity to establish interorganizational and interagency consultations and address potential contributions of agencies and organizations that can most directly affect the factors involved in the emergence and control of infectious disease. A first step would be an interorganizational summit led by WHO to examine potential joint or coordinated programs. The breadth and focus of the summit will depend on the specific programs most compatible with such an approach. WHO has a long record of cooperation with other agencies and organizations, and joint activities are part of its programs addressing infectious disease control (4). Contacts need not be limited to the WHO-affiliated international organizations but can include other groups: WHO will need to evaluate whether participation by such agencies, nongovernmental organizations, and other entities would be beneficial in a particular context, and if so, at what points in the process.

Establishing new connections and building on existing relationships between WHO and other institutions and organizations (e.g., the World Trade Organization, the International Civil Aviation Organization, and the International Maritime Organization) present many benefits: WHO can bring its influence closer to the underlying processes and organizational entities directly involved in disease emergence and control; it can more

effectively use its limited resources; and it can address initiatives in other organizations that may be insufficiently sensitive to key public health concerns. From an international relations perspective, such contacts may ultimately develop into direct links between the administration of WHO regulations and that of other international or multilateral organizations.

World Trade Organization

Health-related trade restrictions are regulated by multilateral organizations and agreements (Table 1), such as the recently established World Trade Organization (WTO) and its related multilateral agreements, including the General Agreement on Tariffs and Trade (GATT) and the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS agreement). Like the WHO regulations, parts of this complex trade regulatory scheme address the health-based trade constraints that countries may implement to protect their citizens and territories from infectious disease threats. Peru's appeal to the GATT Council for assistance in protecting its exports from unjustified rejection during the 1991 cholera outbreak at the same time that PAHO was directly involved in dealing with the epidemic exemplifies this potential overlap of regulation (20).

The WTO agreements specify which health-based trade barriers that would otherwise violate a trade rule of WTO may be justified under the exceptions in GATT article XX(b) for measures "necessary to protect human, animal, or plant life or health" and interalia under the related SPS agreement that governs health measures pertinent to most infectious disease threats. Therefore, a WTO member's "sanitary and phytosanitary measures" (certain health-based trade restrictions) that meet the SPS agreement's detailed requirements are deemed in compliance with GATT's XX(b) exception. The SPS agreement addresses all sanitary and phytosanitary measures that a member may apply that potentially affect international trade (27, arts. 1, 2.4) The WTO agreements, in contrast to the WHO regulations, are not disease-specific. The sanitary and phytosanitary measures described in the SPS agreement focus on risks to humans from diseases carried by animals, plants, and their products; the entry or spread of pests; and additives, contaminants, toxins, and disease-causing organisms in food and beverages (27, Annex A, par. 1). The SPS agreement contains measures focusing on, for example, cholera as transmitted in international trade in food and beverages and insectborne disease risks in international trade. Measures directed against many health risks to animals and plants are also covered (27, Annex A, par. 1). Although the SPS agreement does not refer specifically to epidemics, its stated scope is broad enough to cover them, assuming the agreement's various requirements are met (27, arts. 5.7 and Annex B, pars. 2,6). However, aspects of the relevant applications of the SPS agreement are as yet not entirely clear. The WTO agreement and related Dispute Settlement Understanding have been in force only since 1995. Measures directed at trade-related infectious disease risks not covered under the SPS agreement may be addressed by the Separate Agreement on Technical Barriers to Trade, which concerns product standards, or by the GATT article XX[b] exception itself (28).

Like those of the regulations, the general purposes of the SPS agreement include limiting health-based restrictions to those that are necessary and as minimally burdensome to trade as possible. The SPS agreement provides detailed rules and standards for determining what sanitary or phytosanitary measures are permitted on the basis of scientific support, risk assessment, and other factors (27, arts. 2.2, 3.3, 5 and Annex A par. 4), as well as numerous other provisions. The SPS agreement provides, for members' use, where applicable, certain established "international standards, guidelines or recommendations" (rather than their own standards) to promote international consistency on these measures. A member must justify, scientifically and otherwise, implementation of higher standards if they result in a greater restriction on trade than the stipulated international standard (27, art. 3 and Annex A, pars. 2-3). For example, regarding food safety issues, the SPS agreement designates the "standards, guidelines and recommendations" established by the WHO-Food and Agriculture Organization Codex Alimentarius Commission. The Codex Alimentarius is an extensive code that addresses a broad range of food production issues including food additives, limits on pesticide residues, food labeling requirements, product composition, recommendations on food processing

techniques, and suggested inspection procedures regarding food products and production (27, Annex A, par. 3[a]). However, the regulations do not mention the Codex (although it is a key part of WHO guidelines on food issues).

WTO mechanisms for dispute resolution differ substantially from those of the WHO regulations: among other differences, ultimate adjudication authority remains within WTO. Under GATT articles XXII and XXIII and the Understanding on Rules and Procedures Governing the Settlement of Disputes (Dispute Settlement Understanding), if the disputing parties' consultation and other preliminary steps do not resolve a dispute, they ordinarily resort to adjudication before a WTO three-person panel (29). The panel's report (including its recommendation if a violation has been found) is adopted by the WTO members (sitting as the Dispute Settlement Body), unless there is a consensus to reject it. There is also a potential appellate procedure, before the seven-person Appellate Body, regarding legal issues. If the challenged trade restriction is found to be unjustified (and the related rulings, usually to bring the violating measure into compliance, are not implemented nor is negotiated compensation obtained), the injured member can be authorized to obtain compensation by retaliation: a proportional reduction in a trade concession or obligation owed to the violator. The overall value of this mechanism is controversial. However, since its establishment in 1995, some 50 requests to start consultations on disputes of all kinds have been initiated; several cases are before panels; two panels have completed proceedings; there has been one appellate ruling; and ten disputes have been resolved by consultation without resort to a panel (30).

Given the parallels between the WHO and WTO regulatory systems and the interplay between epidemics and trade, WHO consultations with WTO would enhance coordination (or other more or less formal arrangements) on trade issues related to disease threats. Coordination seems particularly appropriate in light of the current revision of the regulations. As the range of diseases and syndromes covered by the regulations is substantially broadened, areas of potential overlap or parallel may grow (15).

The SPS agreement provides for consultation and coordination between the WTO system and those of other international organizations (27, art. 11.3). The Committee on Sanitary and Phytosanitary Measures established under the SPS agreement has a mandate to consult with other international organizations in the field of sanitary or phytosanitary protection (including the Codex Alimentarius Commission); to obtain scientific and technical advice for administering the agreement; to avoid unnecessary duplication of efforts; and to identify other international standards, guidelines, or recommendations relevant to sanitary and phytosanitary measures that have a major impact on trade. (27, art. 12). Consultation is also recommended for dispute resolution. The SPS agreement encourages dispute resolution panels deciding cases that involve the agreement on scientific or technical issues to seek advice from experts, including the relevant international organizations (27, art. 11.2).

The WTO also has a Committee on Trade and Environment, which addresses the relationship of regulation and policy on trade and Environment issues, including issues concerning the SPS agreement (31-32). In an example of a potential consultation between WHO and WTO, a bulletin of the Committee on Trade and Environment indicates that the Conference of the Parties of the Biodiversity Convention (an environmental treaty) had requested that its Secretariat "liaise with the WTO Secretariat and invite it to provide input in identifying the synergies and relationship between the objectives" of the convention and one of the WTO agreements (33).

Collaboration and consultation on health-related trade issues will depend on accommodating the many differences between the two organizations, as well as WHO's constitutional provisions concerning such relationships (7, arts. 70-71) and the more limited articles in the current regulations on such contacts (1, arts. 46[3], 85). While establishing working relationships with WTO might not be a panacea for the many trade-related concerns under the WHO regulations, it would provide opportunities for reinforcement of the legal and institutional bases for the prevention of inappropriate trade restraints.

International Civil Aviation Organization (ICAO)

Because of the importance of international travel (as well as air transportation of cargo) to disease emergence, ICAO is another important collaborative partner. Unlike WTO, however, ICAO has had a long-standing relationship with WHO, dating from the 1940s and including participation

in creating the regulations (8). The conflicting pressures of globalization of world transport (and commerce) and sovereignty have affected the ability of ICAO and WHO to regulate effectively (34).

In preventing infectious diseases, WHO and ICAO have overlapping areas of interest, such as the disinsectization of aircraft and airport health and sanitary facilities. Under the Convention on International Civil Aviation and related instruments, ICAO addresses a variety of civil aviation issues, including many relating to public health and international transmission of disease. The convention provides that each member state "agrees to take effective measures to prevent the spread by means of air navigation of cholera, epidemic typhus, smallpox, yellow fever, plague, and such other communicable diseases as the contracting States shall ... designate" and to "keep in close consultation with the agencies (such as WHO) concerned with international regulations relating to sanitary measures applicable to aircraft" (35). The specific compulsory "Standards" and related "Recommended Practices" in Annex 9 to the convention include those applicable to public health, infectious disease transmission, and related requirements (36). These provisions are fundamentally tied to WHO recommendations and the regulations in Standard 8.12, which requires ICAO member states to "comply with pertinent provisions of the current edition of the [regulations]." Specific ICAO standards and recommendations also refer to WHO recommendations and regulations in key areas, including aircraft disinsectization, provision of safe food and water at airports and on aircraft at international airports, proper facilities for disposal of refuse, waste, wastewater, and other dangerous matter, and yellow fever certificates.

WHO cooperation with ICAO is exemplified by the participation of ICAO in the 1995 informal WHO consultation regarding revision of the regulations; ICAO was the only such international organization to participate in the consultation (15). The consulting group's recommendations suggested that certain sections of the revised regulations concerning sanitation standards at airports and seaports should refer to the applicable requirements (exceptions being health care services for sick persons on arrival, equipment necessary for disinfection, and disinsectization, and control of animal-borne disease), under other international agreements, such as Annex 9. Although this specific recommendation may have flaws (for example, it effectively renders the regulations incomplete in themselves as a reference or guide to essential rules), it demonstrates ongoing attempts to link the two organizations.

Coordination of WHO and ICAO in dealing with the inappropriate imposition of health measures is particularly relevant now. At a 1995 session of the ICAO division with jurisdiction over such public health issues, "[d]elegates recommended that ICAO work with the World Health Organization ... to draft joint guidelines that, if followed, would prevent adoption by Contracting States of excessive health measures that might disrupt international air transport services in cases of outbreaks or epidemics of diseases" (37).

International Maritime Organization (IMO)

Provisions under the regulations concerning deratting procedures (as noted in the WHO informal consultation on revision of the regulations) (15) and sanitary conditions at seaports also play an important role in maritime health and in containing international disease transmission threats. The regulations also address cholera-contaminated bilge water in certain circumstances on arrival of the ship (1, art. 62[1]). Health authorities are broadly authorized to take measures "to control the discharge from any ship of sewage and refuse which might contaminate the waters of a port, river or canal" (1, art. 29). IMO has recognized the global public health problems of bacterial and viral diseases transmitted in discharges of ballast water and sediment (38). A poll of IMO member states indicated that such transmission is a major international problem expected to worsen (38). The IMO assembly has accordingly adopted (generally nonmandatory) guidelines to prevent the introduction of bacterial and viral pathogens in ballast water and sediment. The IMO resolution traced this concern in part to the 1973 International Conference on Marine Pollution, in which the parties called for WHO, in collaboration with IMO, "to carry out research into the role of ballast water as a medium for the spreading of epidemic disease bacteria" (38).

A sound legal and policy framework is needed to support efforts against emergent infections. Truly intersectoral, interagency, and interorganizational collaboration in addressing the broad factors of emergence and expanded reporting of disease are major steps in this process. The challenge is broad, but in view of the

increased pace of emergence and the globalization of disease, the importance of a comprehensive legal and policy framework cannot be overstated.

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Surface Antigens of the Syphilis Spirochete and Their Potential as Virulence Determinants

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A unique physical feature of *Treponema pallidum*, the venereally transmitted agent of human syphilis, is that its outer membrane contains 100-fold less membrane-spanning protein than the outer membranes of typical gram-negative bacteria, a property that has been related to the chronicity of syphilitic infection. These membrane-spanning *T. pallidum* rare outer membrane proteins, termed TROMPs, represent potential surface-exposed virulence determinants and targets of host immunity. Only recently has the outer membrane of *T. pallidum* been isolated and its constituent proteins identified. Five proteins of molecular mass 17-, 28-, 31-, 45-, and 65-kDa were outer membrane associated. The 17- and 45-kDa proteins, which are also present in greater amounts with the *T. pallidum* inner membrane protoplasmic cylinder complex, had been previously characterized lipoproteins and are, therefore, not membrane-spanning but rather membrane-anchored by their lipid moiety. In contrast, the 28-, 31-, and 65-kDa proteins are exclusively associated with the outer membrane. Both the purified native and an *Escherichia coli* recombinant outer membrane form of the 31-kDa protein, designated Tromp1, exhibit porin activity, thereby confirming the membrane-spanning outer membrane topology of Tromp1. The 28-kDa protein, designated Tromp2, has sequence characteristics in common with membrane-spanning outer membrane proteins and has also been recombinantly expressed in *E. coli,* where it targets exclusively to the *E. coli* outer membrane. The 65-kDa protein, designated Tromp3, is present in the least amount relative to Tromps1 and 2. Tromps 1, 2, and 3 were antigenic when tested with serum from infection and immune syphilitic rabbits and humans. These newly identified TROMPs provide a molecular foundation for the future study of syphilis pathogenesis and immunity.

Despite the fact that *Treponema pallidum* subsp. *pallidum* has remained sensitive to penicillin for more than four decades, syphilis remains an important cause of sexually transmitted disease, accounting for more than 14,000 new cases in 1995 in the United States alone (1). Although syphilis is not an emerging infectious disease, its reemergence, from 1986 to 1990, culminated in more than 100,000 reported cases in 1990 and an 80% increase in the reported rate per 100,000 population. The reemergence of syphilis has also been associated in certain areas with the use of crack cocaine, and syphilis continues to be an important risk factor for acquiring and transmitting human immunodeficiency virus.

Syphilis is characterized by months of clinical disease followed by years of latency with the potential for relapse to debilitating or lethal late disease if left untreated. The chronicity of infection may relate to a striking property of the *T. pallidum* outer membrane, namely that it contains 100-fold less membrane-spanning protein than the outer membranes of typical gram-negative bacteria (2,3). This article reviews recent progress in identifying and characterizing the rare outer membrane proteins (TROMPs) of *T. pallidum*.

The interaction of host and pathogen leading to the establishment of infection generally proceeds through the interactions of their respective surface molecules. An understanding of the molecular basis for syphilis pathogenesis and the identification of corresponding *T. pallidum* surface molecules have long been hampered by the inherent difficulties associated with the study of this organism. In contrast to that of many other pathogenic spirochetes from the genera *Treponema, Borrelia*, and *Leptospira,* the in vitro multiplication of *T. pallidum* is unsuccessful in artificial media and is limited and impractical in tissue culture (4). Further, only limited numbers of *T. pallidum* are

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attainable from testicular cultivation in rabbits. Organisms acquired from rabbit infection are also bound with host material including albumin (5), fibronectin (6), and glycosaminoglycans (7); therefore, the separation and purification of *T. pallidum* molecules are complicated. Host material associated with *T. pallidum* has also been the basis for past theories of molecular mimicry during syphilitic infection (6), of *T. pallidum* adherence to host cells and tissues (8), and of the long recognized antigenic inertness of the surface of this organism.

Past studies showed that the surface of *T. pallidum* behaves as if it lacks proteins and antigens. These findings included the inability to detect antibody bound to the surface of structurally intact treponemas by immunofluorescence (9) or by immunoelectron microscopy (10,11), the limited radiolabeling of surface proteins (12,13), and the observation that only with extended in vitro incubation could proteins of intrinsically radiolabeled *T. pallidum* bind specific antibody (14). One hypothesis generated by these observations was that the surface of *T. pallidum* was antigenically inert because of a limited number of available surface-exposed antigens.

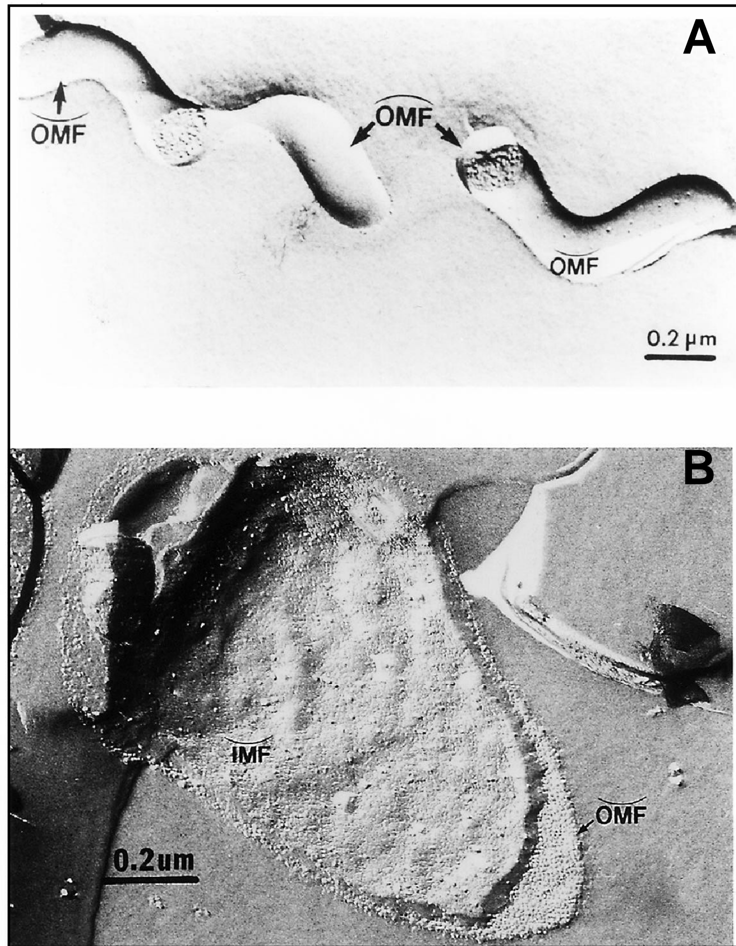

Figure 1. Freeze-fracture electron microscopy of *Treponema pallidum* subsp. *pallidum* (A), and *Escherichia coli* (B). Concave ( ) and convex ( ) outer membrane (OMF) and inner membrane (IMF) fracture faces.

The inability to apply conventional gram-negative cell fractionation procedures to *T. pallidum* to isolate its outer membrane and identify its constituent proteins further hampered the identification of surface-exposed proteins. As a result, alternative methods, which included detergent solubilization, were used to achieve this goal. The detergent Triton X-114 (TX-114) was used with particular interest because of its inherent property of a low temperature cloud point that results in phase separation yielding both aqueous and detergent phases. It was found that 0.1% to 2% TX-114 would selectively solubilize the *T. pallidum* outer membrane and result in the recovery of several prominent detergent-phase proteins (15,16), which were speculated, at the time, to be part of the outer membrane and potentially surface exposed. These proteins were subsequently found to be lipoproteins (17) and were strongly antigenic when tested with serum from syphilitic rabbits and humans (17). While surface exposed lipoproteins have been demonstrated for other pathogenic spirochetes, including *Borrelia* (18) and *Leptospira* (19), a surface exposed location for a strongly antigenic *T. pallidum* lipoprotein was inconsistent with the surface antigenic inertness of this organism.

A major advance in the understanding of the *T. pallidum* outer membrane and cell surface followed the results of freeze-fracture electron microscopy in 1989. The observation that the fracture faces of the *T. pallidum* outer membrane contained only 170 particles/μm^2 (2,3), which is approximately 100-fold less than what is contained in typical gram-negative bacterial outer membranes (Figure 1), together with the finding of freeze-etch analysis (3), has provided the most plausible explanation for the antigenic inertness of the *T. pallidum* surface. The rare particles revealing *T. pallidum* rare outer membrane proteins were designated TROMPs (2,20), and because of their membrane-spanning topology, they were distinct

from lipoproteins, which do not span membranes to form particles in freeze-fracture analyses (21). Moreover, a membrane-spanning protein by definition should possess several surface exposed regions, suggesting that TROMPs had surface exposed antigenic sites. This theory was confirmed by freeze-fracture analysis when it was observed that incubation of *T. pallidum* in serum from syphilitic rabbits immune to reinfection resulted in the aggregation of TROMPs (20) (Figure 2). It was found that 8 to 16 hours of incubation was required for this aggregation to occur, suggesting that antibody-mediated aggregation of TROMPs is the rate limiting step for complement activation and killing of *T. pallidum* (20).

The findings of a potentially protective immunogen in low molar amounts on the outer membrane surface of *T. pallidum* have provided a further attractive explanation for the prolonged period required for the induction of acquired protective immunity during syphilitic infection. This is also in accord with the repeated immunizations required for protective immunity with *T. pallidum* attenuated by gamma irradiation (22), the only method of successful vaccination ever reported. Thus, the identification and study of TROMPs has become essential in understanding syphilis pathogenesis and immunity in molecular terms.

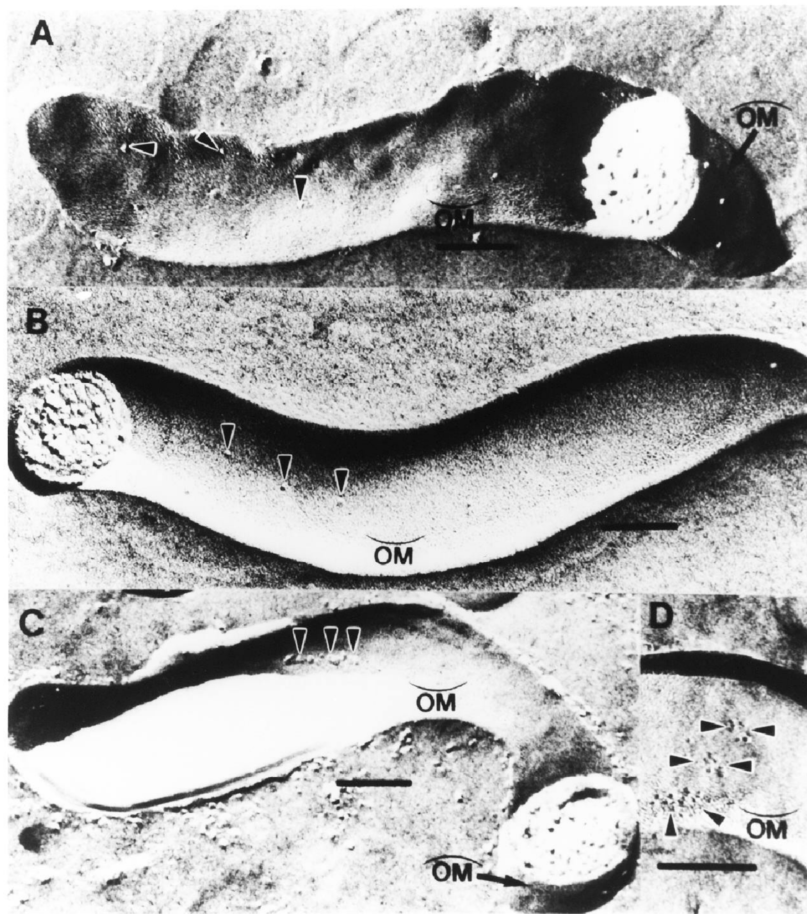

Figure 2. Freeze-fracture electron microscopy of *T. pallidum* subsp. *pallidum* demonstrating TROMP aggregation. Concave ( ) and convex ( ) outer membrane fracture faces (OM). *T. pallidum* incubated in heat-inactivated normal rabbit serum for 16 hours (A), in immune rabbit serum for 2 hours (B), and in immune rabbit serum for 16 hours (C) and (D). Arrows show individual (A&B) and aggregated (C&D) TROMPs. Bar in each micrograph represents 0.1µm. Photograph reprinted with the permission of the Journal of Immunology, copyright 1990, The American Association of Immunologists.

Isolation of the *T. Pallidum* Outer Membrane

In 1994, we developed a procedure to isolate the outer membrane of *T. pallidum* without detergents (23). This method used three key features: 1) a Ficoll step gradient to purify *T. pallidum* from host contaminating material; 2) octadecyl rhodamine B chloride salt, a lipid-conjugated chromophore that intercalates into membranes and provides a visual marker to monitor outer membrane release and recovery; and 3) a hypotonic 0.05 M sodium citrate buffer, pH 3.0, which was found to selectively release the *T. pallidum* outer membrane. Since it has been suggested that endoflagellar filaments physically interact with the outer membrane in the process of motility, the probable mechanism for outer membrane release by this procedure is the depolymerization of endoflagella at low pH.

Purification of *T. pallidum* outer membrane vesicles in sucrose density gradients showed that the membrane banded at the very low density of 1.03 g/ml (7% sucrose). This, however, was expected for properties of a membrane that lacks lipopolysaccharide (24) and contains a small amount of protein. Freeze-fracture electron microscopy demonstrated that the purified membrane vesicles contained extremely rare intramembranous

particles consistent with the low particle density observed for the native outer membrane of *T. pallidum*. The selective isolation of the *T. pallidum* outer membrane was shown by the inability to detect *T. pallidum* penicillin binding proteins, a marker of the cytoplasmic membrane. Further support for the selectivity of the outer membrane isolation was the complete absence of a previously characterized 19-kDa periplasmic protein termed 4D (15) and the abundant 47-kDa lipoprotein (17) and the presence of only trace amounts of endoflagellar protein. The absence of the 47-kDa lipoprotein was particularly important since it is widely accepted that this very abundant protein is exclusively anchored to the inner membrane (25). The finding that the 47-kDa lipoprotein was not detected in the purified outer membrane material indicates that inner membrane anchored lipoproteins were not released by this procedure.

Two dimensional immunoblot analysis was used to identify the protein constituents present in purified *T. pallidum* outer membrane. Two strongly antigenic species were found by syphilitic immune rabbit serum, one very basic protein (pI > 7.0) at 17-kDa and the other having a pI of approximately 4.5 at 45-kDa. The observation that the 17-kDa protein was very basic, showed higher oligomeric forms, and was selectively partitioned into the hydrophobic phase after Triton X-114 detergent extraction was consistent with properties reported for the 17-kDa lipoprotein of *T. pallidum* (26). With specific monoclonal antibodies, the 45-kDa protein was identified as the previously characterized lipoprotein termed TmpA (27). It was also found that most of these two lipoproteins, well over 90%, are associated with the inner membrane protoplasmic cylinder complex. It would appear, however, that the association of the 17- and 45-kDa lipoproteins with the outer membrane is specific, given the absence of the normally abundant 47-kDa lipoprotein. The function of these two outer membrane lipoproteins remains to be determined.

In addition to the strongly antigenic 17- and 45-kDa lipoproteins, gold stained two-dimensional blots of outer membrane showed four additional *T. pallidum* proteins, including one each at 28- and 65-kDa and two at 31-kDa, with close but distinct pI migration patterns. All of these proteins, including the 17- and 45-kDa lipoproteins, were present in approximately equal amounts in the outer membrane. However, in contrast to the 17- and 45-kDa lipoproteins, these four additional proteins required very low dilutions (1:25) of immune syphilitic rabbit serum for detection by immunoblot analysis. Moreover, when compared with stained two-dimensional blots of total *T. pallidum* proteins, these four additional outer membrane proteins were either not detectable, as was the case for the 65-kDa protein and the more basic of the two 31-kDa proteins, or represented extremely minor species, as was the case for the 28-kDa protein and the more acidic 31-kDa protein. In view of the paucity of TROMPs determined from freeze-fracture analysis, these four proteins, which were clearly enriched in our outer membrane preparation, became the leading candidates for membrane-spanning outer membrane proteins of *T. pallidum*.

In 1995, a second *T. pallidum* outer membrane isolation method that used plasmolysis of organisms in 20% sucrose was reported by Radolf et al. (28). Outer membrane isolated by this method was also shown by freeze-fracture analysis to have an extremely low density of membrane-spanning protein. These studies showed that the lipid composition of the *T. pallidum* outer membrane was similar to that of the protoplasmic cylinder inner membrane complex, with the exception of cardiolipin, which was prominently detected only in protoplasmic cylinders. Of further interest was the finding that outer membrane phospholipids and glycolipids did not react with antibody from infection-derived immune serum, suggesting that only outer membrane proteins are target antigens. However, in contrast to the limited number of protein species that we observed, silver stained gels of their outer membrane material showed more than 20 proteins, including the 47-kDa lipoprotein. One interpretation of these results is that many of the proteins identified in their outer membrane preparation are possibly contaminants of the periplasm or inner membrane. Thus, we believe that the small number of protein species identified by our isolation method represent a more accurate account of the outer membrane protein composition of *T. pallidum*.

While lipoproteins are integral membrane proteins, their membrane association extends only to integration of the lipid moiety in the bilayer. In contrast to membrane-spanning proteins, the *T. pallidum* lipoproteins do not span membrane lipid bilayers and, therefore, do not form particles upon freeze-fracture electron microscopy (21). By comparison, one type of membrane-spanning protein common to virtually all gram-negative outer membranes are porins, which form water-filled

channels through membranes allowing for transport of small molecular weight solutes (29). Porin proteins, like almost all outer membrane proteins, span membranes in a beta-pleated sheet topology rather than in alpha helical hydrophobic regions, which are common to inner membrane-spanning proteins (30). Porin activity can be detected by several methods (29), including radioisotope efflux or substrate uptake in proteoliposomes, liposome swelling, and the black lipid bilayer assay, which measures the conductivity of ions through porin channels. In the black lipid bilayer assay, a set voltage is applied across an artificial lipid bilayer which, if a porin protein has been inserted, results in an increase in electrical conductance. The degree of conductance increase for many insertional events is used to calculate the average pore channel size. When the black lipid bilayer assay was used with Triton X-100 detergent solubilized proteins from our purified *T. pallidum* outer membrane, porin activity was demonstrated (23). Two distinct average conductance increases were observed at 0.4 and 0.76 nanosiemens (nS), corresponding to pore channel sizes of 0.35 and 0.68 nm, respectively, which are similar in size to porin channels found for other gram-negative bacteria (29). At the time, the two distinct conductance measurements suggested two different porin species, or alternatively, that the larger activity could be the result of dimeric aggregates of the smaller activity or the smaller activity could be a substate of the larger channel, possibly caused by the application of a voltage (31). Regardless of the exact number of *T. pallidum* porin species, these findings confirmed that at least some of the particles observed by freeze-fracture electron microscopy of the *T. pallidum* outer membrane were membrane-spanning porin proteins. This finding was of particular importance since several gram-negative bacterial porins play a role in pathogenesis by acting as adhesins (32,33) and as surface targets of bactericidal antibody (34-37). Thus, the search for an outer membrane *T. pallidum* porin protein was initiated to identify a first TROMP species.

T. Pallidum Rare Outer Membrane Protein 1 (Tromp1): an Outer Membrane Porin

Since most porin proteins have molecular masses of 28 to 48-kDa (29), our focus was directed toward isolating the 28- and 31-kDa proteins identified in purified *T. pallidum* outer membranes. Internal amino acid sequences from the native 31-kDa protein were used to clone the encoding structural gene, designated tromp1 (38). Antiserum generated to a recombinant Tromp1 fusion protein has shown that both 31-kDa proteins identified by two-dimensional blot analysis of outer membrane are Tromp1. Analysis of the deduced amino acid sequence showed an N-terminal hydrophobic region consistent with a signal peptide. Two potential leader peptidase I cleavage sites are noted, including threonine-histidine-alanine at residues 30 through 32 and alanine-alanine-alanine at residues 38 through 40. Identification of the cleavage site by N-terminal amino acid sequence of the native protein has not been possible because of the limited amount of native protein recoverable. However, other data discussed later in this review suggest that alanine-alanine-alanine is the correct cleavage site. The deduced Tromp1 amino acid sequence also supports the concept that Tromp1 topology is in accord with the structural paradigms of other gram-negative outer membrane proteins. Beta-moment analysis, which shows amphipathic sequence regions, has predicted that Tromp1 has 14 membrane-spanning amphipathic beta-sheet segments typical of gram-negative outer membrane proteins (29,30).

The lethality for *Escherichia coli* transformants harboring the intact tromp1 structural gene, which was found by immunoblot analysis to be expressing Tromp1, is similar to that observed for many recombinant gram-negative porin proteins expressed in *E. coli* (39). The tromp1 gene has recently been found to be part of a putative transport operon that includes an adenosine triphosphate binding protein and a hydrophobic membrane protein (Hardham et al., Gordon Research Conference, 1996). To determine if Tromp1 was a porin, native Tromp1 was purified from Triton X-100 solubilized *T. pallidum* by nondenaturing isoelectric focusing. The demonstration of porin activity, by the black lipid bilayer assay, has confirmed that Tromp1 is a membrane-spanning outer membrane protein of *T. pallidum*, the first such protein to be identified for this organism. Native Tromp1 showed two distinct conductance distributions about means of 0.15 and 0.7 nS. The larger of these channels is similar in mean conductance to the 0.76 nS activity observed by using purified outer membrane. The smaller 0.15 nS conductance channel had not been detected previously, and while two channels of different sizes have been

reported for other porins (40), this may simply be the result of partial damage during isolation.

Because most gram-negative bacterial porins exist in the outer membrane in either a sodium dodecyl sulfate (SDS) unstable or stable trimer conformation (29), it was theorized that Tromp1 would also be organized in an oligomeric form in the outer membrane of *T. pallidum*. Antiserum against a Tromp1 fusion protein has in fact identified two higher molecular mass forms of native Tromp1 on immunoblots of *T. pallidum* with sizes of approximately 55- and 80-kDa. A 98-kDa size is further found to be the only detectable form of native Tromp1 when *T. pallidum* is solubilized at room temperature in a low concentration (0.02%) of SDS. These findings would appear to be consistent with the idea that native Tromp1 exists in the *T. pallidum* outer membrane in an SDS-unstable trimer conformation, a property that would be common to several other gram-negative outer membrane porins (29).

Expression of a Porin Active Recombinant Tromp1 (rTromp1)

Porin proteins of gram-negative pathogens not only function as portals for nutrient acquisition across the outer membrane but also play a role in pathogenesis by acting as adhesins (32,33) and targets for bactericidal antibody (34-37). The membrane-spanning conformation of membrane porins is critical for their biologic function. Many surface-exposed epitopes on porins shown to be targets for bactericidal antibodies are conformational (34,36). Thus, correct outer membrane protein conformation should be a key consideration in the study of porins as they relate to bacterial virulence and host immunity. The previous demonstration of immune serum antibody mediated aggregation of TROMPs, as viewed by freeze-fracture electron microscopy (20), suggested that Tromp1 could be a key surface exposed target for antibody that mediates killing (20,41) or opsonization (42,43) of *T. pallidum*. Because of these possibilities, studies have been initiated to express and isolate a porin form of recombinant Tromp1 (rTromp1) with native conformation and in amounts necessary to conduct functional studies.

The controlled nonlethal expression in *E. coli* of exported rTromp1 was accomplished by inserting the tromp1 gene, including the region encoding its native signal peptide, into a relatively low copy number expression plasmid that has an inducible T7 promoter (44). Uninduced basal levels of T7 RNA polymerase resulted in the stable expression, export, and outer membrane localization of rTromp1. Expression detected in whole cell lysates showed several forms of rTromp1, ranging in molecular mass from 31- to 35-kDa. These different sizes of rTromp1 may represent different conformations of the protein as observed for other recombinant outer membrane proteins expressed in *E. coli*. Only the 35-kDa form of rTromp1 is detected in outer membranes isolated from *E. coli*. One explanation for the higher molecular mass outer membrane form is its possible association with lipopolysaccharides, known to form a complex with gram-negative outer membrane porin proteins (45). Such an interaction would certainly be noteworthy because the *T. pallidum* outer membrane does not contain lipopolysaccharides (24).

The demonstration of porin activity by using rTromp1 isolated from *E. coli* outer membranes has further confirmed the membrane-spanning conformation of Tromp1. Single channel conductance measurements of rTromp1 in the black lipid bilayer assay showed two distinct distributions of activity about 0.4 and 0.8 nS. The 0.8 nS conductance is clearly similar to the 0.7 nS conductance determined for native Tromp1 (38) and the 0.76 nS conductance determined for purified outer membrane, indicating that the pore-forming conformation of a portion of rTromp1 is similar if not identical to that of the native protein. Although it is not known why rTromp1 also showed a 0.4 nS conductance measurement, one possibility is an altered conformation of the protein after the isolation process.

A further question regarding the outer membrane localization of porin active rTromp1 has been whether surface exposed antigenic sites are present. Whole mount immunoelectron microscopy has demonstrated the surface binding of immune rabbit serum antibody on *E. coli* expressing rTromp1 (44). Antibody raised against a soluble fusion protein form of rTromp1, which was found by immunoblot analysis to be 100-fold more sensitive in detecting Tromp1 than the immune rabbit serum antibody, did not show surface binding of antibody on *E. coli* expressing rTromp1. This soluble fusion protein form of rTromp1 does not have porin activity when tested in the black lipid bilayer assay. Thus, a reasonable explanation for the immunoelectron microscopy observations is that the bound immune rabbit serum antibodies

recognize conformational surface epitopes on rTromp1, a possibility which may also extend to native Tromp1 on the surface of *T. pallidum.*

The importance of porin protein conformation to biologic function has been further demonstrated in studies using rTromp1. The primary amino acid sequence of Tromp1 suggests two possible signal peptide processing sites within the first 40 residues at threonine-histidine-alanine and at alanine-alanine-alanine. Signal peptide fusion constructs at these two possible cleavage sites using the OmpT signal peptide of *E. coli* result in both forms of rTromp1 being exported and targeted to the *E. coli* outer membrane. Only the OmpT signal fused at the alanine-alanine-alanine position resulted in an exported product that has a molecular mass closest to that of native Tromp1. Moreover, while the outer membrane form of rTromp1 processed at threonine-histidine-alanine showed porin activity, the average channel conductance was 3.2 nS, which is considerably larger than the 0.7-0.8 nS channels observed for native and recombinant Tromp1 exported with its native signal peptide. The greater lethality and limited recovery of the OmpT-Tromp1 fusion construct processed at alanine-alanine-alanine has precluded at this time the ability to test this outer membrane form for porin activity. These findings suggest that alanine-alanine-alanine is the likely processing site in the Tromp1 sequence for leader peptidase I. More importantly, these findings have indicated that subtle changes in the length of the primary amino acid sequence of Tromp1 can have significant effects upon porin activity and are likely the result of an altered conformation of the protein. For this reason and as described previously, proper Tromp1 conformation is an important consideration for future studies addressing its role in syphilis pathogenesis.

T. Pallidum Rare Outer Membrane Protein 2 (Tromp2)

In the search for additional TROMP species, we have recently focused on the 28-kDa protein identified in purified *T. pallidum* outer membrane preparations (23). As in the protein for Tromp1, internal amino acid sequences from the native 28-kDa protein were used to clone the encoding structural gene, now designated tromp2 (46). Analysis of the deduced amino acid sequence showed a 24-residue N-terminal hydrophobic region consistent with a signal peptide and terminating in a typical leader peptidase I cleavage site of leucine-alanine-alanine. As for Tromp1, the deduced amino acid sequence for Tromp2 is also in accord with the structural paradigms of other gram-negative outer membrane proteins. Beta-moment analysis has predicted that Tromp2 has 9 membrane-spanning amphipathic beta-sheet segments.

Recombinant expression and export of Tromp2 (rTromp2) in *E. coli* has recently been accomplished by using the entire tromp2 structural gene in a relatively low copy number plasmid that has an inducible T7 promoter (46). In contrast to Tromp1, Tromp2 expression was not found to be lethal to *E. coli*, even under maximum inducing conditions. Immunoblot analysis using antiserum generated to rTromp2 has shown that virtually all of the recombinant protein produced is targeted to the *E. coli* outer membrane. When tested in the black lipid bilayer assay, rTromp2 isolated from *E. coli* outer membranes showed only occasional channel formation. The total number of porin insertional events was extremely low when compared with the amount of rTromp2 tested. This is in contrast to both native and recombinant Tromp1 where numerous insertional porin events were readily observed. Thus, whether Tromp2 truly has porin function is not clear at this time.

As for rTromp1, a question regarding the outer membrane localization of rTromp2 was the existence of surface exposed antigenic sites. The use of whole mount immunoelectron microscopy demonstrated the surface binding of immune rabbit serum antibody on *E. coli* expressing rTromp2 (46). By comparison, antibody raised against a denatured form of rTromp2, which by immunoblot analysis was found to be more sensitive in detecting rTromp2 than immune rabbit serum, did not show surface binding on *E. coli* expressing rTromp2. This finding is again similar to that described above for rTromp1 and again suggests that the bound immune rabbit serum antibodies recognize conformational-surface epitopes on rTromp2, a possibility which may extend to native Tromp2 on *T. pallidum*.

T. Pallidum Rare Outer Membrane Protein 3 (Tromp3)

Of the three outer membrane protein species identified (28-, 31-, and 65-kDa), the 65-kDa protein, tentatively termed Tromp3, is present in the least amount on the basis of 2-dimensional SDS-PAGE gold-stained immunoblots of purified outer membrane (23). In addition, the presence of the 65-kDa

protein from one outer membrane preparation to the next has been inconsistent, suggesting that this protein may be differentially expressed. The extremely small amount of Tromp3 has at this time precluded amino acid sequencing and, therefore, the cloning of the gene that encodes this protein.

The Potential Role of TROMPs in Acquired Immunity Against Syphilis

Freeze-fracture electron microscopy has demonstrated the ability of serum from immune syphilitic rabbits to aggregate TROMPs (20). These studies have been extended by using serum from infected rabbits with varying degrees of challenge immunity and have shown that TROMP aggregation correlates directly with the development of challenge immunity (Lewinski et al., unpub. obs., 1994). These findings are in accord with past studies that have demonstrated a significant to complete level of protection of animals after passive immunization with serum from immune donors (47). In a recent study, we have used the small amount of attainable *T. pallidum* outer membrane to immunize mice. We have found that serum from the immunized mice possesses complement-dependent treponemicidal activity (100% killing of *T. pallidum*) that is 30 times greater than that of serum from immune syphilitic rabbits (Blanco et al., unpub. obs.). Immunoblot analysis using this serum has shown that only the outer membrane associated proteins are detected. We have also found that absorption of this serum to remove antibodies to the *T. pallidum* lipoproteins does not diminish the titer of the 100% killing activity. Such levels of serum treponemicidal activity have heretofore never been generated by immunization of mice or rabbits with either native or recombinant *T. pallidum* antigens. These observations are consistent with the idea that TROMPs represent the key surface exposed targets for treponemicidal antibody and perhaps a protective host immune response.

Our recent ability to express in sufficient amounts recombinant Tromp1 and Tromp2 provides an opportunity to address directly the ability of these *T. pallidum* outer membrane proteins to elicit protective immunity in experimental animals. However, the correct outer membrane conformation of the recombinant TROMPs may prove a key factor in whether protective immunity results, as is the case for several bacterial porins. We hope that TROMP immunization will generate a humoral and/or cellular response capable of efficiently killing *T. pallidum* and provide a significant level of acquired resistance.

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Dr. Blanco is a member of the Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles. He has been involved over the past 16 years in syphilis research focusing on structure-function relationships and the immunobiology of surface and subsurface proteins.

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Mycoplasmas: Sophisticated, Reemerging, and Burdened by Their Notoriety

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"Sit down before fact as a little child, be prepared to give up every preconceived notion, follow humbly wherever and to whatever abysses nature leads, or you shall learn nothing."
Thomas Henry Huxley

Mycoplasmas are most unusual self-replicating bacteria, possessing very small genomes, lacking cell wall components, requiring cholesterol for membrane function and growth, using UGA codon for tryptophan, passing through "bacterial-retaining" filters, and displaying genetic economy that requires a strict dependence on the host for nutrients and refuge. In addition, many of the mycoplasmas pathogenic for humans and animals possess extraordinary specialized tip organelles that mediate their intimate interaction with eucaryotic cells. This host-adapted survival is achieved through surface parasitism of target cells, acquisition of essential biosynthetic precursors, and in some cases, subsequent entry and survival intracellularly. Misconceptions concerning the role of mycoplasmas in disease pathogenesis can be directly attributed to their biological subtleties and to fundamental deficits in understanding their virulence capabilities. In this review, we highlight the biology and pathogenesis of these procaryotes and provide new evidence that may lead to increased appreciation of their role as human pathogens.

No other group of procaryotes has been so embroiled in controversy and in establishing a clear pathogenic niche as the mycoplasmas. Their virulence determinants are undeniably complex, and their unique biological properties likely challenge the host differently from typical bacterial pathogens (1,2). Also, numerous *Mycoplasma* species appear to comprise the commensal microbial flora of healthy persons (3), and the association of these mycoplasmas with disease complicates the diagnosis and necessitates extensive and highly specific serologic, nucleic acid, and epidemiologic data. Nonetheless, mycoplasmas by themselves can cause acute and chronic diseases at multiple sites with wide-ranging complications and have been implicated as cofactors in disease. Recently, mycoplasmas have been linked as a cofactor to AIDS pathogenesis and to malignant transformation, chromosomal aberrations, the Gulf War Syndrome, and other unexplained and complex illnesses, including chronic fatigue syndrome, Crohn's disease, and various arthritides (4-8). Even with mounting evidence of their pervasive and pathogenic potential, mycoplasmas still evoke the image of a group of obscure or impotent microorganisms. Yet they are evolutionarily advanced procaryotes (9-11), and their elite status as "next generation" bacterial pathogens necessitates new paradigms in fully understanding their disease potential.

Mycoplasmas, which lack cell walls but possess distinctive sterol-containing plasma membranes, are taxonomically separated from other bacteria and belong to the class Mollicutes (*mollis*, soft; *cutis*, skin). Mollicutes, a term that includes the cell wall-less procaryotes assigned to numerous genera under the class Mollicutes and is frequently used interchangeably with mycoplasmas, are unusual for other biological reasons

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as well. They are evolutionary descendants of the low G+C containing gram-positive bacteria and, through chromosome reduction, represent the smallest self-replicating life forms. Their streamlined genome size, which illustrates extreme biological gene economy, imposes complex nutritional requirements, such as dependence on external supplies of biosynthetic precursors, including amino acids, nucleotides, fatty acids, and sterols. This limited coding capacity dictates for mycoplasmas a parasitic way of life that few pathogenic microorganisms can claim. Therefore, the view that pathogenic mycoplasmas can grow "independently" requires an appreciation of their fastidious nature and their intimate dependence upon the host. Because of these properties, pathogenic mycoplasmas are among the most difficult microorganisms to grow from clinical specimens and remain frequent contaminants of primary and continuous eucaryotic cell lines and tissue cultures (12). In some instances, mycoplasma contamination is obvious since infected eucaryotic cells exhibit aberrant growth, metabolism, and morphology. However, mycoplasmas often establish covert and chronic infections of target cells that lead to either invalid and misleading data or introduction of mycoplasmas or their products into reagents dedicated to therapeutic or research purposes. The recent emphasis on isolating viral agents, such as human immunodeficiency virus (HIV)-1, from human primary lymphocytic cells has also demonstrated the frequent cocultivation of mycoplasmas of human origin. Often, the unwanted sources of exogenous mycoplasmas are serum products and filter-"sterilized" (450 nm) solutions; cross-contamination by already infected cell cultures, viral stocks, or immunologic preparations; breaks in technique, including aerosols from the respiratory tract or by mouth pipetting; ignorance of the mycoplasma problem; or scientific indifference.

Detailed up-to-date reviews describing the biological and pathogenic properties of mycoplasmas have been published (1,2,13,14). Our intention here is to provide a concise historical perspective of the role of mycoplasmas in human disease; highlight the discoveries of new *Mycoplasma* species and their association with human illness and host conditions that present problems in detection and treatment; describe selected biological properties of mycoplasmas consistent with their intimate host relationship and possible mechanisms of pathogenicity; and address recent controversies associated with mycoplasmas as emerging infectious agents. Renewed attention to these issues may provide the impetus to demystify mycoplasmas and improve their standing as genuine, card-carrying pathogens.

Historical Perspectives

The earliest reports of mycoplasmas as infectious agents in humans appeared in the 1930s and 1940s. At that time, primary atypical pneumonia was associated with an infectious agent that because of its minute size and innate biological properties unknown at that time, passed through bacteria-retaining filters, resisted penicillin and sulfonamide therapies, and adapted to growth in embryonated eggs and tissue culture cells. Correlations between the etiologic agent of "walking pneumonia" with viruses, L-forms, and pleuropneumonialike agents (referred to as PPLOs in publications and textbooks of that era) were frequent and often misleading. Finally, definitive studies in the early 1960s established *Mycoplasma pneumoniae* as the singular cause of cold agglutinin-associated primary atypical pneumonia (2). Today *M. pneumoniae* remains an important cause of pneumonia and other airway disorders, such as tracheobronchitis and pharyngitis (13,14), and is associated with extrapulmonary manifestations, such as hematopoietic, exanthematic, joint, central nervous system, liver, pancreas, and cardiovascular syndromes (15).

The confusion associated with *M. pneumoniae*-mediated infections has recurred many times with other mycoplasmas, whose detection in clinical specimens through culture, antibody, or DNA-based testing is frequently dismissed as "only mycoplasmas" even when they appear to be the primary pathogens. Two mycoplasmas commonly found in the urogenital tracts of healthy persons are *Mycoplasma hominis* and *Ureaplasma urealyticum*. However, over the years, the pathogenic roles of these mycoplasmas have been proven in adult urogenital tract diseases, neonatal respiratory infections, and a range of other diseases usually in immunocompromised patients (2).

Several recent examples illustrate the increasing impact of *Mycoplasma* species on emerging diseases. *Mycoplasma fermentans* strains were first isolated from the lower genital tract of both adult men and women in the early 1950s, but their role in classic lower genital tract disease has not been established (16). Reports in the 1970s of *M. fermentans* in the joints of rheumatoid arthritis patients and in the bone marrow of

children with leukemia raised expectations for its pathogenic potential (17,18); these findings have not been adequately confirmed. Sufficient evidence, however, has accumulated recently to establish an important and emerging role for *M. fermentans* in human respiratory and joint diseases. For example, *M. fermentans* has been detected by specific gene amplification techniques such as polymerase chain reaction (PCR) in the synovial fluid of patients with inflammatory arthritis, but not in the joints of patients with juvenile or reactive arthritis (19). In two other studies using PCR, *M. fermentans* was identified in the upper respiratory tract of 20% to 44% of both healthy and HIV-infected patients (20,21) and was associated with acute respiratory distress syndrome in nonimmunocompromised persons (22).

Mycoplasma genitalium was detected in the urogenital tract of two patients with nongonococcal urethritis in 1981 (23), but for more than a decade, very little was known about its host distribution and pathogenicity. Early experimental studies established that the organism caused lower genital tract infections in both male and female chimpanzees, with extensive urethral colonization in males and apparent tissue invasion, eventually leading to overt bacteremia (24). However, the fastidious growth requirements of *M. genitalium* from human hosts severely limited further study until the advent of molecular detection techniques. Specific sequences in the 140 kDa adhesin protein gene of *M. genitalium* were selected as targets in a PCR-based detection assay (25,26). Subsequent application of these techniques in cases of acute nongonococcal urethritis, not including those of patients colonized or infected with *Chlamydia trachomatis,* has provided mounting evidence for the involvement of *M. genitalium* as an etiologic agent of this disease (27-29). Also, *M. genitalium* has been suspected in chronic nongonococcal urethritis and pelvic inflammatory disease (30).

The discovery in 1988 of *M. genitalium* strains in human nasopharyngeal throat specimens, where they were frequently mixed with strains of *M. pneumoniae*, not only changed dramatically the concept of host distribution of *M. genitalium* but also prompted critical questions about the role of this mycoplasma in human respiratory disease (31). However, the immunologic cross-reactivity between *M. genitalium* and *M. pneumoniae* and the inability of most conventional diagnostic serologic tests to conclusively identify *M. genitalium* have complicated its delineation in acute human respiratory disease. PCR assays specific for the organism have detected *M. genitalium* in throat specimens of patients infected with HIV-1 (32). However, these probes have not been applied to control groups and patients in outbreaks of acute respiratory disease and/or pneumonia to determine whether *M. genitalium* alone is an etiologic agent in respiratory infections.

M. genitalium has been implicated as an etiologic agent in certain human joint diseases. This clinical correlation began with the observation of a mixed infection of *M. pneumoniae* and *M. genitalium* in synovial fluid specimens of a nonimmunocompromised patient after an acute respiratory infection (33). A predominant role was not established for either *Mycoplasma* species in the initial respiratory disease or in the joint manifestations, although evidence to implicate postinfectious autoimmunity to both organisms was described. These findings prompted a PCR assay on synovial fluids from patients with various arthritic syndromes, which presented case reports on two of 13 patients with *M. genitalium* detected in joint fluids (34).

Another area of emerging mycoplasmal infections concerns immunodeficiency. Although patients with congenital or acquired disorders of antibody production are susceptible to a wide variety of microbial infections, the unique susceptibility of such patients to mycoplasmal infections is a growing concern, especially considering the number of occurrences, the types of mycoplasmas involved, and the difficulties posed in the therapeutic management of such infections. In addition, the increased use of prolonged or permanent immunosuppressive chemotherapy required for patients undergoing tissue or organ transplantation or treatment of various malignant diseases has also increased the risk for mycoplasmal infections—from mycoplasmas that are part of the normal human mollicute flora to those acquired through animal contact.

The association between immunodeficiency and mycoplasmal infections was first reported in the mid 1970s in patients with primary hypogammaglobulinemia and infection with *U. urealyticum*, *M. pneumoniae*, *Mycoplasma salivarium*, and *M. hominis* that localized in joint tissue, frequently with destructive arthritis. Similar joint infections in hypogammaglobulinemic patients with these mycoplasmal species continue to be reported (35). Since most of these mollicutes, with the possible

exception of *M. pneumoniae*, occur as part of the normal human flora, the origin of such joint infections is considered endogenous. Patients with hypogammaglobulinemia and other antibody deficiencies are also especially susceptible to mycoplasmal infections of the upper respiratory and urinary tracts caused most frequently by *M. pneumoniae* or *U. urealyticum*, respectively (36).

Mycoplasmal infections following organ transplantation and immunosuppressive chemotherapy were observed in the early 1980s, with both *M. hominis* and *U. urealyticum* reported most often (37-39). Although these infections most likely originated from the patient's normal microbial flora, a recent report of donor transmission of *M. hominis* to two lung allograft recipients (40) suggests that donor tissue may be a more important factor in transplant infections than currently recognized.

While patients with antibody defects or those receiving immunosuppressive drugs appear to be the most susceptible to infections with mycoplasmas present in healthy tissues, emerging evidence indicates that contact with other mycoplasmas in the environment is an important hazard. For example, the direct isolation of a feline mycoplasma (*M. felis*) from the joint of a hypogammaglobulinemic patient with septic arthritis was recently reported (41), with suspected transmission occurring through a cat bite 6 months before the onset of arthritis. Other examples include fatal septicemia caused by *M. arginini*, a common animal mycoplasma, from blood and multiple tissue sites in a slaughter house employee who had advanced non-Hodgkin's lymphoma and hypogammaglobulinemia (42), and a septicemic infection with a canine mycoplasma (*M. edwardii*) in a patient with advanced AIDS (M.K. York, pers. comm.).

One of the most critical aspects of mycoplasmal infections in immunodeficient patients is the frequent inability to control such infections with appropriate broad spectrum antibiotics. Although the tetracyclines and erythromycins are effective chemotherapeutic agents for many mycoplasmal infections, *M. fermentans* and *M. hominis* strains are usually resistant to erythromycin, and tetracycline-resistant strains of *M. hominis* and *U. urealyticum* have been reported from the lower urogenital tract of patients. However, these antibiotics and most other broad spectrum agents have limited mycoplasmacidal activity in vivo, and their efficacy eventually depends on an intact host immune system to eliminate the mycoplasmas. Most hypogammaglobulinemic patients lack the ability to mount a strong antibody response. Guidelines for managing such mycoplasmal infections in patients with immune defects should include immediate in vitro testing of the isolated mollicute against a wide range of antibiotics; expeditious administration of the antibiotic by the most appropriate route (intravenously, if warranted); prolonged therapy terminated only if there is no rapid clinical or microbiological response; and possibly administration of intravenous immunoglobulin (35,36). Clinical management of mycoplasmal infections in transplant patients is more difficult since immunoglobulins may enhance graft or organ rejection. In the absence of suitable mycoplasmacidal chemotherapeutic agents, vigorous and sustained chemotherapy with the most active antibiotic is the current method of choice.

Mechanisms of Pathogenicity

Many mycoplasmal pathogens exhibit filamentous or flask-shaped appearances and display prominent and specialized polar tip organelles that mediate attachment to host target cells (43,44). These tip structures are complex, composed of a network of interactive proteins, designated adhesins, and adherence-accessory proteins (Figure 1, [14,43]). These proteins cooperate structurally and functionally to mobilize and concentrate adhesins at the tip and permit mycoplasmal colonization of mucous membranes and eucaryotic cell surfaces, probably through host sialoglycoconjugates and sulfated glycolipids (Figure 2, [14,43,45]). It appears that mycoplasmal cytadherence-related proteins represent a superfamily of genes and proteins that have been conserved through horizontal gene transfer from an ancestral gene family. This protein network resembles a specialized cytoskeletonlike apparatus, which may represent the precursor to mammalian cytoskeletal and extracellular matrixlike complexes (14). Other *Mycoplasma* species lack distinct tip structures yet are capable of cytadherence, and they may use related genes or proteins or alternative mechanisms of surface parasitism.

The family of mycoplasmal genes and proteins involved in cytadherence has been studied most extensively in *M. pneumoniae* (14,43,46-48). Noncytadhering phenotypes that arise through spontaneous mutation at high frequency have been categorized into mutant classes on the basis

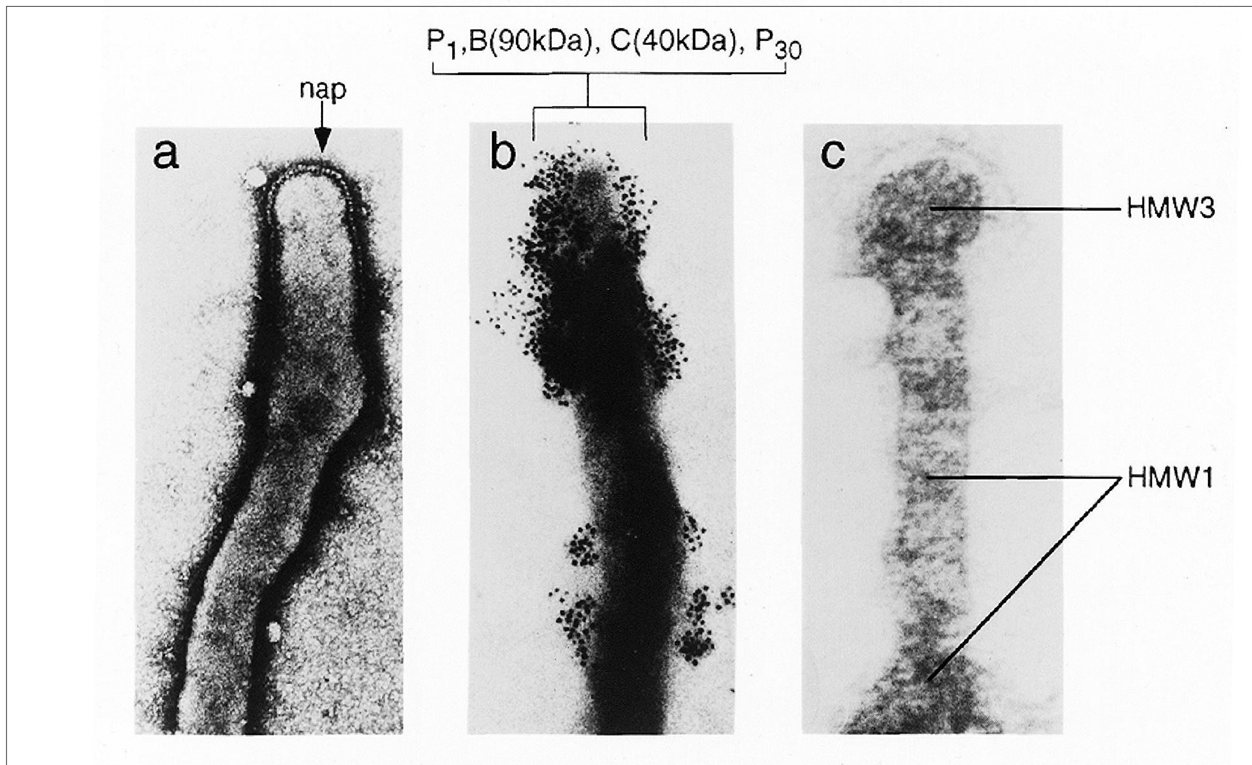

Figure 1. Transmission electron photomicrographs of the specialized tip organelle of cytadherence-positive *M. pneumoniae* demonstrating a) truncated structure with nap, b) clustering of cytadherence-related proteins (P1, B, C, P30) at the tip based on immunolabeling with ferritin and colloidal gold and crosslinking studies, and c) Triton X-100–resistant, cytoskeleton-like, structure with distinct bleb and parallel filaments (14,43,45,46).

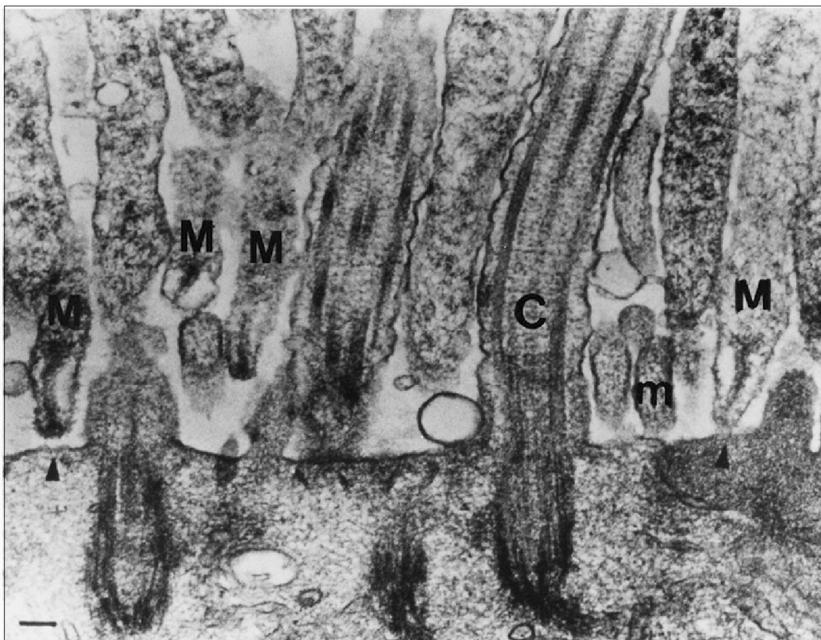

Figure 2. Transmission electron photomicrograph of a hamster trachea ring infected with *M. pneumoniae* (43). Note the orientation of the mycoplasmas through their specialized tiplike organelle, which permits close association with the respiratory epithelium. M, mycoplasma; m, microvillus; C, cilia.

of distinct protein profiles. These noncytadhering mycoplasmas cannot synthesize specific cytadherence-related proteins or are unable to stabilize them at the tip organelle, which leads to abnormal anatomical tip structures and avirulence (43). Spontaneous reversion to the cytadhering phenotype is accompanied by the reappearance of the implicated proteins, restoration of structurally and functionally intact tips, and return of full infectivity (43). Similar cytadherence-related genes and proteins have been reported for *M. genitalium* on the basis of biochemical, immunologic, and genetic analyses (25,49,50). Furthermore, striking similarities exist in the order of operons that comprise the cytadherence-related genes and the organization of these genes within each operon of *M. pneumoniae* and *M. genitalium* (14,50,51). These similarities reinforce the unexpected coisolations of *M. genitalium*, along with *M. pneumoniae*, from the nasopharyngeal throat swabs of patients with acute respiratory diseases and from synovial fluids of patients with arthritis as described in the previous section (31,33). The isolation of *M. pneumoniae* from the human urogenital tract (52) further suggests that these mycoplasmas have evolved parasitic strategies that include overlapping tissue tropisms as determined by the genetic and chemical relatedness of their cytadherence genes and proteins (14,25,43,50,51). The recent use of transposon mutagenesis to generate *M. pneumoniae* and *M. genitalium* transformants displaying cytadherence-deficient phenotypes should further clarify the relationships between the cytadherence-related genes and proteins and identify additional sites previously unlinked to cytadherence (46,53).

An interesting feature of specific *M. pneumoniae* and *M. genitalium* adhesins is their multiple gene copy nature (14,43,54,55,56). Although only one full-length copy of the adhesin structural genes exists in adhesin-related operons, precise regions of these adhesin genes are detected as single genomic copies, while other regions occur as closely homologous, but not identical, multiple copies. In other words, multiple truncated and sequence-related copies of the adhesin genes are dispersed throughout the genome, which could generate adhesin variation through homologous recombination. Consistent with this possibility is the existence of restriction fragment length polymorphisms in the adhesin genes of human clinical isolates of *M. pneumoniae* and *M. genitalium*, reflected by sequence divergence in the multiple-copy regions of the adhesin genes (56-59). It appears that a repertoire of partial adhesin-related gene regions serves as a reservoir to regulate the structural and functional properties of mycoplasmal adhesins through recombination events, which may lead to circumvention of the host immune response. Mechanisms of phase and antigenic variation are likely to occur in which mycoplasmal adhesins exhibit altered specificities and affinities, as determined by the organization of constant and variable adhesin gene sequences. Therefore, despite their small genomes, pathogenic mycoplasmas facilitate DNA rearrangements through repetitive gene sequences, thus promoting genetic diversity and maximizing the coding potential of their limited genomes. The immunodominant epitopes of the mycoplasmal adhesins appear not to be identical to the adherence-mediating domains (13). The latter are in part encoded by single copy regions of the adhesin genes and are highly conserved, which reinforces their essential role in mycoplasmal recognition of host cell receptors and colonization (60,61). Host immunoresponsiveness directed at the noncytadherence-mediating variable regions is unlikely to generate effective cytadherence-blocking antibodies, which may in part clarify the observed high reinfection rates of patients. Thus, the grouping of clinical isolates of *M. pneumoniae* into two categories, on the basis of sequence divergence in the multiple-copy regions of the adhesin gene (56-59), along with the immune status of the population, may explain the epidemiologic patterns of *M. pneumoniae* reported over the years.

Another characteristic of the cytadherence-related proteins is their proline-rich composition, which markedly influences protein folding and binding. Several reports have established the importance of these proline-rich domains in mycoplasmal cytadherence and virulence (47,48,62,63), and recent evidence further suggests that mycoplasmal peptidyl-prolyl isomerases, i.e., cyclophilins, are critical in regulating the conformation and function of the mycoplasmal cytadherence-related tip organelle, colony morphology, and growth (14,64). In addition to this proline-rich property, one of the most unusual features of the adhesins is their extensive sequence homology to mammalian structural proteins (1,14,33,43,47,48). This molecular mimicry is especially interesting since it has been suggested for decades that mycoplasmas provoke an anti-self response that

triggers immune disorders, although the basis for the induction has been elusive (65). Patients with documented *M. pneumoniae* respiratory infections demonstrate seroconversion to myosin, keratin, and fibrinogen (33) and exhibit extrapulmonary manifestations, such as exanthems and cardiac abnormalities. Furthermore, a classic example of bacteria-mediated autoimmune disorders is the development of acute rheumatic fever following streptococcal infection (66). Antistreptococcal antibodies reactive against α-helical coiled-coil regions of the M protein cross-react with heart myosin, tropomyosin, and mycoplasmal adhesins (14,66). In the latter case, these mycoplasmal adhesins exhibit amino acid sequence homologies with human CD4 and class II major histocompatibility complex lymphocyte proteins, which could generate autoreactive antibodies and trigger cell killing and immunosuppression (67,68). Also, mycoplasmas may serve as B-cell and T-cell mitogens and induce autoimmune disease through the activation of anti-self T cells or polyclonal B cells. The multiorgan protean manifestations of mycoplasmal infections in humans are consistent with the pathogenesis of autoimmunity. Furthermore, the ability of mycoplasmas to induce a broad range of immunoregulatory events, mediated by cytokine production and direct effects on macrophages, B and T cells, and glial cells, is evidence that mycoplasmas possess the attributes of primary mediators of pathogenesis (1,2,12,69). For example, cytokine production and lymphocyte activation may either minimize disease through the activation of host defense mechanisms or exacerbate disease through lesion development (69,70). Also, a superantigen derived from *Mycoplasma arthritidis*, a mycoplasma pathogenic for rodents, induces arthritis and chronic disease manifestations (69). It has been suggested that related superantigen-like molecules may exist in mycoplasmas of human origin triggering autoimmune and other inflammatory pathologies.

It appears that cytadherence is the initial step in the virulence process of pathogenic mycoplasmas (Figure 2) and precedes a spectrum of subtle or overt host cell responses. In specific instances, distinct cytopathology correlates with the infecting *Mycoplasma* species, the number of adherent mycoplasmas, the length of coincubation, the induction of proinflammatory cytokines, and the age and immune status of the patient. For example, the exacerbation of clinical syndromes may correlate with a history of mycoplasmal infection as observed in patients with recurrent *M. pneumoniae* exposures (2,13). Also, the elevated expression of proinflammatory cytokines associated with mycoplasmal disease pathogenesis may coincide with the intensity of the symptoms. In other cases, chronic disease or no obvious signs or symptoms of disease accompany mycoplasmal infection.

Other biological properties of mycoplasmas have been implicated as virulence determinants and include 1) generation of hydrogen peroxide and superoxide radicals by adhering mycoplasmas, which induces oxidative stress, including host cell membrane damage; 2) competition for and depletion of nutrients or biosynthetic precursors by mycoplasmas, which disrupts host cell maintenance and function; 3) existence of capsule-like material and electron-dense surface layers or structures, which provides increased integrity to the mycoplasma surface and confers immunoregulatory activities; 4) high-frequency phase and antigenic variation, which results in surface diversity and possible avoidance of protective host immune defenses; 5) secretion or introduction of mycoplasmal enzymes, such as phospholipases, ATPases, hemolysins, proteases, and nucleases into the host cell milieu, which leads to localized tissue disruption and disorganization and chromosomal aberrations; and 6) intracellular residence, which sequesters mycoplasmas, establishes latent or chronic states, and circumvents mycoplasmicidal immune mechanisms and selective drug therapies (1,2,71,72). Whether pathogenic mycoplasmas enter and survive within mammalian cells has been debated for many years. Consistent with this possibility, mycoplasmas exhibit limited biosynthetic capabilities; are highly fastidious and dependent upon the host microenvironment and complex culture medium for growth; have been observed in intimate contact with mammalian cell surfaces and within target cells; may be capable of initiating fusion with host cells through their cholesterol-containing unit membranes; and survive long-term recommended antimicrobial treatment in humans and tissue cultures. Recent sightings of intact mycoplasmas throughout the cytoplasm and the perinuclear regions of tissue cells from infected patients and in cell cultures, along with evidence that

mycoplasmas are capable of long-term intracellular survival and replication in vitro, offer an additional dimension to the pathogenic potential of mycoplasmas (4,14,72,73).

The Latest Controversies: Food for Thought or the Twilight Zone

On the basis of the above information, the virulence strategies displayed by mycoplasmas are likely the summation of a multitude of biological activities (1). Since no obvious single or group of mycoplasmal properties inextricably correlates with disease manifestations, the proof that mycoplasmas are card-carrying pathogens necessitates thorough and highly specific microbiological, epidemiologic, and diagnostic criteria; detailed descriptions of biochemical, genetic, and immunologic characteristics that distinguish virulent and avirulent mycoplasmas; and reproducibility of the symptoms of disease in experimental animal models or in the natural spread of infection among susceptible populations. The portfolio of available evidence concerning mycoplasma-mediated disease pathogenesis is limited. These scientific shortcomings precipitate misconceptions concerning mycoplasmas as singular agents of infectious diseases, as putative cofactors in the progression of other diseases, and as universal contaminants of cell cultures. Clearly, multiple pathways of interaction with target cells appears to be the *modus operandi* of the *Mycoplasma* species. With this conceptual scientific framework as a background, five recently proposed and controversial associations of mycoplasmas to human diseases are worth noting.

AIDS

The role of mycoplasmas in accelerating the progression of AIDS could not have begun under more baffling and circuitous conditions. A viruslike agent that arose through transfection of NIH 3T3 cells with DNA from Kaposi sarcoma tissues of AIDS patients was later shown to be *M. fermentans*. The spotted history of *M. fermentans* in rheumatoid arthritis and leukemia and its frequent contamination of cell cultures, along with its contemporary link to AIDS, have been considerable impediments to overcome in its elevation to pathogenic status. However, careful and convincing independent studies by several laboratories have implicated *M. fermentans* as a cause of systemic infections and organ failure in AIDS patients (4,74). The isolation of *M. fermentans* from blood and urine samples of HIV-infected persons, its detection by PCR and immunohistochemistry in multiple tissue sites at various stages of AIDS, and its ability to stimulate CD4+ lymphocytes and other immunomodulatory activities implicate this *Mycoplasma* species as a cofactor in AIDS. Consistent with this possibility, *M. fermentans* has been shown to act synergistically with HIV to enhance cytopathic effects on human CD4+ lymphocytes. Coincident with these studies, a new *Mycoplasma* species, *Mycoplasma penetrans*, also has emerged as a potential cofactor in AIDS progression (75,76). Its isolation almost exclusively from the urine of HIV-infected patients, the extraordinarily high prevalence of antibodies against this mycoplasma in HIV-infected patients and not in HIV-seronegative persons, and its capacity to invade target cells and activate the immune system of HIV-infected patients at various stages of disease correlate with a synergistic role with HIV. Other mycoplasmas, including *M. genitalium* and *Mycoplasma pirum*, have also been isolated from AIDS patients and implicated as potential cofactors. However, the proposed role of mycoplasmas as infectious agents and cofactors in AIDS-related disorders still remains a hypothesis without definitive proof. If cofactors of HIV are essential to the development of late stages of HIV-mediated disease, mycoplasmas possess all the prerequisite properties of the consummate helper. Their ability to establish covert or overt chronic and persistent infections with concomitant activation of the immune system, stimulation of cytokine production, and induction of oxidative stress correlate with increased HIV replication and disease progression. Are mycoplasmas irrelevant to AIDS, or are the clinical and microbiological correlations sufficient to imply intimate relationships between HIV and mycoplasmas, especially as the infected host undergoes immunologic distress?

Malignant Transformation

As early as the mid-1960s, mycoplasma-infected cell lines were associated with chromosomal aberrations, altered morphologies, and cell transformation (77,78). These abnormal oncogenic cell traits continued even after the apparent elimination of mycoplasmas, and evidence implied increased tumorigenicity of these transformed cells in animals. This issue has been revisited in studies demonstrating that long-term, persistent

mycoplasmal infection of mouse embryo cells initiated a multistage cellular process that resulted in irreversible cell transformation, karyotypic alterations, and tumorigenicity in nude mice (6). Do these oncogenic events associated with mycoplasma-mammalian cell coincubation relate to the ontogeny of human cancers?

Gulf War Syndrome

One of the most controversial current medical issues is whether the multiple acute and chronic symptoms found in veterans of the Persian Gulf War were caused by chemical exposure, infectious agents, or psychological problems, or whether a Gulf War Syndrome exists at all. The clinical illness comprises a collection of symptoms, including chronic fatigue, joint pain, headaches, and skin rashes. One study suggests that pathogenic mycoplasmas are responsible for a large number of cases among veterans, on the basis of DNA hybridization and the responsiveness of veterans to prolonged antibiotic treatment (5). Even though the experimental evidence is sparse and incomplete and well-controlled and detailed studies by independent laboratories are needed, if the Gulf War Syndrome has infectious causes, mycoplasmas with their requisite biological credentials are potential candidates.

Crohn's Disease

Several epidemiologic studies correlate respiratory infections with exacerbation of Crohn's disease and other chronic inflammatory bowel diseases (7,79). Acute onset gastrointestinal symptoms in patients with these diseases are accompanied by seroconversion to specific viral or *M. pneumoniae* antigens. As indicated earlier, mycoplasmas can elicit pleiotropic immune responses and are difficult to eliminate in patients despite appropriate antibiotic treatment. Steroid therapy to control gastrointestinal symptoms in these patients, along with the multifaceted biological properties associated with pathogenic mycoplasmas, may precipitate the onset of acute exacerbations of chronic inflammatory bowel disease.

Rheumatoid Arthritis and Other Human Arthritides

The occurrence of various *Mycoplasma* and *Ureaplasma* species in joint tissues of patients with rheumatoid arthritis, sexually transmitted reactive arthritis, and other human arthritides can no longer be ignored (8). A clinical trial of long-term (6 to 12 months) antibiotic (doxycycline) therapy before cartilage destruction might prove beneficial in managing such frequent and often debilitating infections.

Extensive clinical and microbiological evidence indicates that mycoplasmas alone can elicit a spectrum of illness for which no other agents are incriminated. The eradication of these pathogenic mycoplasmas from various tissue sites requires an intact and functional immune system, although persons with fully competent immune systems may have difficulty eliminating mycoplasmas, even with recommended prolonged drug therapy. Nonetheless, mycoplasmas are still viewed as subordinates to other infectious agents and are relegated to a category of commensals that unwittingly cause disease in patients whose immune systems offer little resistance to microbial stress and overload.

The fundamental importance of mycoplasmas in specific diseases of humans, animals, insects, and plants is irrefutable, and their unique biological properties are consistent with their intimate association with host target cells. These remarkable bacteria must continue to receive the scientific attention of mycoplasmologists, cell culturists, clinicians, immunologists, and DNA sequencers who most recently are compiling extensive databases that may eventually dissect every approachable mycoplasmal element that defines their biological and genetic being. Nonetheless, mycoplasmas remain mysterious and enigmatic, and the available data and proposed hypotheses that correlate mycoplasmas with disease pathogenesis range from definitive, provocative, and titillating to inconclusive, confusing, and heretical. Controversy seems to be a recurrent companion of mycoplasmas, yet good science and open-mindedness should overcome the legacy that has burdened them for decades.

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Fluoroquinolone Resistance in *Neisseria gonorrhoeae*

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Fluoroquinolones and broad-spectrum cephalosporins are the most effective antimicrobial agents for the treatment of gonorrhea. However, clinically significant resistance to fluoroquinolones has emerged in *Neisseria gonorrhoeae*. Fluoroquinolone-resistant strains account for approximately 10% of all gonococcal strains in Hong Kong and the Republic of the Philippines. As many as 50% of strains from some Far Eastern countries exhibit decreased susceptibility (intermediate resistance) to fluoroquinolones. Strains with intermediate resistance and clinically significant resistance are being isolated sporadically in North America, where resistant strains have been associated with an outbreak and with failure of infections to respond to treatment with doses of ciprofloxacin and ofloxacin recommended by the Centers for Disease Control and Prevention; strains exhibiting decreased susceptibility to these agents are endemic in at least one metropolitan area. Monitoring for fluoroquinolone resistance is now critical for ensuring adequate treatment of infections with resistant strains and for maximizing the time during which fluoroquinolones may be used to treat gonorrhea.

Gonorrhea is among the most prevalent sexually transmitted diseases throughout much of the world. The emergence of resistance to antimicrobial agents in *Neisseria gonorrhoeae* is a major obstacle in the control of gonorrhea. In 1989 and 1993, in response to the increasing frequency of isolation of penicillin-, tetracycline-, and spectinomycin-resistant strains of *N. gonorrhoeae* in the United States, the Centers for Disease Control and Prevention (CDC) recommended the use of broad-spectrum cephalosporins or fluoroquinolones for the primary treatment of uncomplicated gonorrhea (1,2). However, resistance to fluoroquinolones has now emerged in *N. gonorrhoeae* (3-8). Because ciprofloxacin and ofloxacin are more frequently used to treat gonorrhea than other fluoroquinolones, this synopsis will focus on these agents.

Fluoroquinolone Therapeutic Regimens, Therapy Failure, and Susceptibility Tests

In 1993, CDC recommended single-dose, oral therapy with ciprofloxacin (500 mg) or ofloxacin (400 mg) as two of the primary regimens for the treatment of uncomplicated gonorrhea (2). Enoxacin (400 mg), lomefloxacin (400 mg), and norfloxacin (800 mg) were recommended among alternative regimens (2). In some countries, gonococcal infections have been treated with a single, orally administered dose of 250 mg ciprofloxacin (8). The failure of gonococcal infections to respond to treatment with 250 mg ciprofloxacin has been reported in the United Kingdom since 1990 (8-11). The failure of infections to respond to single-dose therapy with 500 mg ciprofloxacin or 400 mg ofloxacin has been reported in the United Kingdom, Australia, Canada, Hong Kong, and the United States (3-7,12).

Different methods for determining in vitro antimicrobial susceptibilities of *N. gonorrhoeae* have been developed in several countries. Differences between these methods—the medium on which the susceptibilities are determined, the concentrations of antimicrobial agents tested, the concentration of antimicrobial agents in disks used to determine zone inhibition diameters, or the inoculum size—complicate the interpretation of susceptibility test results. For example, in the United Kingdom, Australia, and Hong Kong, susceptibilities are usually

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determined on Isosensitest medium (Oxoid, Basingstoke, United Kingdom) (3,8,13-15). In the United States (where criteria for interpreting susceptibilities of *N. gonorrhoeae* are established by the National Committee for Clinical Laboratory Standards [NCCLS]) and Canada, susceptibilities are determined on a Difco or Becton Dickinson base medium (Difco Laboratories, Detroit, MI; Becton Dickinson, Cockeysville, MD) (16-19). Antimicrobial susceptibilities determined on Oxoid base medium may be one concentration lower than those determined on supplemented Difco or Becton Dickinson base media (15). Neither method is right or wrong; nor is one method better than the other; they are different. The method used in different countries is influenced by the commercial availability of the base medium. Thus, criteria for interpreting susceptibilities in the United States and Canada may differ slightly from those used to interpret susceptibilities in the United Kingdom, Australia, and Hong Kong, reflecting the difference in susceptibilities obtained on different media. Similarly, when susceptibilities are measured on media different from those described above it may be necessary to establish "local" criteria using reference strains with known susceptibilities.

Gonococcal strains associated with therapy failure to 250 mg ciprofloxacin have had minimum inhibitory concentrations (MICs) of ≥0.06 to 0.25 µg/ml of ciprofloxacin (8,11); posttreatment strains from infections that failed to respond to treatment with 500 mg ciprofloxacin or 400 mg ofloxacin have had MICs ≥1.0 µg/ml and ≥2.0 µg/ml, respectively (4-6,12,15).

Criteria for Interpreting Fluoroquinolone Resistance

Before the emergence of fluoroquinolone resistance in *N. gonorrhoeae*, NCCLS established interpretive criteria to differentiate between susceptible strains and those exhibiting decreased susceptibility to selected fluoroquinolones including ciprofloxacin, ofloxacin, lomefloxacin, and enoxacin (16,17). Criteria for the interpretation of clinically significant resistance of gonococcal strains to 500 mg ciprofloxacin and 400 mg ofloxacin have been proposed (6,18).

Criteria for interpreting susceptibilities of *N. gonorrhoeae* to antimicrobial agents should be based on treatment outcome and strain susceptibility data. However, because many infections caused by fluoroquinolone-resistant strains were treated with broad-spectrum cephalosporins and few observations linked fluoroquinolone therapy outcome and antimicrobial susceptibilities (MICs), CDC proposed criteria for interpreting fluoroquinolone resistance in *N. gonorrhoeae* that were based on theoretical predictions of the MICs at which gonococcal infections may fail to respond to CDC-recommended doses of selected fluoroquinolones (18). On the basis of the therapeutic index (calculated by dividing the peak level of the agent in serum by the MIC of the infecting strain), CDC proposed criteria for interpreting susceptibilities to selected fluoroquinolones (Table) (18,20). These criteria were consistent with the MICs of isolates from the observed treatment failures to 500 mg ciprofloxacin and 400 mg ofloxacin documented at the time of the study (4,5,12). Recently, Kam et al. proposed interpretive criteria based on the susceptibilities of many strains that did not respond to treatment with ofloxacin in Hong Kong (Table) (6). CDC and Kam et al. proposed identical MICs for interpreting resistance to ciprofloxacin and ofloxacin: MICs of ≥2.0 µg/ml and ≥1.0 µg/ml of ofloxacin and ciprofloxacin, respectively. These criteria have also been adopted in Australia (15).

In the United Kingdom, where 250 mg ciprofloxacin has been used to treat gonorrhea, strains with MICs of 0.06 to 0.25 µg/ml have been isolated from infections that did not respond to treatment (8,11). Ciprofloxacin in a 250-mg dose produces a peak serum level of approximately 1.2 µg/ml; thus, strains with MICs of ≥0.25 to 0.5 µg/ml would produce therapeutic indices of 4.8:1 and 2.4:1, respectively. These calculated MICs suggest that strains with MICs ≥0.25 µg/ml should be considered resistant to treatment with 250 mg ciprofloxacin. Criteria for interpreting susceptibilities of gonococcal strains to treatment with 250 mg ciprofloxacin have been determined to ciprofloxacin (1 µg disks) or nalidixic acid (30 µg disks) (10,21). Measurement of nalidixic acid resistance may be useful for detecting strains causing infections that may not respond to treatment with 250 mg ciprofloxacin. However, because naladixic acid-resistance indicates decreased susceptibility to ciprofloxacin and ofloxacin, this test does not differentiate between strains with intermediate resistance and clinically significant resistance to treatment with 500 mg ciprofloxacin or 400 mg ofloxacin (18).

Table. Criteria for interpreting susceptibilities of *Neisseria gonorrhoeae* strains to the fluoroquinolones ciprofloxacin, ofloxacin, enoxacin, lomefloxacin, and norfloxacin[a,b]

Therapeutic agent, Dose	Agent Tested, Disk Content (µg)	Zone Inhibition Diameters[c] nearest whole mm			Equivalent MIC[c] (mg/ml)			Reference
		R	I	S	R	I	S	
Ciprofloxacin, 250 mg	Cip, 1	≤24			≥0.03			8
Ciprofloxacin, 500 mg	Cip, 5	≤29	30-35	≥36	≥1.0	0.13-0.5	≤0.06	18
	Cip, 5	≤22			≥1.0			6
Ofloxacin, 400 mg	Ofx, 5	≤24	25-30	≥31	≥2.0	0.5-1.0	≤0.25	18
	Ofx, 5	≤24			≥1.0			6
Enoxacin, 400 mg	Enx, 10	≤31		≥32	≥1.0	0.5	≤0.25	18
Lomefloxacin, 400 mg	Lom, 10	≤26	27-35	≥36	≥2.0	0.25-1.0	≤0.125	18
Norfloxacin, 800 mg	Nor, 5	≤32	33-35	≥36	≥1.0	0.5	≤0.25	18

[a]These criteria have not been recommended by the National Committee for Clinical Laboratory Standards (NCCLS); they are provided to guide interpretation of susceptibilities until the NCCLS establishes criteria.
[b]Abbreviations: MIC, minimal inhibitory concentration (µg/ml); R, resistant; I, intermediate resistance; S, susceptible; Cip, ciprofloxacin; Ofx, ofoxacin; Enx, enoxacin; Lom, lomefloxacin; Nor, norfloxacin.
[c]With the exception of norfloxacin, criteria for the susceptible categories are those designated by the NCCLS (16,17); criteria for the interpretation of the susceptible category for norfloxacin were proposed by CDC (18).

Geographic Distribution, Frequency, and Diversity of Fluoroquinolone-resistant *N. gonorrhoeae*

Strains exhibiting intermediate resistance (MICs 0.125 to 0.5 µg/ml of ciprofloxacin; MICs 0.5-1.0 µg/ml of ofloxacin) have been reported from many geographic areas including Australia, Canada, the Canary Islands, Hong Kong, Japan, the Republic of the Philippines, mainland Spain, Thailand, the United Kingdom, the United States, and the West Indies (4,7,9-15,21-32). Fluoroquinolone resistance (MICs of ≥1.0 µg/ml or ≥2.0 µg/ml of ciprofloxacin or ofloxacin, respectively) has been reported most frequently from the Far East, Republic of the Philippines, Hong Kong, Japan) and less frequently from Australia, Canada, Spain, Thailand, United Kingdom, and the United States (3,4,6,7,9,12,15,22-32). Ofloxacin- and ciprofloxacin-resistant strains had been isolated sporadically in Hong Kong, the Republic of the Philippines, and Thailand for some years (22-24). The frequency of fluoroquinolone-resistant strains has increased dramatically since the early 1990s. For example, in Hong Kong, fluoroquinolone-resistant strains were isolated intermittently during 1990 to 1992 (13) but have increased dramatically from an estimated 0.5% in late 1992 to 10.4% in late 1994 (14).

From 1994 to 1995, strains exhibiting decreased susceptibility to ciprofloxacin and ofloxacin accounted for approximately 36%, 54%, and 22% of strains in Hong Kong, the Republic of the Philippines, and Thailand, respectively (13,14,29,31,32). During the same period, fluoroquinolone-resistant strains accounted for approximately 10%, 12%, and 1% of all strains in Hong Kong, the Republic of the Philippines, and Thailand, respectively (13,14,29,31,32). Strains with ciprofloxacin MICs of ≥8.0 µg/ml were first isolated in 1994 (10,12,29,31).

In other geographic areas, strains exhibiting intermediate resistance and resistance have been isolated only sporadically, although with increasing frequency. In Sydney, Australia, fluoroquinolone-resistant strains were isolated infrequently in 1991 to 1994, but with dramatically increasing frequency in early 1995 (15). The pattern of isolation of fluoroquinolone-resistant strains in Australia, i.e., infrequent and sporadic isolations for a number of years followed by increasing frequency of isolation over a short period, may be anticipated in other countries unless the fluoroquinolone-resistant strains are controlled in the Far East, where they are now prevalent. In the CDC-sponsored Gonococcal Isolate Surveillance Project in the United States, the frequency of strains with intermediate resistance has increased significantly from 0.3% (17/5,238) in 1991 to 1.3% (65/4,996) in 1994 ($p \leq 0.001$); however, resistant strains accounted for only 0.04% (2/4,996) of strains in 1994 (33,34). In the United States, the increase in strains with intermediate resistance is associated largely, but not exclusively, with the persistence of such strains in Cleveland, Ohio. First detected in 1992, these strains accounted for 16% to 17.5% of isolates in Cleveland in 1994 (26,35). In addition, a sustained outbreak caused by ciprofloxacin-resistant strains has been reported from Seattle, Washington, in 1995; these strains had MICs of 8.0 µg/ml of ciprofloxacin and ofloxacin (5).

Many different strains, as defined by penicillin/tetracycline resistance phenotype and auxotype/serovar (A/S) class, exhibit intermediate resistance and resistance to fluoroquinolones (4,5,15,25,26,31,32,36). Fluoroquinolone resistance has been identified frequently in strains that produce ß-lactamase and strains exhibiting chromosomally mediated resistance to penicillin and tetracycline and less frequently in strains that are susceptible to penicillin and tetracycline (4,31,32). Fluoroquinolone resistance has not been documented in strains possessing the 25.2-megadalton TetM-containing plasmid (TRNG) alone or in TRNG strains possessing a ß-lactamase plasmid (31). It should not, however, be assumed that these strains may not develop fluoroquinolone resistance, but rather that resistant strains have not been detected at this time. Many strains, as defined by A/S class, are associated with fluoroquinolone resistance; fluoroquinolone resistance is not associated with the epidemic spread of one or two strains (15,31,32). In Australia, strains exhibiting intermediate resistance and resistance to fluoroquinolones have belonged to 27 different A/S classes; strains with MICs 8.0-16.0 µg/ml of ciprofloxacin have belonged to 6 A/S classes (15). In the Republic of the Philippines, strains belonging to 27 A/S classes exhibited decreased susceptibility to ciprofloxacin; strains belonging to 10 A/S classes had MICs ≥1.0 µg/ml of ciprofloxacin (31). In Thailand, strains belonging to 13 A/S classes exhibited decreased susceptibility to ciprofloxacin (32).

The Gonococcal Isolate Surveillance Project monitors the susceptibilities of *N. gonorrhoeae* isolates to fluoroquinolones in the United States and provides prototype reference strains for quality assurance of susceptibility testing of these agents. Information about antimicrobial resistance in *N. gonorrhoeae* in the United States is available on the Internet at http://www.cdc.gov/ncidod/dastlr/gcdir/gono.html.

Mechanisms of Resistance to Fluoroquinolones

Fluoroquinolones inhibit the replication of DNA; they are believed to bind to the GyrA region of DNA gyrase, which is attached to DNA, and inhibit the enzyme from supercoiling the DNA (37). Resistance to fluoroquinolones in *N. gonorrhoeae* is associated with mutations that result in amino acid changes in the A subunit (GyrA) and the B subunit (GyrB) of the DNA gyrase, and in the *parC*-encoded subunit of topoisomerase IV (37-40). Although mutations in *gyrB* confer low-level resistance to naladixic acid, high-level quinolone resistance is associated with mutations in the quinolone resistance-determining region of *gyrA* (41). Topoisomerase IV, encoded by *parC* and *parE* in *Escherichia coli* and believed to be located in the cytoplasmic membrane, is involved in DNA replication but is not as sensitive to fluoroquinolone inhibition as is DNA gyrase (37). No *parE* analog has been detected in *N. gonorrhoeae* (37). Mutations in *gyrA* and *parC* are most relevant when considering clinically significant levels of fluoroquinolone resistance in *N. gonorrhoeae* (37,39,40). Similar results have been obtained in studies of *gyrA* mutations in both laboratory-adapted strains and clinical isolates (37,39,40): ciprofloxacin-susceptible strains (MICs, <0.03 µg/ml) had no mutations in *gyrA* and strains with MICs, ≥0.5 µg/ml of ciprofloxacin may have changes in nucleotides 272 and 283 of *gyrA*. In addition, strains with MICs ≥2.0 had mutations in *parC*. Mutations in *parC* were observed only in strains with at least one mutation in *gyrA* (37,39) and appeared to be associated with an MIC higher than would be expected with the *gyrA* mutation alone (39). Mutations in *gyrA* and *parC* may be characterized by polymerase chain reaction and DNA sequencing (37,39). The transfer of *gyrA* and *parC* mutations between gonococcal strains has been demonstrated in vitro (37). The presence of transformation sequences just downstream from the *gyrA* sequences suggests that transformation may play a role in the spread of *gyrA* mutations between gonococcal strains in vivo (37). The opportunity for transformation of genes between gonococcal strains, which depends on concurrent infections with multiple strains, has been documented for women and homosexual men (42,43).

In addition to mutations in *gyrA* and *parC*, reduced permeability of the cytoplasmic membrane may contribute to low-level resistance to fluoroquinolones in *N. gonorrhoeae,* e.g., increasing the MIC of one recipient strain from ≤0.002 to 0.06 µg/ml of ciprofloxacin (3,44). This resistance may also be transferred between gonococcal strains by transformation (3).

Guidance

Continued reports of the isolation of fluoroquinolone-resistant strains of *N. gonorrhoeae* indicate the need for heightened awareness of the potential for increasing prevalence of strains with

clinically significant fluoroquinolone resistance. In areas where a fluoroquinolone is used to treat gonorrhea, the following steps are recommended to monitor and control the spread of fluoroquinolone-resistant strains of *N. gonorrhoeae*.

- Susceptibility testing should be performed to detect fluoroquinolone resistant strains. In geographic areas where interpretive criteria have not been proposed, criteria should be developed for results obtained with local test procedures that use strains with known susceptibilities to ciprofloxacin or ofloxacin.
- Ideally, routine surveillance for emerging fluoroquinolone resistance should be performed in centers where fluoroquinolones are used widely to treat gonorrhea; e.g., a sample of approximately 20 to 50 consecutive isolates should be tested periodically. If ciprofloxacin-resistant isolates are detected, the use of alternative therapies for gonorrhea should be considered; if isolates are ciprofloxacin-susceptible, ciprofloxacin or ofloxacin may be used to treat gonorrhea.
- Susceptibilities of isolates from individual patients whose infections did not respond to treatment with a fluoroquinolone should be determined.
- A cluster of infections unresponsive to fluoroquinolone therapy may indicate an outbreak caused by a resistant strain.
- If treating gonorrhea with a fluoroquinolone, e.g., ciprofloxacin or ofloxacin, never use less than the recommended dose. In the United States, 500 mg of ciprofloxacin or 400 mg ofloxacin should be used.
- Because gonococcal infections caused by fluoroquinolone-resistant strains have been acquired frequently in the Far East, clinicians may wish to treat infections possibly acquired in the Far East with 125 mg ceftriaxone, intramuscularly, or 400 mg cefixime, orally, the current CDC-recommended doses for treating gonococcal infections.

The importance of the emergence and spread of fluoroquinolone resistance in *N. gonorrhoeae* cannot be overstated. Of antimicrobial agents available for treating gonorrhea, broad-spectrum cephalosporins are the only agents to which *N. gonorrhoeae* is not resistant, and exclusive use of these agents, particularly the orally administered cephalosporins such as cefixime, may result ultimately in the emergence of resistance to these agents. Thus, it is critical to take measures to ensure that fluoroquinolones remain effective for the treatment of uncomplicated gonorrhea for as long as possible.

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Infectious Diseases and Immunity: Special Reference to Major Histocompatibility Complex

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Human leukocyte antigens (HLAs) are an inherent system of alloantigens, which are the products of genes of the major histocompatibility complex (MHC). These genes span a region of approximately 4 centimorgans on the short arm of human chromosome 6 at band p 21.3 and encode the HLA class I and class II antigens, which play a central role in cell-to-cell interaction in the immune system. These antigens interact with the antigen-specific cell surface receptors of T lymphocytes (TCR) thus causing activation of the lymphocytes and the resulting immune response. Class I antigens restrict cytotoxic T-cell (CD8+) function thus killing viral infected targets, while class II antigens are involved in presentation of exogenous antigens to T-helper cells (CD4+) by antigen presenting cells (APC). The APC processes the antigens, and the immunogenic peptide is then presented at the cell surface along with the MHC molecule for recognition by the TCR. Since the MHC molecules play a central role in regulating the immune response, they may have an important role in controlling resistance and susceptibility to diseases. In this review we have highlighted studies conducted to look for an association between HLA and infectious diseases; such studies have had a variable degree of success because the pathogenesis of different diseases varies widely, and most diseases have a polygenic etiology.

Major Histocompatibility Complex (MHC) and Immune Response

Because of its remarkable power to deal with infection, the immune system is central to the prevention and control of infectious disease. Immune responsiveness is affected, even controlled, by gene products of the major histocompatibility system (1). Many diseases are associated with human leukocyte antigens (HLAs) (2,3). Moreover, in some infectious diseases (4-6), the host immune reactivity, which is responsible for the pathologic manifestation of disease, has been correlated with HLA specificities.

The discovery of the human MHC dates from the mid 1950s when leukoagglutinating antibodies were found in the sera of patients who received multiple transfusions and in the sera of 20% to 30% of multiparous women. In humans, the entire histocompatibility complex is termed the HLA complex. Genes coding for HLAs occupy a segment of approximately 4 centimorgans on the short arm of chromosome 6. The HLA-A, -B, and -C genetic loci determine class I antigens; HLA-DR, -DP, -DQ genetic loci determine class II antigens. Class I antigens are found on virtually every human cell; class II antigens are found chiefly on the surfaces of immunocompetent cells, including macrophages/monocytes, resting T lymphocytes, activated T lymphocytes, and particularly B lymphocytes.

The MHC molecule provides a context for the recognition of antigens by T lymphocytes. The polymorphic binding site of MHC class I and class II molecules is composed of a ß-pleated sheet flanked by two alpha helixes. They form a groove that accommodates one single microbial peptide ligand.

MHC class I molecules bind to peptides produced by the intracellular degradation of viral proteins and display them on the cell surface for recognition by CD8+ T lymphocytes. A class of white blood cells, the CD8 T lymphocytes, bear receptors specific for the HLA class I antigens

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and route pathogens such as viruses. Surface expression of class I MHC molecules depends on the availability of peptides that bind MHC molecules in the endoplasmic reticulum. A peptide transporter, associated with antigen processing (TAP), plays an important role in maintaining adequate levels of peptide (7). The transporter is a heterodimer encoded by two genes, TAP1 and TAP2, located in the MHC class II region. TAP genes belong to the adenosine triphosphate (ATP) binding cassette super family of transport proteins, which have two ATP-binding cassette domains and two transmembrane domains. TAP genes are polymorphic (8), and allelic MHC differences may be associated with disease by altering the peptides that bind class I MHC molecules. Since human TAP genes are located between HLA-DP and HLA-DQ, TAP alleles could result in an apparent disease association with class II HLA alleles. Class I, i.e., HLA-A, -B, and -C molecules, play an important role in viral infections in the lysis of target cells by cytotoxic killer T lymphocytes.

MHC class II molecules are highly polymorphic membrane glycoproteins that bind peptide fragments of proteins and display them for recognition by CD4+ T lymphocytes. The white blood cells known as CD4 T lymphocytes are of central importance in defeating the bacteria and other parasites that live within cells. The CD4 T lymphocytes are called helper T cells because they secrete substances that amplify and control virtually all aspects of immunity. These T cells have receptor molecules that can recognize one particular peptide-HLA class II antigen combination. The binding capability of any given peptide to MHC class II molecules depends on the primary sequence of the peptide and allelic variation of the amino acid residues in the binding site of the MHC receptor. Anchor residues defining allele-specific peptide motifs have been identified in the class II binding peptides. The proposed anchor residues combining with MHC pockets through their side chains seem to be a primary requirement for peptide-MHC interaction. The invariant chain (Ii) plays a critical role in the assembly, intracellular transport, and function of MHC class II molecules (9). In intracellular parasites (e.g., *Leishmania* infections of macrophages), it is the class II MHC molecules that specifically bind to receptors on these microbes.

HLA Association with Infectious Diseases

Infectious diseases are associated with impaired immunity. Some persons mount very effective immune responses when given vaccines, while others respond to vaccines poorly or not at all. The level of response is determined by several factors: intensity of infection, factors related to the intensity of the host immune response, T-cell state, T-cell function, and perhaps most important, the genetic factor that interacts with the other factors to determine the outcome of the disease. Infectious disease research is now focusing on genetic markers such as allelic forms of HLA molecules.

HLA Association with Mycobacterial Infections

Genetic factors may control host responses to *Mycobacterium tuberculosis* (10-12). Several investigators have conducted population studies to determine an association between pulmonary tuberculosis (TB) and HLA specificities. HLA-DR2 is associated with the development of multibacillary forms of both TB and leprosy (13,14); molecular subtyping of DR2 showed that the majority of the allele in patients and controls was DRB1*1501 and DRB1*1502. The frequency of these molecular subtypes of DR2 in patients was not skewed, suggesting that the entire DR2 molecule or its closely linked gene(s) may govern patient susceptibility to pulmonary TB and, particularly, to drug-resistant TB. When the three-dimensional structure of the HLA-DR molecule is elucidated (15), sequencing of class II alleles in patients with pulmonary TB and drug-resistant TB could identify an amino acid residue(s) critical for the binding of a *M. tuberculosis*-derived pathogenic peptide(s) responsible for the detrimental or protective immune response.

HLA alleles also modulate the immune response that determines the form of leprosy (a heterogeneous disease caused by *Mycobacterium leprae)* that develops in each patient (16,17). At one pole of the spectrum of leprosy are the multibacillary lepromatous leprosy (LL) patients, who are anergic to the antigens of *M. leprae,* and at the other extreme are the paucibacillary tuberculoid leprosy (TT) patients, who exhibit a good cell-mediated immune response. Humoral immunity is present throughout the spectrum but does not seem to provide protection. Between the two poles are patients with intermediate features as seen in the borderline lepromatous,

borderline leprosy (BB), and borderline tuberculoid forms (18). An increased frequency of HLA-DR2 and -DQ1 in LL patients (19) and of HLA-DR3 in TT patients has been reported (20). These antigens can be further subdivided into alleles defined by their amino acid sequence. A single amino acid substitution may give rise to alleles with different immunologic properties. The allele DRB1*1501 showed a stronger association with LL patients than with TT patients ($p < 0.00001$). In addition, DQB1*0601 was found significantly more often in LL patients than in controls ($p < 0.00001$); DQA1*0103 was more frequent in the LL group than in the tuberculoid leprosy group; and DQA1*0102 was selectively increased in patients with borderline lepromatous leprosy (Table 1). However, DRB1*0701, DQB1*0201, and DQA1*0201 were decreased in LL patients compared with TT patients and controls, and DQB1*0503 was selectively decreased in TT patients, suggesting that these alleles might modulate the immune response that determines the form of leprosy that develops in each patient (21).

Table 1. Frequency of HLA class II alleles with significant differences between leprosy patients and healthy controls

HLA alleles	Healthy controls N=47		Leprosy patients N=93			
	N	%	N	%	RR[a]	p
DRB1*15	10	21.3	70	75.3	11.3	<0.00001
DRB1*1501	6	12.8	49	52.7	7.6	<0.00001
DRB1*1502	5	10.6	26	27.9	3.2	<0.05
DRB5*0101	6	12.8	49	52.7	7.6	<0.00001
DRB5*0102	5	10.6	26	27.9	3.2	<0.05
DQA1*0102	9	19.1	38	40.9	2.9	<0.05
DQA1*0103	13	27.6	48	51.6	2.8	<0.05
DQB1*0601	8	17.0	56	60.2	7.4	<0.00001
DRB1*0404	5	10.6	0	0.0	0.04	<0.01
DRB1*0701	13	29.8	11	11.8	0.3	<0.05
DRB1*1401	4	8.5	0	0.0	0.005	<0.05
DQB1*0503	16	36.2	14	16.1	0.3	<0.05

[a]RR = relative risk

HLA Association with Parasitic Infections

Because there are significant differences between malaria-exposed and -unexposed populations in the frequencies of HLA genes at the A and B loci, the HLA complex may protect populations in endemic-disease areas who are exposed to malaria parasites. The adaptative mechanisms may be expressed by HLA-associated genes that control immune responsiveness to malaria antigens. The association between the HLA class I antigen HLA-B53 and protection from severe malaria has been well established (5). This link might be mediated by HLA class I restricted cytotoxic T lymphocytes (CTL) during the liver stage of the parasite's life cycle (22). The protective association between HLA-B53 and severe malaria was investigated by sequencing peptides eluted from this molecule before testing candidate epitopes from preerythrocytic-stage antigens of *Plasmodium falciparum* in biochemical and cellular assays. Among malaria-immune Africans, HLA-B53 restricted CTL recognized a conserved nonamerpeptide from liver stage-specific antigen, but no HLA-B53 restricted epitopes were identified in antigens from other stages (5). These findings indicate a possible molecular basis for this HLA disease association and support the candidacy of liver stage-specific antigen as a malaria vaccine component.

The association between HLA-DR/-DQ phenotypes and immune response to circumsporozoite protein of the human malaria parasite were investigated in Thai adults (23). Evidence suggests that human T- and B-cell responses to a major *P. falciparum* antigen (Pf RESA) in persons primed by repeated infections are genetically regulated (24). To associate T-cell and antibody responses with the donors' MHC class II genotypes, genomic HLA class II typing of DQ antigens of leukocytes from 145 donors living in endemic-disease regions of Africa were performed by restriction fragment length polymorphism (24). These data imply that the impact of MHC class II gene products on specific immune responses to Pf 155/ RESA epitopes is weak and hard to demonstrate in outbred human populations naturally primed by infection. The relationship between class II HLA and immune recognition of three candidate antigens for a vaccine against *P. falciparum* was investigated in persons extremely heterozygous for HLA class II alleles living in an endemic-disease area of West Africa (25). One class II DQA-DQB combination (serologic specificity DQw2) was particularly common among these persons. This haplotype was significantly associated with higher than average levels of antibody to a peptide epitope (EENV)6 of Pf RESA. There was little evidence of association between HLA class II genotype and cellular proliferation responses to the antigen tested.

The frequency of HLAs was studied in 62 patients with scabies and 27 patients with

cutaneous leishmaniasis to evaluate the role of HLA antigens as genetic markers in the pathogenesis of these parasitic skin diseases. A significant statistical association was found between HLA-A11 antigen and scabies and between HLA-A11, -B5, and -B7 antigens and diffuse cutaneous leishmaniasis (26). In another study, 24 families with one or more cases of localized cutaneous leishmaniasis from an endemic-disease region with the highest incidence of localized cutaneous leishmaniasis in Venezuela were typed for HLA-A, -B, -C, -DR, and -DQ antigens and complement factors. The parental HLA haplotypes segregated at random among healthy and affected siblings, but in backcross families significantly higher frequencies of HLA-A28, -Bw22 or -DQw8 were present in infected compared with healthy siblings (27). In addition, HLA-B15 showed a higher frequency among healthy siblings. Haplotypes Bw22, DR11, DQw7 were also significantly more frequent in infected than in healthy siblings. No HLA linkage with a putative localized cutaneous leishmaniasis susceptibility gene(s) could be demonstrated in this study (27). A case/control comparison of 26 unrelated localized cutaneous leishmaniasis patients and healthy persons of the same ethnic origin confirmed the association of HLA-Bw22 and -DQw3 with this disease. The relative risk reached 12.5 for Bw22 and 4.55 for DQw3. HLA-DQw3 apparently makes the major contribution as a genetic risk factor for localized cutaneous leishmaniasis at the population level. In another study, a statistically significant association was found between HLA-B5 and -DR3 and schistosomiasis (28).

A study of the association of HLA class I antigen frequencies in 52 patients with kala-azar and 222 unrelated healthy controls in Iran found HLA-A26 to be statistically significant ($p = 0.004$) (29). This indicates a high risk of contracting the disease for HLA-A26 positive persons and a remarkable influence of this antigen on the prevalence rate of kala-azar.

The significance of susceptibility/protection correlations between HLA and parasitic diseases has been established by serologic typing methods. To improve the accuracy of MHC-disease associations, we have used a DNA-based HLA typing method, namely polymerase chain reaction with sequence specific oligonucleotide probes, for the molecular typing of kala-azar patients in India (30). To study the possible association at the molecular level of HLA class I (A and B) as well as class II (DR) antigens in kala-azar patients, we typed patients with kala-azar by polymerase chain reaction with sequence specific oligonucleotide probes and compared the antigen frequencies with healthy family-based controls. On the basis of the distribution of alleles in each sample, percentage phenotype and genotype frequencies were calculated for both control and kala-azar patients. Statistical analysis using the Transmission Disequilibrium Test was carried out to assess the association of different HLA allelic specificities with kala-azar patients. No significant association between any of the HLA class I or class II antigens was found. We will conduct a linkage analysis study based on the data from typing the above-mentioned case/controls. The findings might lead to a new dimension in the study of HLA association with parasitic infections: genetic markers, such as HLA, that are sufficiently polymorphic (as measured by their heterozygosities) can be used in linkage and association analysis to detect Mendelian segregation underlying disease phenotypes (31).

Comprehensive analysis of HLA associations with infectious diseases has allowed precise definition of susceptibility and protective alleles in large populations of different ethnic origins. Of great interest in the fine dissection of molecular mechanisms leading to parasitic diseases, these studies also provide the genetic basis for identification of the subset of persons at risk for subsequent infection. Infectious diseases may have exerted significant pressure on the development and maintenance of HLA polymorphism (32). Widespread and frequently fatal parasitic diseases such as malaria have selectively maintained certain gene frequencies in endemic-disease areas (33).

Although HLA associations with parasitic diseases have provided clues to pathogenesis, the molecular basis of these associations has not yet been defined. The determinant selection hypothesis, which states that associations result from the ability of a particular HLA type to present a critical antigenic peptide, has been difficult to investigate because, for most disease associations, the relevant antigen is unknown. Recently, the identification of characteristic sequence features in peptides eluted from HLA class I molecules (34,35) suggested that the relevant antigen might be identifiable by assessing cellular immune responses to peptides containing such motifs among antigens that are candidates for mediating

HLA disease associations. With the development of modern techniques of the HLA assembly assay (36), relevant peptides can be synthesized; it can then be determined whether they have any function as CTL epitopes during immune responses. Such studies will elucidate the HLA associations with parasitic infections and the molecular basis of these associations and facilitate the development of vaccines for these infectious diseases.

HLA Association with Viral Infections

The associations of viral diseases with HLA alleles have not been studied extensively. However, mechanisms by which HLA molecules determine the immune response to viral peptides have been well studied as part of efforts to develop safe and efficient virus vaccines. Successful development of vaccines against viral infections depends on the ability of inactivated and live virus vaccines to induce a humoral immune response and produce antiviral neutralization antibodies. Additionally, virus vaccines that induce a cellular immune response leading to the destruction of virus-infected cells by CD8+ CTLs may be needed to provide protection against some viral infections. Antiviral CD8+ CTLs are induced by viral peptides presented within the peptide binding grooves of HLA class I molecules on the surface of infected cells. Studies in the last decade have provided an insight into the presentation of viral peptides by HLA class I molecules to CD8+ T cells.

Herpesvirus saimiri, an oncogenic, lymphotropic, gamma-herpesvirus, transforms human and simian T cells in vitro and causes lymphomas and leukemias in various species of New World primates. An open reading frame of the *H. saimiri* genome encodes a heavily glycosylated protein that is secreted and binds to heterodimeric MHC class II HLA-DR molecules (37). These results indicate that the open reading frame can function as an immunomodulator that may contribute to the immunopathology of *H. saimiri* infection.

Cytotoxic T cells that recognize dengue virus peptides have been reported (38). Analysis of HLA class I haplotype-restricted peptides showed that HLA-A2 and -A68 motifs were abundant compared with nonpeptides with HLA-A24, -B8, and -B53 motifs. Studies by Zeng et al. (39) suggest that the T-cell response to dengue virus is restricted by the HLA-DR15 allele. Becker (40) developed an approach to priming antiviral CD8+ CTLs that may provide cellular immune protection from flavivirus infection without inducing the humoral immune response associated with dengue fever shock syndrome. He proposed using synthetic flavivirus peptides with an amino acid motif to fit with the HLA class I peptide binding group of HLA haplotypes prevalent in a given population in an endemic-disease area as an immunogen. These synthetic viral peptides may be introduced into the human skin by using a lotion containing the peptides (Peplotion) and substances capable of enhancing the penetration of these peptides into the skin to reach Langerhans cells. The peptide-treated Langerhans cells, professional antigen presenting cells, may bind the synthetic viral peptides by their HLA class I peptide binding grooves. Antigens carrying Langerhans cells can migrate and induce the cellular immune response in the lymph nodes.

Transmission of human immunodeficiency virus 1 (HIV-1) from an infected woman to her offspring during gestation and delivery is influenced by the infant's MHC class II DRB1 alleles. Forty-six HIV-infected infants and 63 seroreverting infants, born with passively acquired anti-HIV antibodies but not becoming detectably infected, were typed by an automated nucleotide-sequence-based technique (41). One or more DR-13 alleles, including DRB1*1301, 1302, and 1303 were found in 15.2% of those becoming HIV-infected and 31.7% of seroreverting infants ($p = 0.048$); this association was influenced by ethnicity. Upon examining for other allelic associations, only the DR2 allele DRB1*1501 was associated with seroreversion in Caucasian infants. Among these infants, the DRB1*03011 allele was positively associated with HIV infection.

Molecular mimicry, where structural properties borne by a pathogen "imitate" or "simulate" molecules of the host, also appears to be an important mechanism in the association of HLA molecules with viral disease. Molecular mimicry takes different forms in the molecular biology of HIV-1 (42). Molecular mimicry between HIV envelope proteins and HLA class II molecules may lead to autoimmunity against CD4+ T cell expressing class II molecules (43). Bisset (44) states that both the HIV-1 gp 120 envelope and *Mycoplasma genitalium* adhesion proteins share an area of significant similarity with the CD4-binding site of the class II MHC proteins. Interaction with this triad could contribute to T-

cell dysfunction, T-cell depletion, Th1-cell–Th2-cell shift, B-cell proliferation, hyperglobulinemia, and antigen-presenting cell dysfunction.

HLA-DR has been evaluated as a marker for immune response related to human cytomegalovirus infection (45); this virus plays a role in chronic inflammatory reaction in inflammatory abdominal aortic aneurysm. In the fibrously thickened adventitia of this aneurysm, human cytomegalovirus-infected cells and HLA-DR positive cells were more frequently encountered than in that of atherosclerotic aneurysms and control cases ($p < 0.01$).

An estimated 250 million people throughout the world are chronically infected with hepatitis B virus, the primary cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in endemic-disease areas (46,47). Because HLA class I antigens contain viral peptides, they may be important targets for immune mediated hepatocytolysis by CD8+ CTLs in hepatitis B virus infection (48). Davenport et al. (49) have shown that HLA-DR13 is associated with resistance to hepatitis B virus infection. Prognosis may be quite different among patients infected with hepatitis C virus: a chronic liver disease occurs in half the patients, while the other half exhibits no signs of histologic progression of liver damage. The host immune responses may play an important role in such different outcomes. To identify human CTL epitopes in the NS3 region of hepatitis C virus, Kurokohchi et al. (50) modified an approach using recombinant protein and the ability of short peptides to bind to class I MHC molecules. They identified a cytotoxic T-cell epitope presented by HLA-A2 in the hepatitis C virus NS3 region. A study conducted by Peano et al. (51) establishes that HLA-DR5 antigen appears as a protective factor against a severe outcome of hepatitis C virus infection.

Epstein-Barr virus, a member of the herpesvirus family, has been associated with virus replication (infectious mononucleosis, oral hairy leukoplakia) as well as neoplastic conditions such as nasopharyngeal carcinoma, B-cell lymphoma, and Hodgkin disease associated with viral latency. An influence of CTL response on Epstein-Barr virus evolution was first suggested by the finding that virus isolates from highly HLA-A11–positive Asian populations were specifically mutated in two immunodominant A11 restricted CTL epitopes (52). Additionally, B35.01-restricted CTL responses in white donors reproducibly map to a single peptide epitope (53). However, most Epstein-Barr virus isolates from a population where B35.01 was prevalent (in the Gambia) either retained the CTL epitope sequence or carried a mutation that conserved antigenicity; changes leading to reduced antigenicity were found in only a minority of cases. Two epitopes for Epstein-Barr virus specific CTLs restricted by the common allele HLA-B7 were identified by Hill et al. (54).

The level of serum HLA class I antigens markedly increases during the course of viral infections such as those caused by cytomegalovirus, hepatitis B virus, hepatitis C virus, HIV-1, and varicella-zoster virus (55-57). During HIV-1 infection, the level of serum HLA class I antigens correlates with disease stage and represents a good prognostic marker of disease progression (55).

HLA Association with Bacterial Infections

Vaccines based on recombinant attenuated bacteria represent a potentially safe and effective immunization strategy. A carrier system was developed by Verjans et al. (58) to analyze in vitro whether foreign T-cell epitopes, inserted in the outer membrane protein PhoE of *Escherichia coli* and expressed by recombinant bacteria, are efficiently processed and presented through HLA class I and II molecules by infected human macrophages.

A well-defined HLA-B27 restricted cytotoxic T-cell epitope and an HLA-DR53 restricted T-helper epitope of the fusion protein of measles virus were genetically inserted in a surface-exposed region of PhoE, and the chimeric proteins were expressed in *E. coli* and *Salmonella typhimurium.* Macrophages infected with recombinant bacteria presented the T-helper epitope to a specific CD4+ T-cell clone but failed to present the CTL epitope to the specific CD8+ T-cell clone. Phagocytic processing of intact bacteria within infected macrophages was essential for antigen presentation by HLA class II. Nascent HLA class II molecules were also required for the presentation of the T-helper epitope to the CD4+ T-cell clone by infected macrophages.

HLA associations may also link various diseases; for example the HLA-B27 association for ankylosing spondylitis, Reiter disease, reactive arthropathy, and acute anterior uveitis indicate that these disorders may share a pathogenic pathway. According to the molecular mimicry hypothesis, antigens carried by a

particular pathogen may resemble a certain HLA allomorph. As the person carrying this allomorph is unresponsive to it, it is susceptible to the disease caused by the pathogen. For example, some investigators believe that one of the antigens of *Klebsiella* resembles HLA-B27 and that pathogen is responsible for ankylosing spondylitis (59). In most patients who have an acute attack of anterior uveitis, a common ocular disease characterized by inflammation of the iris and ciliary body, the only clues to the pathogenesis of this disease are its close association with the genetic marker HLA-B27 and the likely triggering role of a variety of gram-negative bacteria (60). HLA-B27 acute anterior uveitis appears to be a distinct clinical entity frequently associated with the seronegative arthropathies, such as ankylosing spondylitis and Reiter syndrome.

Sasazuki (61) showed that low responsiveness to streptococcal cell wall antigen was inherited as an HLA-linked dominant trait. The immune suppression gene for streptococcal cell wall was in strong linkage disequilibrium with particular alleles of the HLA-DQ locus. This shows that the HLA-linked immune suppression genes exist in humans to control low response to natural antigens.

Table 2 lists the associations that have been established between various HLA factors and certain infectious diseases. Only the antigens showing statistically significant associations are indicated. Because some persons are unresponsive to certain critical epitopes of the pathogens presumably responsible for certain infectious diseases, particular HLA alleles occur more frequently in patients with certain infectious diseases than in healthy persons; therefore, researchers associate these diseases with certain HLA alleles. This article has summarized the findings from population genetic analysis and from studies of the association of immune response mechanisms of infectious diseases and HLA.

Table 2. Association between human leukocyte antigen (HLA) and some infectious diseases

Disease	HLA Association
Bacterial	
Ankylosing spondylitis	B27
Reiter disease	B27
Acute anterior uveitis	B7
Mycobacterial	
Tuberculosis and leprosy	DR2
(multibacillary forms)	(DRB1*1501, 1502)
lepromatous leprosy	DR2 and DQ1
paucibacillary tuberculoid	DR3
Viral	
Dengue fever virus	DR15
Human immunodeficiency virus 1	DR13 (DRB1*1301, 1302, 1303)
	DR2 (DRB1*1501)
	DRB1*03011
Hepatitis B virus	DR13
Hepatitis C virus	A2
	DR5
Epstein-Barr virus	B35.01
	A11
	B7
Parasitic	
Malaria	B53
Scabies	A11
Diffuse cutaneous leishmaniasis	A11, B5, B7
Localized cutaneous leishmaniasis	A28, Bw22, DQw8
	Bw22, DR11, Qw7
	Bw22, Dqw3
Schistosomiasis	B5, DR3
Visceral leishmaniasis	A26

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Dr. Singh is a scientist in the Department of Biochemistry, Central Drug Research Institute, Lucknow. Her research interest is visceral leishmaniasis, or kala-azar, as it is known in eastern India, where the disease is now epidemic. She is developing PCR-based diagnostics for the disease focusing on kinetoplast DNA, studying the molecular mechanisms of drug resistance, and striving to answer the most important question: are host genetic factors, like HLA, involved in susceptibility to kala-azar in India.

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Cryptosporidiosis: An Emerging, Highly Infectious Threat

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Cryptosporidium parvum, a leading cause of persistent diarrhea in developing countries, is a major threat to the U.S. water supply. Able to infect with as few as 30 microscopic oocysts, *Cryptosporidium* is found in untreated surface water, as well as in swimming and wade pools, day-care centers, and hospitals. The organism can cause illnesses lasting longer than 1 to 2 weeks in previously healthy persons or indefinitely in immunocompromised patients; furthermore, in young children in developing countries, cryptosporidiosis predisposes to substantially increased diarrheal illnesses. Recent increased awareness of the threat of cryptosporidiosis should improve detection in patients with diarrhea. New methods such as those using polymerase chain reaction may help with detection of *Cryptosporidium* in water supplies or in asymptomatic carriers. Although treatment is very limited, new approaches that may reduce secretion or enhance repair of the damaged intestinal mucosa are under study.

An emerging infection comes to our attention because it involves a newly recognized organism, a known organism that newly started to cause disease, or an organism whose transmission has increased. Although *Cryptosporidium* is not new, evidence suggests that it is newly spread (in increasingly used day-care centers and possibly in widely distributed water supplies, public pools, and institutions such as hospitals and extended-care facilities for the elderly); it is newly able to cause potentially life-threatening disease in the growing number of immunocompromised patients; and in humans, it is newly recognized, largely since 1982 with the AIDS epidemic. *Cryptosporidium* is a most highly infectious enteric pathogen, and because it is resistant to chlorine, small and difficult to filter, and ubiquitous in many animals, it has become a major threat to the U.S. water supply. This article will focus on the recognition and magnitude of cryptosporidiosis, the causative organism and the ease with which it is spread, outbreaks of cryptosporidiosis infection, and its pathogenesis, diagnosis, and treatment.

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Recognition and Magnitude of Cryptosporidiosis

First recognized by Clarke and Tyzzer (1) at the turn of the century and well known to veterinarians, *Cryptosporidium* was reported as a human pathogen in 1976 by Nime (2). From 1976 until 1982, seven cases of cryptosporidiosis were reported in humans, five of which were in immunosuppressed patients. Since 1982, cryptosporidiosis has been increasingly recognized as a cause of severe, life-threatening diarrhea in patients with AIDS as well as in previously healthy persons (3). Of the first 58 cases of cryptosporidiosis described in humans by 1984, 40 (69%) were in immunocompromised patients who contracted severe, often irreversible, diarrhea (lasting longer than 4 months in 65%); of these 40 patients, 33 (83%) had AIDS (4-6); 55% of the 40 immunocompromised patients died.

A review of 78 reports of more than 131,000 patients and more than 6,000 controls showed *Cryptosporidium* infection in 2.1% to 6.1% of immunocompetent persons in industrialized and developing countries, respectively, vs. 0.2% to 1.5% in controls (Table 1). A review of an additional 22 reports of nearly 2,000 human immunodeficiency virus (HIV)-infected persons showed *Cryptosporidium* infection in 14% to 24% of HIV-infected persons with diarrhea vs. 0% to 5% of HIV-infected controls without diarrhea (7). Seroepidemiologic

Table 1. Rates of *Cryptosporidium* infection among immunocompetent and HIV-positive persons in industrialized and developing areas[abc]

	Patients with diarrhea	Controls without diarrhea
Immuno-competent		
Industr. areas	2.2%(0.26%-22%) [n=2232/107,329]	0.2%(0%-2.4%) [n=3/1941]
Developing areas	6.1%(1.4%-40.9%) [n=1486/24,269]	1.5% (0%-7.5%) [n=61/4146]
HIV-positive		
Industr. areas	14%(6%-70%) [n=148/1074]	0%(0%-0%) [n=0/35]
Developing areas	24%(8.7%-48%) [n=120/503]	5%(4.9%-5.3%) [n=5/101]

[a]From 100 reports of 133,175 patients with diarrhea and 6,223 controls. [b]Ranges given in parentheses. [c]Data from reference (7).

studies suggest that 17% to 32% of nonimmunocompromised persons in Virginia, Texas, and Wisconsin, as well as nonimmunocompromised Peace Corps volunteers (before travel), have serologic evidence of *Cryptosporidium* infection by young adulthood. In contrast, more than half of the children in rural Anhui, China, had serologic evidence of cryptosporidial infection by 5 years of age, and more than 90% of children living in an impoverished area of Fortaleza, Brazil, had serologic evidence of cryptosporidial infection in their first year of life (Figure) (8-11).

The Organism

Among protozoa, *C. parvum* is the major human pathogen that is also found in numerous mammals. It is slightly smaller than the murine *Cryptosporidium*, *C. muris*, and is also distinguished from the other *Cryptosporidium* species commonly seen in birds, turkeys, snakes, and fish. Infection begins when a person ingests chlorine-resistant, thick-walled oocysts (7). These hardy oocysts appear to be infectious, with an estimated ID_{50} (from studies in humans) of one isolate containing only 132 oocysts (12). Infections may occur with ingestion of as few as 30 oocysts; some infections have occurred with just one oocyst (13).

When the oocysts reach the upper small bowel, the proteolytic enzymes and bile salts enhance the excystation of four infectious sporozoites, which enter the brush border surface epithelium and develop into merozoites capable of replicating either asexually or sexually beneath the cell membrane (but extracytoplasmically) in the brush border epithelial cell surface. Sexual stages combine to form new oocysts, some of which (perhaps 20% as thin-walled oocysts) may sporulate and continue infection in the same person, while others (thick-walled oocysts) are excreted. Although few organisms may enter through M cells, systemic infection essentially does not occur; the occasional biliary tract or respiratory tract infections in immunocompromised patients probably reached these sites through the lumenal surface.

Cryptosporidiosis Outbreaks

Numerous well-documented outbreaks of cryptosporidiosis have occurred. Most of these often waterborne outbreaks have involved subtle problems in the flocculation and/or filtration process (17-21). These outbreaks culminated in the huge waterborne outbreak in Milwaukee, which was initially thought to be viral gastroenteritis, reported to the State Health Department on April 5, 1993, diagnosed on April 7, and followed by an advisory note that evening to the public to boil all drinking water (Table 2). This became the largest waterborne outbreak in U.S. history and affected an estimated 403,000 persons, thus constituting a 52% attack rate among those served by the South Milwaukee water works plant. Several immunocompromised patients died, and many previously healthy persons became ill. The mean duration of illness was 12 days with a range of 1 to 55 days, and the average maximum number of watery diarrheal stools was 19 per day at the peak of illness. While watery diarrhea was the predominant symptom among 93% of confirmed

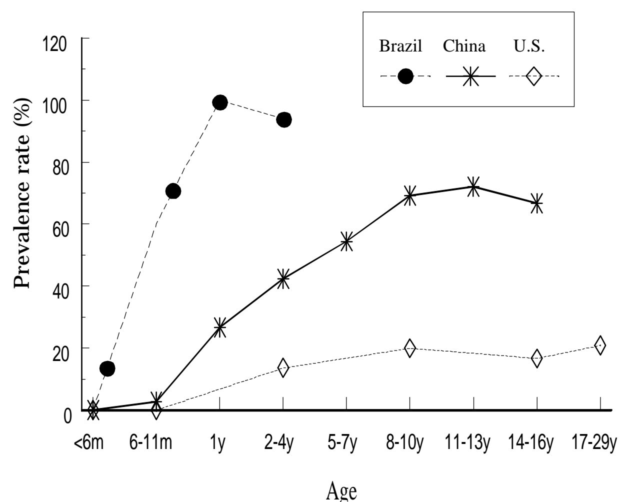

Figure. Prevalence of IgG antibodies to *Cryptosporidium parvum,* by age, in Brazil, China, and the United States.

Table 2. Symptoms of 205 patients with confirmed cases of cryptosporidiosis during the Milwaukee outbreak[a]

Symptom	Percent(%)
Watery diarrhea	93
mean=12d; med=9d (1-55d)	
mean=19/d; med=12/d (1-90)	
39% recurred after 2d free	
Abdominal cramps	84
Weight loss	75
(med=10lb, 1-40lb)	
Fever	57
(med=38.3°, 37.2°-40.5°)	
Vomiting	48

[a]Data from reference (21)

cases, other symptoms such as abdominal pain, low-grade fever, and vomiting were not infrequent; 75% of infected nonimmunocompromised persons had an average 10-lb weight loss.

Additional outbreaks involving public swimming pools and wade pools have further documented the ability of *Cryptosporidium* to cause infection even when ingested in relatively small amounts of fully chlorinated water (22-26). While the leading causes of 129 drinking and recreational water outbreaks in the United States from 1991 through 1994 were *Giardia* and *Cryptosporidium*, cryptosporidiosis accounted for substantially more cases (even if the Milwaukee outbreak were excluded) (23,24,26). In addition, although *Cryptosporidium* oocysts cannot multiply in the environment, an outbreak of foodborne cryptosporidiosis, affecting 54% of those ingesting incriminated freshly pressed apple cider, has been reported (27). In this outbreak, *Cryptosporidium* oocysts were found in the cider press, as well as in a calf on the farm from which the apples were obtained. There was also a 15% secondary attack rate in households involved in this outbreak. The apparent person-to-person spread in households and institutions such as day-care centers and hospitals further documents the highly infectious nature of *Cryptosporidium.* In an urban slum area in northeastern Brazil, secondary household infections occurred in 58% of households with an infected child (index case) despite the 95% prevalence of antibody in children more than 2 years of age (28).

The spread of cryptosporidiosis in day-care centers is well documented, with 14 outbreaks reported in the United States, as well as others in the United Kingdom, France, Portugal, Australia, Chile, and South Africa (29). Illnesses usually occurred in the summer and early fall, especially during August and September in the United States and Portugal. Attack rates were 13% to 90%, with the highest rates found among nontoilet-trained toddlers and staff caring for children in diapers. Overall prevalence rates were usually in the 1.8% to 3.8% range; however, rates as high as 30% in day-care homes were reported (30). During outbreaks, 3.7% to 22.9% of infected children may not have diarrhea; infectious oocysts may be excreted for up to 5 weeks after diarrheal illness ends (31). In addition, numerous nosocomial outbreaks of cryptosporidiosis have occurred among health-care workers as well as patients in bone marrow transplant units, pediatric hospitals, and patient wards with HIV-infected patients (32-37). Furthermore, elderly hospitalized patients may also be at risk for *Cryptosporidium* infection (38). In one Pennsylvania hospital, 45% of nurses, medical students, and house staff caring for an HIV-positive patient with cryptosporidiosis seroconverted (39).

Numerous potential animal and water sources have been found to be infected with *Cryptosporidium*. In the Gonçalves Dias slum in Fortaleza, Brazil, 10% of animals (including dogs, pigs, donkeys, and goats), 6.3% during the dry season to 14.3% during the wet season, had *Cryptosporidium* in their stool specimens. In addition, 22% of drinking water sources studied were infected with *Cryptosporidium* oocysts (40). Furthermore, LeChavalier et al. have documented that *Cryptosporidium* oocysts were present in 27% of 66 drinking water samples obtained from 14 states and one Canadian province (mean of 0.18 NTU) (41,42).

Pathogenesis and Impact

C. parvum does not infect tissue beyond the most superficial surface of the intestinal epithelium; however, it can derange intestinal function. Although a parasite enterotoxin has been extensively sought and some reports have suggested that one may exist (43), this issue remains controversial, and the source of substances in the stools of infected animals and patients that induce secretion remains unclear (44). Extensive studies in a piglet model of cryptosporidiosis by Argenzio and colleagues demonstrate the loss of vacuolated villus tip epithelium (approximately two-thirds of the villus surface area), accompanied by an approximate 50% reduction in glucose-coupled sodium cotransport. What remains is a predominance of transitional junctional epithelium, in which increased glutamine metabolism drives a sodium-hydrogen exchange, to which is coupled chloride transport. Thus, glutamine drives neutral

sodium chloride absorption in an apparent prostaglandin-inhibitable manner in *Cryptosporidium*-infected piglet epithelium (45). Furthermore, Argenzio and colleagues have demonstrated increased macrophages that produce increased tumor necrosis factor (TNF) in the lamina propria of *Cryptosporidium*-infected piglets (46). Although TNF did not directly affect epithelial transport, when a fibroblast monolayer was added, an indomethacin-inhibitable secretory effect was noted with TNF (46). Consequently, the researchers propose a prostaglandin-dependent secretory effect, which occurs 1) through a bumetanide-inhibitable chloride secretory pathway, predominantly from crypt cells; and 2) through the inhibition of neutral sodium chloride absorption through the amiloride-sensitive sodium:hydrogen exchanger, predominantly in the junctional or transitional epithelium during active cryptosporidial infection. Reduced xylose and B-12 absorption are among the effects described in humans and animals with cryptosporidiosis (47-49). Disruption of intestinal barrier function with strikingly increased lactulose to mannitol permeability and absorption has been documented during active symptomatic cryptosporidial infection in children and in HIV-infected adults (Lima et al., unpublished observations) (50).

Cryptosporidium appears to be one of the leading causes of diarrhea, especially persistent diarrhea, among children in northeastern Brazil (51,52). In addition, the incidence of diarrhea has been nearly double for many months in young children after symptomatic cryptosporidial infections, suggesting that the disrupted barrier function in infected children leaves residual damage resulting in increased susceptibility of injured epithelium to additional diarrheal illnesses (Agnew et al., unpub. obs.).

Recognition and Diagnosis

The diagnosis of *C. parvum* in patients with diarrhea is usually made by using acid-fast or immunofluorescence staining on unconcentrated fecal smears. Appropriate concentration methods may enhance detection when small numbers of oocysts are present, but some methods such as formalin-ethyl acetate concentration may result in loss of many oocysts (52,53). While several enzyme-linked immunosorbent assay methods are available for detection of fecal cryptosporidial antigen with 83% to 95% sensitivity in diarrheal specimens, these methods are less sensitive in formed specimens and require more time. Microscopy using immunofluorescence antibody is slightly more sensitive and may be faster (54,55).

Polymerase chain reaction (PCR) provides a new method that may help detect *Cryptosporidium* in water supplies or asymptomatic carriers. A genomic DNA library has been constructed in the plasmid pUC18 for propagation in *Escherichia coli*. After sequencing a 2.3 kilobase *C. parvum*-specific fragment, a 400-base sequence with a unique *Sty* I site has been amplified by using primers of 26 nucleotides each (56). Laxer et al. then used a 20-base probe labeled with digoxigenin-11-dUTP to detect *C. parvum* DNA in fixed, paraffin-embedded tissue (57). In addition, primers for a 556 BP *Cryptosporidium*-specific region of the small subunit 18s ribosomal RNA gene have been used to produce a PCR product with unique Mae 1 sites that distinguish *C. parvum* from *C. baileyi* and *C. muris* (58). Available methods for detection of viable oocysts in environmental samples are relatively insensitive and under active investigation.

Treatment and Prevention

Despite numerous attempts at examining transfer factor, hyperimmune colostral antibody, and more than 100 antiparasitic and antimicrobial agents, few agents have shown modest benefit in controlled studies; paromomycin is one of them. Although this agent does not eradicate the parasite in immunocompromised patients, it slightly reduces parasite numbers (from 314×10^6 to 109×10^6 oocysts shed per day) and decreases stool frequency, with a tendency toward improved Karnofsky scores and reduced stool weight (59). In view of its effectiveness in driving sodium cotransport (60) and its success in studies of experimental animals, we are examining a new approach to speeding repair of disrupted intestinal barrier function by using glutamine and its derivatives.

The ability of the thick-walled oocysts to persist and spread in the environment and their well-documented resistance to chlorine are responsible for the spread of *Cryptosporidium* even in fully chlorinated water supplies that meet existing turbidity standards in drinking water and swimming pools. Although some scientists have noted that 9,600 parts per million (mg/l) of chlorine for one minute of exposure are required to decontaminate water (14), others have noted that even after exposure to full-strength household bleach (5.25% sodium hypochlorite; 50,000 parts per million) for

2 hours, the oocysts still remained infectious for experimental animals (15). While *Giardia* are 14 to 30 times more susceptible to chlorine dioxide or ozone, respectively, ozone is probably the most effective chemical means of inactivating *Cryptosporidium* oocysts (16). Consequently, eradication of the organism from drinking water supplies depends on adequate flocculation and filtration, rather than chlorination. Although previous turbidity requirements were based on the removal of larger parasite cysts such as those of *Giardia lamblia* or *Entamoeba histolytica*, the smaller *C. parvum* oocysts are more difficult to remove. Several waterborne outbreaks, including the recent outbreak in Milwaukee, have occurred with turbidity levels in the 0.45 to 1.7 nephelometry turbidity units (NTU) range. In a study of waterborne cryptosporidiosis, predominantly among HIV-positive adults in Clark County, Nevada, Goldstein et al. (1996) report that the outbreak was associated with a substantial number of deaths and that the turbidity of the implicated water never exceeded 0.17 NTU (much lower than the new standard of 0.5 NTU required for 95% of measurements each month, with no spikes over 1.0 NTU) (5).

New approaches to the eradication of infectious oocysts from water supplies are needed, possibly using reverse osmosis, membrane filtration, or electronic or radiation methods, instead of the ineffective chemical or difficult filtration techniques currently used. Ideally, these new methods would provide low cost, effective treatment that could be applied in developing areas as well. Meanwhile, an improved understanding of the pathogenesis and impact of *Cryptosporidium* infections should aid the development of improved treatment and control of this ubiquitous, highly infectious threat to the water supply and to the people it serves, especially malnourished children and immunocompromised patients around the world.

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Isolation and Phylogenetic Characterization of Ebola Viruses Causing Different Outbreaks in Gabon

Three outbreaks of Ebola hemorrhagic fever have recently occurred in Gabon. Virus has been isolated from clinical materials from all three outbreaks, and nucleotide sequence analysis of the glycoprotein gene of the isolates and virus present in clinical samples has been carried out. These data indicate that each of the three outbreaks should be considered an independent emergence of a different Ebola virus of the Zaire subtype. As in earlier Ebola virus outbreaks, no genetic variability was detected between virus samples taken during an individual outbreak.

Since the first recognized outbreaks of human Ebola virus hemorrhagic fever in Africa in the 1970s, biological and genetic differences have been described among the four distinct Ebola viruses isolated: Zaire, Sudan, and Côte d'Ivoire, all isolated from humans, and Reston virus isolated from macaque monkeys from the Philippines (1-5).

Serologic evidence has suggested the presence of Ebola virus in Gabon since 1982 (6). Since late 1994, three apparently independent outbreaks of Ebola virus hemorrhagic fever have occurred among humans in northeastern Gabon, in the forested areas of equatorial Ogooué-Ivindo province. The first, which started in December 1994 in gold-panner encampments of far northeastern Gabon, in the Minkouka area (between 1°24' and 1°44' North and 12°49' and 12°59' East, Figure 1) near the Nouna River, had several laboratory-confirmed cases (7). The second, which began in early February 1996 in Mayibout village (1°07' North, 13°06' East, Figure 1) on the Ivindo River, resulted in 37 Ebola hemorrhagic fever cases (8). The only means of transportation between these two areas is by boat; Makokou, the closest town to them (0°33' North, 12°50' East, Figure 1), has the provincial hospital to which patients and contacts were transferred. The third outbreak, which is ongoing, started in July 1996 in the village of Booué (0°06' South, 11°57' East, Figure 1), where most of the cases occurred; however, scattered cases have been diagnosed in surrounding villages and towns. Some patients have even been transported to Libreville, probably during the incubation period of the disease. One patient was treated in South Africa, where a fatal nosocomial infection was subsequently reported in a health care worker (9,10); 43 deaths due to Ebola hemorrhagic fever have already been reported during this prolonged outbreak (11).

We report here the Ebola virus glycoprotein (GP) gene sequences obtained from human samples collected during each of the three Gabonese epidemics. The Gab280 sequence comes from blood collected 1 day before the death of a patient, from the Nouna area, during the 1994 outbreak. The Gab282 and Gab283 sequences were derived from blood collected during the spring 1996

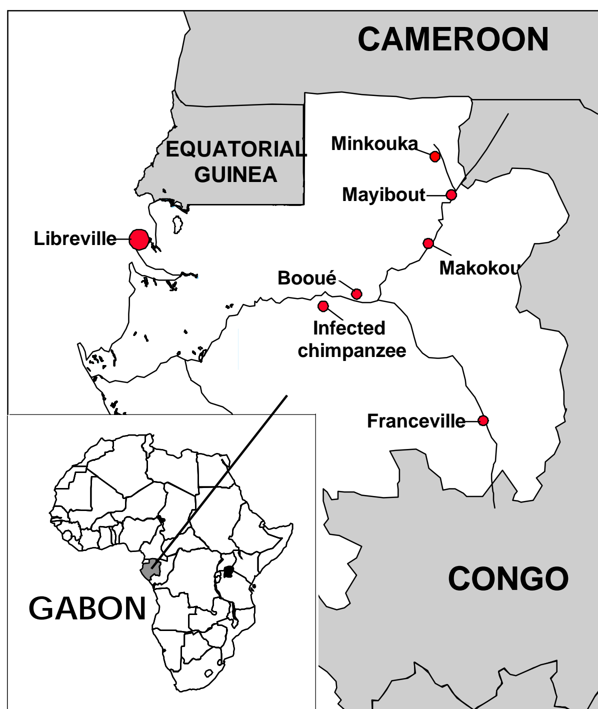

Figure 1. Geographic distribution of the three Ebola virus hemorrhagic fever epidemics and site of the infected chimpanzee in Gabon.

outbreak, from two primary patients who were infected while butchering a chimpanzee they found dead in the forest. The Gab281 sequence was derived from blood collected from what appears to have been a secondary case during the same outbreak; the patient was probably infected by contact with one of the index patients while visiting a traditional doctor (nganga) who lived near Mayibout village. Sequences Gab275 to Gab279 were derived from blood specimens collected from two patients during the fall 1996 outbreak: Gab275, Gab276, and Gab277 sequences are from one patient (a serum specimen collected on October 27, a primary tissue culture passage from that specimen, and a primary tissue culture passage from another blood sample drawn on October 25, respectively). The Gab278 and Gab279 sequences were derived from plasma (collected during the acute phase of illness) from another patient and its primary tissue culture passage, respectively. The isolation of Ebola virus in Vero E6 cell culture from human blood samples collected during the three different outbreaks was easily accomplished in a single passage.

RNA was extracted from blood or primary tissue culture samples (Table 1) by using a commercial kit (RNaid Kit , BIO 101, Inc., Vista, CA) as directed by the instruction manual. Viral sequences were amplified from RNA by using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique, and DNA products were sequenced as described elsewhere (5). Briefly, amplified products were subjected to agarose electrophoresis and were stained and visualized with ethidium bromide; DNA bands were then excised and extracted by using the QIAEX II kit (QIAGEN, Inc., Chatsworth, CA). In some cases, nested PCR with internal primers was performed, using first-round products. Amplified products were directly sequenced by using an automated nonisotopic method (ABI PRISM dye-terminator cycle sequencing kit, Perkin-Elmer, Foster City, CA). Centri Sep columns (Princeton Separation, Inc.) were used to remove excess dye-labeled dideoxynucleotide terminators, and reaction products were analyzed on an ABI 377 or 373 DNA sequencer (Perkin-Elmer).

Initially, a 783-base-pair fragment of the GP gene, which encompasses the most variable region of the gene, was sequenced. A consensus sequence was established by aligning all the Ebola from Gabon, the Zaire 1976 and 1995 Ebola virus sequences, as well as the sequence of the virus obtained from a nurse in South Africa who was infected by a patient from Gabon in November 1996 (10). Viruses from the same outbreak had identical sequences, but unique virus sequences were found in each of the three different outbreaks in Gabon. Although the viruses causing the Gabonese outbreaks clearly belong to the Zaire subtype, they were distinct from viruses that had caused disease in Zaire. No differences were observed between tissue-culture-passaged and clinical-material-derived sequences or between primary or secondary case sequences. RNA extracted from a single representative of each outbreak (sequences Gab277, Gab280, and Gab283) was then used to generate the entire GP gene sequence for the Gabon Ebola viruses. The GP from the Gabon spring 1996 viruses differed from the sequence of the Gabon fall 1994 viruses by four nucleotides. The GP sequence from the Gabon fall 1996 viruses differed from the sequence of the Gabon spring 1996 virus by four additional nucleotides (Figure 2).

Maximum parsimony analyses were performed by using the entire coding regions of the GP genes of the Gabon viruses, together with those of

Table 1. Origin and identification of the sequence used in the analysis

Outbreak	Patient #	Specimen	Date of original specimen	Laboratory #
Gabon, Fall 94	1	Blood	Dec 28,94	Gab280
Gabon, Spring 96	1	Blood	Feb 20,96	Gab281
	2	Blood	Feb 8,96	Gab282
	3	Blood	Feb 8,96	Gab283
Gabon, Fall 96	1	Serum	Oct 27,96	Gab275
		Vero E6 Passage 1	Oct 27,96	Gab276
		Vero E6 Passage 1	Oct 25,96	Gab277
	2	Plasma	Oct 27,96	Gab278
		Vero E6 Passage 1	Oct 27,96	Gab279
South Africa, Fall 96	1	Vero E6 Passage 1	Nov 9,96	SA253

previously characterized Ebola viruses (5). In addition, the sequence of a 1980 Marburg virus isolate (Musoke strain) was included to provide an outgroup in the analysis. The Phylogenetic Analysis Using Parsimony version 3.1.1 software, using the branch-and-bound search option (12), was run on a Power Macintosh 8100/110 (Apple Computer, Inc.). A 4:1 transversion:transition weighting was used in analyses. A single most parsimonious tree was obtained (Figure 2), and bootstrap analysis strongly supports a common evolutionary origin for the viruses associated with disease in Gabon and Zaire. Overall, these data indicate that the three Gabon outbreaks should be considered independent events, likely originating from different sources.

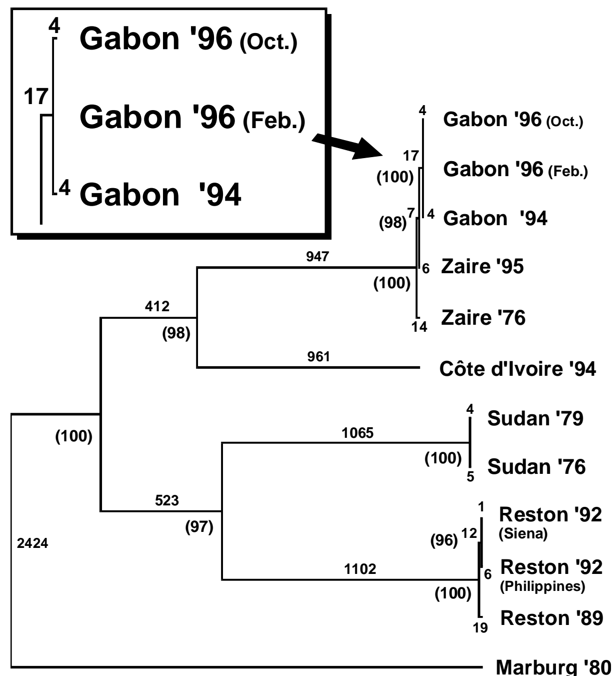

Figure 2. Phylogenetic tree showing the relationship between the Ebola viruses that caused outbreaks of disease in Gabon and previously described filoviruses (5). The entire coding region for the glycoprotein gene of the viruses shown was used in maximum parsimony analysis, and a single most parsimonious tree was obtained. Numbers in parentheses indicate bootstrap confidence values for branch points and were generated from 500 replicates (heuristic search). Branch length values are also shown.

The presence of stable virus sequences and the lack of genetic variability between strains isolated within an outbreak was previously seen during the outbreak of Ebola hemorrhagic fever in Kikwit, Zaire, in 1995 (Rodriguez et al., unpublished data), and despite the small number of isolates tested, is again suggested in Gabon.

During a 20-month period, Gabon has had three different outbreaks of Ebola virus hemorrhagic fever. The first and the second episodes apparently started during the rainy season (December and February), while the third began during the dry season (July). The deaths of nonhuman primates were associated with all three outbreaks. Minkouka area inhabitants reported finding dead chimpanzees and gorillas in the forest during the fall of 1994. All the primary human patients in the spring 1996 outbreak were infected while butchering dead chimpanzees. For the third outbreak, the investigation has indicated an index patient who was a hunter, living in a forest camp in the Booué area. During the same period, an Ebola virus-infected dead chimpanzee was found in the forest (0°11' South, 11°36' East) near Booué; Ebola virus infection in the chimpanzee was confirmed using a newly developed immunohistochemical Ebola skin biopsy test (Figure 3).

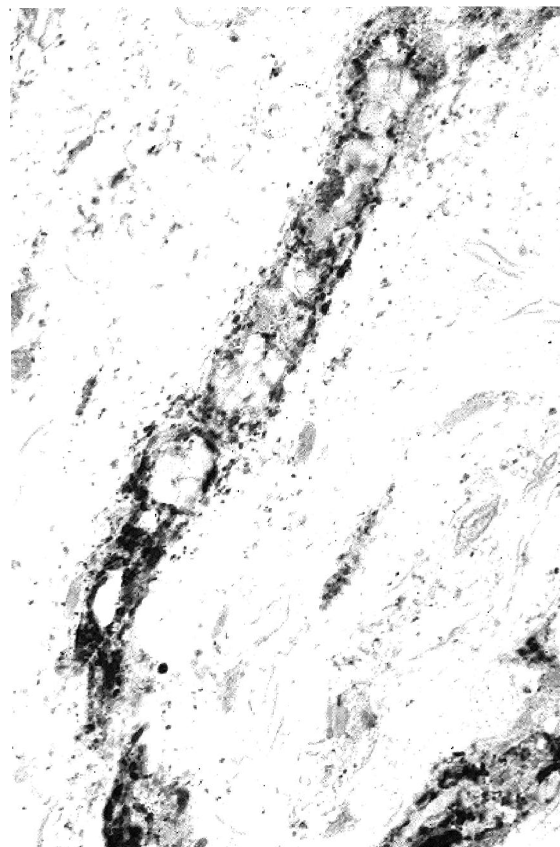

Figure 3. Immunostaining of Ebola virus antigens (red) within vascular endothelial cells in skin biopsy of the chimpanzee found dead in the forest near Booué. Note also the presence of extracellular viral antigens. (Rabbit anti-Ebola virus serum, napthol/fast red with hematoxylin counterstain, original magnification x250).

A long term surveillance for Ebola virus using this test is under way in Africa (13,14).

In Côte d'Ivoire in 1994, an investigator was infected with Ebola virus while performing necropsy on a dead chimpanzee (15). Primates are unlikely to be the reservoir of Ebola virus since experimental or natural infection is quickly fatal. A better knowledge of the ecology of great apes, particularly their food preferences and habitats, may lead to the identification of the virus reservoir. Gabon's equatorial forests, where three independent outbreaks have occurred in less than 2 years, offer an excellent opportunity for these investigations.

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The Epidemiology of Creutzfeldt-Jakob Disease in Canada: A Review of Mortality Data

Creutzfeldt-Jakob disease (CJD), and particularly its transmissibility through blood and blood products, has become a focus of concern in Canada. The recent identification of new variant CJD led to a review of the Canadian mortality database to identify any clustering of CJD by age, sex, or geographic location.

The study by Holman and colleagues (1), which uses mortality data and active surveillance methods, has provided further information on the epidemiology of Creutzfeldt-Jacob disease (CJD). The study concluded that the incidence of the disease in the United States has remained stable and is similar to crude incidence worldwide at about one case per million annually. In addition, the study found no evidence that new variant CJD is present in the U.S. population. These findings are of interest because the epidemiology of CJD in Canada is not well described, and concerns have been raised there about the transmissibility of CJD through transfusion of blood and blood products or through tissue and organ transplants. In addition, many Canadians travel to and from the United Kingdom, where new variant CJD was first identified and linked to the bovine spongiform encephalopathy epidemic (2).

We report here our findings on the epidemiology of CJD in Canada, which are derived from published mortality data (underlying cause of death by standard 5-year age group and sex, for all Canadian residents). The Statistics Canada mortality reports for the years 1979 to 1993 were reviewed for CJD deaths by sex and age group for each province and territory. Reports before 1979 were not used because CJD (ICD-9 code 46.1) was not listed as a cause of death before this time.

Overall, 334 deaths attributed to CJD were recorded in Canada in the 15-year period from 1979 to 1993, ranging from 14 to 34 deaths per year, with a 1.1:1 male-to-female ratio (Figure 1). Eighty-five percent of the deaths were among persons at least 60 years of age and 50% among the 60- to 69-year-old age group, which corresponds to the peak age of onset of sporadic-type CJD (3-5). Eleven deaths (3%) were reported among persons 30 to 44 years of age. Of these, one death was reported in the 30- to 34-year-old age group, four in the 35- to 39-year-old group, and the remaining six in the 40- to 44-year old age group. No more than one CJD-attributed death was reported per year in the 30- to 44-year-old age groups, with the exception of two deaths reported in 1993. No CJD deaths have been reported among persons under 30 years of age.

Figure 2 shows the mortality rate for CJD in Canada, standardized to the 1979 population. The age-adjusted mortality rate increases from a low of 1.1 deaths per million population in 1979 to a high of 2.1 per million in 1992, dropping to 1.8 per million in 1993. The increase is most marked from 1986 onward. This may be related to increased case recognition following publication of the discovery that CJD was linked to human growth hormone of pituitary origin. A real increase in incidence may also be present, but this graph must be interpreted with caution as the absolute numbers are small and the validity of CJD on death certificates has not been determined in Canada.

Familial-type CJD has been documented in Canada (6,7). As in other countries, most cases of CJD in Canada are of the sporadic type (3-5). One case of iatrogenic CJD was reported in Canada, in a dura mater recipient (8). A review of the

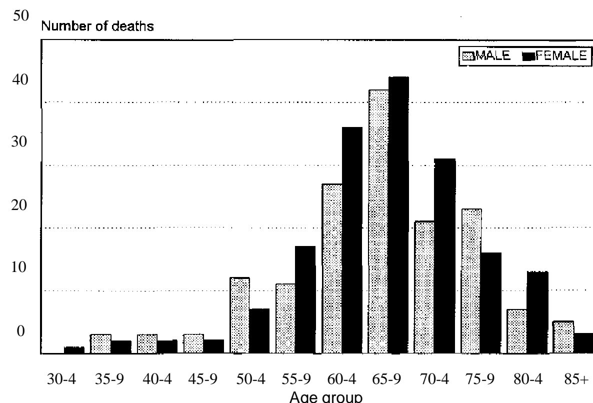

Figure 1. Age and sex distribution for Creutzfeldt-Jakob disease in Canada, 1979–1993.

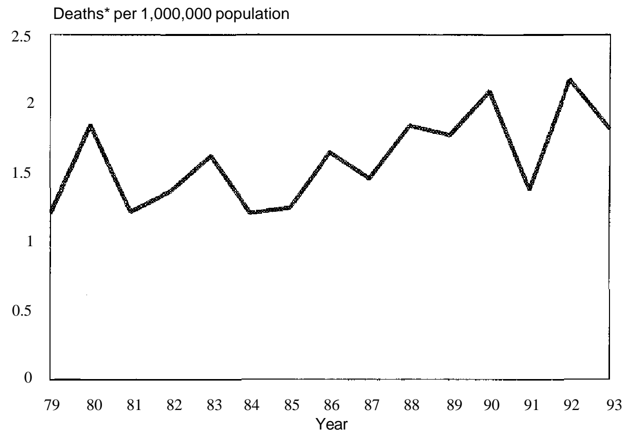

Figure 2. Age-adjusted mortality rate for Creutzfeldt-Jakob disease in Canada, 1979–1993.

Canadian growth hormone recipient database, containing information on the 800 patients who received human growth hormone shows no cases of CJD (Dr. Heather Dean, pers. comm., 1996). Human growth hormone was used in Canada from 1965 until April 1985 (9).

There is one report of a possible cluster of CJD cases in Canada; between April 1989 and October 1990, six cases were reported in the province of Ontario, from a population of 9.5 million (1986 census figure). Two of the patients had come from areas of Czechoslovakia with a high incidence of familial-type disease, but no other risk factors were associated with these cases (7).

In conclusion, the epidemiology of CJD in Canada is not well defined, as current data sources are limited to aggregated mortality data and the annual total case numbers are small. However, several projects have been initiated to provide further information on the transmission of the disease, including an examination of death certificates to identify space/time clustering, active surveillance for CJD and new variant CJD, and a case control study of CJD and blood transfusion.

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Exudative Pharyngitis Possibly Due to *Corynebacterium pseudodiphtheriticum*, a New Challenge in The Differential Diagnosis of Diphtheria

Corynebacterium pseudodiphtheriticum has rarely been reported to cause disease in humans, despite its common presence in the flora of the upper respiratory tract. We report here a case of exudative pharyngitis with pseudomembrane possibly caused by *C. pseudodiphtheriticum* in a 4-year-old girl. The case initially triggered clinical and laboratory suspicion of diphtheria. Because *C. pseudodiphtheriticum* can be easily confused with *Corynebacterium diphtheriae* in Gram stain, clarification of its role in the pathogenesis of exudative pharyngitis in otherwise healthy persons is of public health importance. Simple and rapid screening tests to differentiate *C. pseudodiphtheriticum* from *C. diphtheriae* should be performed to prevent unnecessary concern in the community and unnecessary outbreak control measures.

Among the pathogenic nondiphtheria corynebacteria, *Corynebacterium pseudodiphtheriticum* has rarely been reported to cause disease in humans, despite its frequent presence in the flora of the upper respiratory tract (1,2). *C. pseudodiphtheriticum* was first isolated in humans by Von Hoffmann-Wellenhof from the throat of a patient in 1888 (2). The organism exhibits little pleomorphism. It appears as short gram-positive rods that often lie in parallel rows on smear preparation. We report here a case of exudative pharyngitis in a 4-year-old girl with a pseudomembrane possibly caused by *C. pseudodiphtheriticum,* which initially triggered suspicion of diphtheria, and provide a summary of previously published case reports.

Case Report

On November 6, 1994, a 4-year-old girl was admitted to the Emergency Room of St. Mary's Hospital in Rogers, Arkansas, with fever and generalized lymphadenopathy. Two other children in her home day care, including the patient's sibling, also had lymph node enlargement and fever. Her mother reported that the patient had not been immunized against any of the common childhood diseases but had an unremarkable medical history, with the exception of hand-foot-and-mouth disease at 3 years of age; she had never received antibiotics. Her developmental milestones were achieved on time. The family included the patient, her mother, father, and a 3-year-old sibling.

The patient's posterior pharynx was erythematous with swelling of the posterior palate and with a thick grayish white membranous exudate adhering to the posterior wall of the pharynx. The pseudomembrane had almost completely detached and was gone, leaving behind ulcerated tissue. A discrete adenopathy in the neck and palpable nodes in the axillary, epitrochlear, and inguinal regions were observed.

The initial white blood cell count was 7,000 per ml, with 50% polymorphonuclear cells and 17% lymphocytes. A rapid screening test for group A streptococci and a Paul-Bunnell test for Epstein-Barr virus had negative results. Routine throat and nasopharyngeal swabs were collected and submitted to the hospital's clinical microbiology laboratory, where they were injected into blood agar and blood chocolate agar (special media for diphtheria culture were unavailable). From the throat swab, there was light growth of *Streptococcus pneumoniae* and light growth of gram-positive rods; a mixture of normal flora was also present. Gram-positive rods were also isolated from the nasopharyngeal swab. Subsequently, when subcultured on cystine-tellurite agar, these gram-positive rods grew as black colonies. Subcultures were then sent to the state laboratory, which confirmed them as *C. pseudodiphtheriticum*. After ambulatory treatment with cefprozil and erythromycin, the patient recovered in 4 days.

Investigation of Contacts

Because of its resemblance with *C. diphtheriae*, a *C. pseudodiphtheriticum* isolate was not immediately identified, and the case was initially

thought to be diphtheria. Consequently, 36 children and adults who had recently been in contact with the patient in day care, at the fitness center, or at the church were cultured for diphtheria. *C. diphtheriae* was not found, but *C. pseudodiphtheriticum* was found in the patient's 3-year-old sibling and in three additional persons: a 1-year-old girl and her 3-year-old brother, who lived in the same building as the patient and shared her day care, and a 54-year-old woman who attended the same church.

Microbiology and Molecular Characterization

The *C. pseudodiphtheriticum* isolate from the initial patient was forwarded to the Centers for Disease Control and Prevention (CDC), where it was assayed by phenotypic and genotypic methods. The identification was confirmed on the basis of growth characteristics and biochemical properties. The isolate was assayed by polymerase chain reaction (PCR), which detects 248 base pair fragments of the A subunit of the diphtheria toxin gene (3), and the result was negative. Additional PCR assays were performed with 12 sets of primers that cover the entire diphtheria toxin gene (tox) and diphtheria toxin regulatory element (dtxR); 560 base pairs of the dtxR were detected. However, the PCR results of its five prime and three prime ends were negative, indicating that the gene was nonfunctional.

Previous Case Reports[1]

Since 1932, only 83 cases of disease possibly caused by *C. pseudodiphtheriticum* in humans have been reported. Until 1981, the only reported disease associated with this organism was endocarditis in the presence of anatomic abnormalities of the heart. Since then, this organism has been increasingly recognized as a pathogen of the lungs and bronchi, particularly in patients with underlying immunosuppressive conditions and preexisting pulmonary diseases and in patients undergoing endotracheal intubation.

Among reported cases, 19 were endocarditis (4-17); 61 involved infections of the lungs, trachea, or bronchi (18-32); one was a urinary tract infection (33); one involved a skin infection (34); and one was a vertebral discitis (35). All but three patients had underlying medical conditions, including functional or anatomic abnormalities of the heart (N=27), lung and tracheobronchial diseases (N=28), endotracheal intubations (N=3), and immunosuppressive conditions including prolonged steroid use (N=6) and AIDS (N=4).

Most patients showed good clinical response to various antimicrobial drugs: penicillin, ampicillin, cefazolin, vancomycin, gentamicin, tobramycin, norfloxacin, and others. Nineteen (23%) deaths were reported overall. Information on sex was available for 80 cases; 54 (68%) were in males. Only five (6%) cases occurred in children <18 years of age; no cases were reported among children <5 years of age.

Since the early 1990s, diphtheria has returned in epidemic proportions in the former Soviet Union and is likely to be imported into other countries. Two cases of exudative upper respiratory tract infections with a pseudomembrane, in which the main organism isolated was *C. pseudodiphtheriticum*, have been reported. The first case was in a 54-year-old man with necrotizing tracheitis in 1991 (32). Santos et al. (36) reported isolation of *C. pseudodiphtheriticum* from a 32-year-old male Uzbek national who had a severe sore throat and dysphagia of 2 days duration. His tonsils were enlarged bilaterally, and a grayish-white exudate extended from the tonsil to the posterior pharyngeal wall. The uvula and soft palate were erythematous and edematous, and tender cervical lymphadenopathy was present. The presumptive clinical diagnosis in this patient was respiratory diphtheria.

Although the presence of a grayish pseudomembrane in the upper respiratory tract associated with isolation of *C. pseudodiphtheriticum* in these two cases and in the case we described is not proof that the isolated corynebacterium caused the pharyngitis or tracheitis, these accumulating case reports suggest that *C. pseudodiphtheriticum* may have played a pathogenic role.

The isolate from our patient was nontoxigenic by PCR. The diphtheria toxin is considered to be the main virulence factor of *C. diphtheriae* that causes the formation of the pseudomembrane in faucial diphtheria (37). Several possibilities could explain the presence of a pseudomembrane in infections with nontoxigenic corynebacterium strains. First, toxigenic and nontoxigenic colonies

[1]A table listing all published case reports is available upon request from Dr. Hector Izurieta, Mail Stop A32, Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30333.

of the same species can coexist in a patient; this phenomenon has been reported in diphtheria (38). Multiple colonies would have to be tested to confirm this hypothesis in this case. Second, nontoxigenic forms of *C. diphtheriae* can produce invasive disease (39,40). It is possible that the symptoms in our patient were caused by a nontoxigenic *C. pseudodiphtheriticum* and that the pseudomembrane was simply an inflammatory exudate. This has been reported for *Arcanobacterium haemoliticum* (formerly *Corynebacterium haemoliticum*), which has also been associated with production of a grayish pharyngeal pseudomembrane (41,42). In 1960, Barksdale et al. reported a pseudomembrane in two laboratory workers contaminated with known nontoxigenic forms of *C. diphtheriae* (43). Third, we cannot exclude the possibility that the symptoms were caused by other organisms, including *S. pneumoniae*, which was found in the throat culture of our patient (29). However, this is unlikely because the growth of *S. pneumoniae* in our patient's throat culture was light, and the organism was not found in the nasopharyngeal swab. Furthermore, *S. pneumoniae* has not been reported to produce a pseudomembrane (44,45).

Because several other *Corynebacterium* spp. have been identified as potentially pathogenic for humans, routine screening for *C. diphtheriae* and other members of the *Corynebacterium* spp. in clinical samples of patients with respiratory infections should be encouraged (46). Full identification of gram-positive rods isolated from the respiratory tract, especially when they appear on original plates as the predominant organisms or copredominant with another species, should be carried out at the local, state, or reference level. Simple screening tests, such as negative cysteinase and positive pyrazinamidase, in addition to inability to ferment glucose, maltose, and sucrose quickly differentiate *C. pseudodiphtheriticum* from *C. diphtheriae*. These tests should be a part of improved training of laboratory personnel. Clinical and laboratory experience gained through this process will not only provide information about death rates for these organisms, but would be invaluable in preventing unnecessary concern in the community and the extraordinary measures needed to control dissemination of diphtheria.

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Mycoplasmal Conjunctivitis in Wild Songbirds: The Spread of a New Contagious Disease in a Mobile Host Population

A new mycoplasmal conjunctivitis was first reported in wild house finches (*Carpodacus mexicanus*) in early 1994. The causative agent was identified as *Mycoplasma gallisepticum* (MG), a nonzoonotic pathogen of poultry that had not been associated with disease in wild songbirds. Since the initial observations of affected house finches in the mid-Atlantic region, the disease has become widespread and has been reported throughout the eastern United States and Canada. By late 1995, mycoplasmal conjunctivitis had spread to an additional species, the American goldfinch (*Carduelis tristis*). This new disease exemplifies the rapid spread of a pathogen following introduction into a mobile wildlife population and provides lessons that may apply to emerging human diseases.

In February 1994, house finches with swollen or crusty eyelids and impaired vision were observed at backyard bird feeders in suburban Washington, D.C. (1). Severely affected birds lingered on bird feeders or on the ground surrounding the feeders. These birds had chronic lymphoplasmacytic conjunctivitis, sinusitis, and rhinitis. Microorganisms resembling mycoplasmas were adhering to conjunctival epithelium, and *Mycoplasma gallisepticum* (MG) was isolated from affected tissues (2,3). A Maryland field survey of MG in house finches in late 1994 showed a strong association between conjunctivitis and MG by culture and polymerase chain reaction (PCR) (3). Subsequently the disease was reproduced by inoculation of unaffected house finches with a finch-derived MG isolate (Fischer, unpublished data).

Since the first reports from the mid-Atlantic states, mycoplasmal conjunctivitis in house finches has spread rapidly to the north, south, and west. The disease was first monitored by wildlife biologists with state and federal wildlife resource agencies, and by October 1994, affected house finches had been reported in nine states in the mid-Atlantic region (Figure). Beginning in November 1994, backyard sightings of healthy and diseased house finches have been recorded by private citizens participating in a survey conducted by the Cornell Laboratory of Ornithology (4). The percentage of participants reporting diseased house finches has steadily increased since the survey began: House finches with conjunctivitis were reported by 11% of 1,413 participants in November 1994, by 17.3% of 1,239 participants in March 1995, by 28.1% of 769 participants in November 1995, and by 35.8% of 1,047 participants in March 1996. Most reports in November 1994 came from the mid-Atlantic region, and 30% to 40% of survey participants in this area had reported diseased finches through March 1996. The survey also has documented the dramatic spread of disease to house finches in the Midwest and Southeast (Figure). In November 1994, diseased finches were reported by only 0.4% of 229 participants in these regions, but by March 1996, the percentage had climbed to 37% of 257 participants. Mycoplasmal conjunctivitis now has been reported almost throughout the eastern population of house

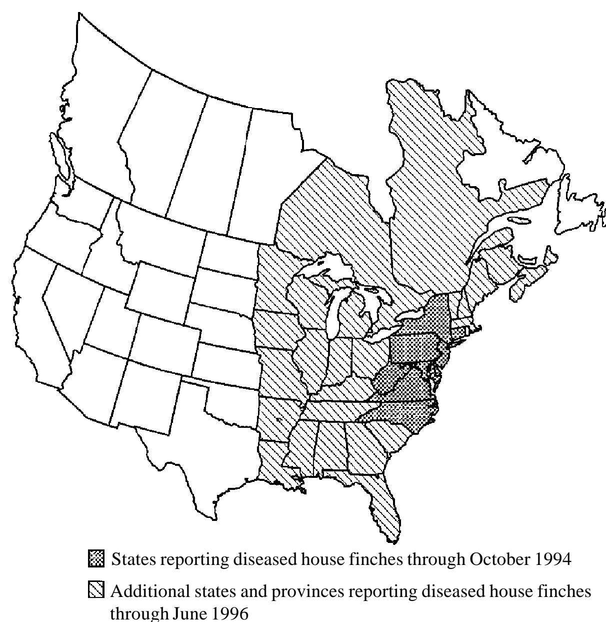

Figure. Case distribution of house finches with conjunctivitis, October 1994–June 1996.

Table. Test results for *Mycoplasma gallisepticum* in house finches and American goldfinches, Southeastern Cooperative Wildlife Disease Study, May 1994–June 1996

State of Origin	Species	Number	Conjunctivitis		Rhinitis	*Mycoplasma gallisepticum*[a]		
			Bilateral	Unilateral		Isolation	PCR	Serology[b]
AL	House finch	1	1	0	1	0/1	1/1	0/0
GA	House finch	6	3	3	2	3/6	3/3	6/6
	Am. goldfinch	2	1	1	0	0/2	2/2	2/2
KY	House finch	2	1	1	2	1/2	2/2	2/2
MD	House finch	9	4	5	6	0/7	6/6	6/6
	Am. goldfinch	1	1	0	0	0/1	1/1	1/1
OH	House finch	1	1	0	1	1/1	0/0	0/0
SC	Am. goldfinch	1	1	0	0	0/1	1/1	0/0
TN	House finch	12	9	3	6	2/7	2/2	4/6
	Am. goldfinch	1	1	0	1	0/1	0/1	0/0
TOTALS	House finch	31	19	12	18	7/24	14/14	18/20
	Am. Goldfinch	5	3	1	1	0/5	4/5	3/3

[a] Data are presented as number positive/number tested.
[b] Positive = 2+ on rapid plate agglutination test.

finches. No diseased birds have been reported within the western population of house finches, which occupies the historic range of this species.

House finches are native to the western United States, where they were captured during the 1930s for sale in eastern pet shops. House finches were released in New York City in the early 1940s when regulations regarding the commercial trade of domestic songbirds changed. The first nesting pair of house finches was observed on Long Island in 1943, and by 1951 the area held an estimated 280 birds (5). The eastern house finch population has expanded dramatically from the original limited number of birds in New York; it currently contains millions of finches over the entire eastern and midwestern United States and southeastern Canada (6).

Mycoplasmal conjunctivitis apparently has spread to another wild finch species in the eastern United States. During the winter and spring of 1995 to 1996, American goldfinches with inflamed eyelids were reported within the range of diseased house finches in Georgia, Maryland, North Carolina, South Carolina, and Tennessee. The goldfinches had lesions identical to those of house finches with mycoplasmal conjunctivitis, and in March 1996, MG was isolated from two diseased goldfinches from North Carolina (7).

House finches and goldfinches were submitted for diagnostic laboratory examinations during this epornitic. At the Southeastern Cooperative Wildlife Disease Study, gross and microscopic postmortem examinations, serologic tests for antibodies against MG (3), mycoplasmal cultures, and PCR testing for MG (3) were performed on 31 house finches and five goldfinches from seven states (Table). Consistent pathologic findings in both species included moderate to severe lymphoplasmacytic conjunctivitis often with hyperplasia of associated lymphoid and epithelial tissues, and rhinitis. Occasionally, keratitis and tracheitis were observed. Antibodies against MG were detected by rapid plate agglutination tests in 91% of tested birds, and positive PCR products were obtained from 95% of birds tested. MG was isolated from only 24% of birds cultured; this percentage probably reflects the fastidiousness of the strain affecting wild finches and the condition of the specimens.

MG is a major pathogen of domestic poultry and causes infectious sinusitis in turkeys, chronic respiratory disease in chickens, and subclinical infections (8). MG also has been associated with conjunctivitis in chickens (9) and farmed gamebirds (10). Clinical disease had not been associated with MG in wild passerine birds, although MG (11) and antibodies against MG (12) have been detected in these birds. Infections in poultry persist despite antimicrobial treatment or the development of antibody response; MG can be transmitted by direct contact, by airborne droplets or dust, and vertically through eggs (8). Like other mycoplasmas, MG can alter its antigenic surface components in vivo. This feature may contribute to its ability to persistently infect by adapting to the host environment and evading the host immune response (13). The possible role of this antigenic variation in the adaptation to new host species is unknown.

The source of the MG affecting house finches and goldfinches and its introduction into the eastern house finch population are under investigation. Field isolates of MG collected during 1994 through early 1996 from house finches, goldfinches, and domestic poultry; three poultry vaccine strains of MG; and three poultry reference strains of MG were analyzed by random amplification of polymorphic DNA (7). Molecular characterization showed that isolates from both finch species over a wide geographic range had identical or nearly identical random amplification of polymorphic DNA banding patterns, but they differed from reference strains, vaccine strains, and isolates from commercial poultry (7). These results suggested that the finch epornitic arose from a single source and was caused by an MG strain that differed from strains commonly associated with poultry disease or vaccination. The ultimate source of the outbreak strain remains unknown; however, molecular studies have not shown any relationship between wild finch and poultry strains of MG (7). Possible sources of MG include unrecognized reservoirs of MG in the wild or small poultry operations with poor biosecurity, such as backyard chicken flocks.

The remarkable spread of mycoplasmal conjunctivitis in wild finches probably reflects both bird behavior and human activity. House finches are well adapted to human land use practices; they nest and feed in open areas around buildings and farms, as well as in suburban settings with ornamental trees and shrubbery. They eat weed and grass seeds, as well as fruits and berries of flowering trees and shrubs. During the winter, house finches flock around backyard bird feeders (5). Although precise MG transmission modes are unknown, the propensity of this highly gregarious species to assemble at bird feeders may enhance contact with infected birds or with surfaces contaminated with the causative agent. Unlike their western counterparts, house finches within the eastern range of the species may migrate several hundred miles (14) and thus disseminate an infectious agent over a large geographic area.

Intentional and unintentional ecologic changes may have contributed significantly to the current mycoplasmal conjunctivitis outbreak. The affected species was introduced into a new range, and the birds have flourished because they prefer the areas that humans increasingly provide. Although the eastern house finch population is large and widely distributed, the limited genetic pool from which it descended may have contributed to its apparently high susceptibility to MG. The causative agent is best known for its association with domestic poultry, and its spread through house finches appears to have been enhanced by bird feeders, which not only provide an opportunity for increased contact between infected and uninfected birds, but also may prolong the lives of infectious, diseased birds that otherwise would not have been able to feed. The combination of these and other unknown factors has resulted in the emergence of a severe infectious disease that has spread rapidly through the susceptible host population and could become permanently established in house finches and possibly other species.

Even though human activity has contributed to the emergence of this disease, human efforts to control it have not been successful. Treatment and immunization strategies to control infectious human and domestic animal diseases are not available, and if they were available, they would likely be impractical and costly. Treatment and subsequent release of individual birds in rehabilitation facilities is of questionable value because the effect may be minimal on a population basis. Furthermore, it is unknown if birds released after treatment remain persistently infected with MG. Moreover, the presence of multiple avian species in a rehabilitation clinic may enhance the transmission of MG to other species (2).

The current epornitic of mycoplasmal conjunctivitis in house finches has some interesting parallels to emerging human diseases, particularly regarding the substantial roles that human activity, ecologic changes, and the introduction of exotic species may play in the emergence of infectious disease. Additionally, the impressive speed at which this pathogen moved through the house finch population illustrates how rapidly a pathogen can be disseminated throughout a large geographic area within a highly gregarious and mobile host population. Finally, the lack of suitable control methods for MG in house finch populations and the inability to correct the ecologic conditions that contributed to its emergence provide strong support for preventive rather than reactive measures in dealing with the next potential wildlife or zoonotic disease.

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Knowledge-Based Patient Screening for Rare and Emerging Infectious/Parasitic Diseases: A Case Study of Brucellosis and Murine Typhus

Many infectious and parasitic diseases, especially those newly emerging or reemerging, present a difficult diagnostic challenge because of their obscurity and low incidence. Important clues that could lead to an initial diagnosis are often overlooked, misinterpreted, not linked to a disease, or disregarded. We constructed a computer-based decision support system containing 223 infectious and parasitic diseases and used it to conduct a historical intervention study based on field investigation records of 200 cases of human brucellosis and 96 cases of murine typhus that occurred in Texas from 1980 through 1989. Knowledge-based screening showed that the average number of days from the initial patient visit to the time of correct diagnosis was significantly reduced (brucellosis—from 17.9 to 4.5 days, $p = 0.0001$, murine typhus—from 11.5 to 8.6 days, $p = 0.001$). This study demonstrates the potential value of knowledge-based patient screening for rare infectious and parasitic diseases.

Knowledge-based medical decision support systems allow the user to relate clinical and laboratory manifestations from which a list of possible diagnoses can be generated. Studies have demonstrated that such a list is extremely useful for alerting clinicians and epidemiologists to diseases seen rarely, if ever, and to unusual clinical manifestations of diseases (1,2).

This report describes the development and laboratory testing of a medical decision support system designed to facilitate early recognition of rare and unusual infectious and parasitic diseases. The assumption is made that early diagnosis can improve the course of treatment as well as the overall health of the patient.

Constructing a Knowledge Base

In this study, the knowledge base was restricted to the 223 diseases common to humans and animals. Several disease information axes were identified for inclusion in the medical knowledge base. For each disease, nomenclature along with any recognized synonyms, etiology, epidemiologic descriptions, diagnostic procedures, diagnostic procedure findings, and clinical signs and symptoms were gathered and abstracted from recent editions of standard medical texts. In addition, occupational risks, food consumption data, travel history, and animal/insect exposure were included. Finally, basic treatment, management, and prevention protocols were also recorded (3-10). Bibliographic citations for all entries into the knowledge base were tied to each information axis to authenticate the content and provide a quick reference for additional reading. Glossaries of knowledge base entries (e.g., signs, symptoms, occupation, diagnostic procedure results, geography, animal/insect exposure) to be used in generating a differential diagnosis were created to ensure the consistency of the lexicon. Audits were performed to validate the knowledge base entries against the glossaries. The resulting knowledge base contains the entries listed in Table 1. A batch

Table 1. Knowledge base of 223 diseases at the time of case study

Information axis	Disease entities	Lines of text
Etiology	410	
Epidemiology[a]		9724
Immunodeficiency info		352
Diagnostic procedures	306	
Diagnostic procedure	637	
Occupational links[b]	37	
Geographic links (countries)[c]	191	
Vectors/Invertebrate hosts	703	
Symptoms/Clinical signs	1422	
Treatment/Prevention		3162
Special considerations[d]		308
Bibliographic citations	838	
Descriptive notes[e]	322	

[a]Includes specific etiologic information, reservoirs, portal of entry/escape, mode of transmission/dissemination, susceptible hosts, incubation period, seasonal distribution, age/sex/race/religion/occupation relationships, mortality and zoonotic classification.
[b]Lists occupations with increased potential for exposure.
[c]Lists countries where the disease is known to occur.
[d]Describes additional disease attributes not fitting other categories.
[e]Pop-up definitions of medical terms and concepts in the knowledge base.

procedure indexing all findings to diseases for use by the inference engine resulted in more than 12,000 finding/disease links. The inference engine is the portion of the decision support system used to derive useful clinical information (e.g., possible diagnoses). It is composed of nine program modules containing more than 10,000 lines of C language code linked into one executable program.

Differential Diagnosis Methods

The goal of the system is to remind the user of relationships between clinical signs, symptoms, and findings and the diseases themselves. Therefore, a simple, intuitive pattern-matching technique was employed. The user sets a "closeness-of-fit" parameter in the system that determines how tight the relationship between case signs/findings and diseases must be before a disease can become a candidate for the differential diagnosis list. A simple ratio is created with signs/findings entered that fit each disease as the numerator and the total number of signs/findings entered as the denominator. The ratio ranges from zero to 1.00 with 1.00 implying a perfect fit. If this value is equal to or greater than the "closeness-of-fit" parameter set by the user (also ranging from zero to 1.00), the disease is entered in the differential diagnosis list as a candidate. The ratio for each disease shows how close the clinical manifestations are to previously published descriptions of the disease. For example, if seven clinical signs and findings are collected on a case and six fit a particular disease by the pattern-matching algorithm, the case fit would be 0.86. If the case fit parameter in the system is set to 0.86 or greater, that disease would be added to the differential diagnosis list. If the case fit parameter is set to 1.00, only diseases that fit the clinical manifestations perfectly would become differential diagnosis candidates. Signs and findings are not weighted by the system.

Decision Support System Trial on Murine Typhus Cases

The purpose of this trial was to determine if the intervention of the support system at the time of the first physician visit would significantly alter the mean number of days to the correct diagnosis. Investigation records of 96 cases of murine typhus occurring in Texas from 1979 to 1987 were obtained from the Texas Department of Health. All records were complete and were used in the study. Each record contained the date of the first contact with a physician (dp). The date on which the indirect fluorescence antibody test for *Rickettsia typhi* was ordered was defined as the date the correct diagnosis was suspected (ds). Therefore, the number of days from the first contact with a physician (dp) to the correct diagnosis without decision support system intervention (dc_{ni}) was defined as follows: $dc_{ni}=ds-dp$.

The number of days till the correct diagnosis was suspected (dc_{ni}) was calculated for all cases without support system intervention. A case was classified as missed if dc_{ni} >13 days. This number was derived from the worst-case scenario for a murine typhus test turnaround from the Texas Department of Health. During 1979-1987, the department had the only laboratory in Texas that used the indirect fluorescence antibody test for confirming murine typhus. On the basis of this criterion, 23 of the 96 original cases fell into the missed category.

The clinical history, symptoms, physical findings, and some preliminary laboratory findings as noted in the investigation records for the 23 missed patients were entered in the support system and processed at case fit parameter values of 0.25, 0.50, 0.75, and 1.00. Specific historical data such as occupation, travel, and animal/insect exposure, if available, were also entered in the support system. The support system was said to have suggested the correct diagnosis of murine typhus if the disease was included in a differential diagnosis list of five or fewer diseases at a case fit parameter of 0.50 or greater.

Testing for murine typhus was assumed to be ordered immediately if the disease was suggested by the support system. This effectively set the number of days to suspect the correct diagnosis with intervention (dc_{wi}) to 1 day under these conditions.

The distributions of dc_{ni} and dc_{wi} were found to be Gaussian (PROC UNIVARIATE, SAS). Therefore, parametric statistical techniques were used for data analysis. A paired t-test was run on μ_{ni} and μ_{wi} (mean time to suspecting murine typhus with and without the system, respectively) to see if there was a significant difference in the means (PROC MEANS, SAS) (14).

In 11 (48%) of the 23 cases defined as missed, murine typhus was clearly suggested by the support system in a differential list of five or fewer possibilities by using only clinical history, signs, symptoms, and preliminary laboratory data if available. In six (26%) of these cases, murine typhus was the only disease suggested by the support system. The support system did not suggest the correct disease in 12 (52%) of the cases classified

as missed. Table 2 lists the most common diseases that also appeared in the differential diagnosis lists for murine typhus cases.

Table 2. Other diseases commonly listed in the differential diagnosis of murine typhus

1. Lyme borreliosis	7. Relapsing fever
2. Tularemia	8. Avian chlamydiosis
3. Brucellosis	9. Leptospirosis
4. Zoonotic epidemic typhus	10. Salmonellosis
5. Rocky Mountain spotted fever	11. Babesiosis
	12. Malaria
6. Rat bite fever	13. Q fever

The mean number of days to suspect the correct diagnosis without support system intervention (μ_{wi}) was 11.5 days. The mean number of days to suspect the correct diagnosis with support system intervention (μ_{wi}) was 8.6 days, an improvement of 2.9 days ($p = 0.001$).

Decision Support System Trial on Brucellosis Cases

Investigation records of 342 cases of brucellosis occurring in Texas from 1980 to 1989 were also obtained from the Texas Department of Health. Of these records, 13 were incomplete, and 129 involved cases of recrudescence of the disease or had no specific date of onset of illness and were excluded from the study. The remaining 200 records contained the date of onset of illness. However, the day of the first physician visit was not available and had to be extrapolated from the day of onset of illness. The average number of days from onset of illness to the first physician visit was assumed to be 4 days, on the basis of a study in Texas for patients with murine typhus (12).

The day that the correct diagnosis was suspected was defined as the day on which the serum agglutination test or a bacterial culture for brucellosis was ordered. The number of days from the first contact with a physician to the day the correct diagnosis was suspected without support system intervention (dc_{ni}) was calculated as indicated above.

A case was classified as missed if $dc_{ni} > 11$ days. This number was derived from the worst-case scenario for a brucellosis test turnaround from the Texas Department of Health. By this liberal criterion, 98 of the 200 original cases still fell into the missed category.

The clinical history, symptoms, physical findings, and some preliminary laboratory findings as noted in the investigation records for the 98 missed patients were entered in the support system and processed at case fit parameter values of 0.25, 0.50, 0.75, and 1.00. Specific historical data such as occupation, travel, and animal/insect exposure, if available, were also entered in the support system. By definition, the system gave the correct diagnosis of brucellosis if the disease was included in a differential diagnosis list of five or fewer diseases at a case fit parameter of 0.50 or greater.

It was assumed that the testing for brucellosis would be ordered immediately if the disease was suggested by the support system. This effectively sets the number of days to suspect the correct diagnosis with intervention (dc_{wi}) to 1 day under these conditions. The distributions of dc_{ni} and dc_{wi} were also Gaussian and analyzed by paired t-tests on μ_{ni} and μ_{wi} (14).

In 86 (88%) of the 98 cases defined as missed, brucellosis was clearly suggested by the support system in a differential list of five or fewer possibilities by using only clinical history, signs, symptoms, and preliminary laboratory data if available. In 69 (70%) of the cases, brucellosis was the only disease suggested. The support system did not suggest the correct disease in only 12 (12%) of the cases classified as missed. Table 3 lists the most common diseases that also appeared in the differential diagnosis of brucellosis.

Table 3. Other diseases commonly listed in the differential diagnosis of brucellosis

1. Toxoplasmosis	6. Rat bite fever
2. Q fever	7. Listeriosis
3. Viral hepatitis A	8. Blastomycosis
4. Salmonellosis	9. Trichinellosis
5. Histoplasmosis	10. Lyme borreliosis

The mean number of days to suspect the correct diagnosis without support system intervention (μ_{ni}) was 17.9 days. The mean number of days to suspect the correct diagnosis with support system intervention was 4.5 days, an improvement of 12.9 days ($p=0.0001$). However, data were derived by using an extrapolated day of first physician visit, and that may have affected these comparisons.

The brucellosis investigation records screened showed that of nine cases, three involved apparently healthy pregnancies, one a premature delivery, one a miscarriage, one seizures, one weakness and chronic headaches, and two deaths. In eight of the nine cases, the support system correctly suggested brucellosis. In addition, 59 (30%) of the patients with brucellosis were hospitalized, some for extended periods during the diagnostic phase.

Twenty-one of 23 murine typhus patients were hospitalized, some for extended periods during the diagnostic phase of the case. Furthermore, many patients had to undergo expensive diagnostic testing, including 19 electrocardiograms, four computer-aided tomography scans, and a various other procedures such as bone scans, liver scans, barium enemas, and renal and pelvic sonograms. Earlier diagnoses of brucellosis and murine typhus could have eliminated the need for many of the diagnostic procedures, shortened hospital stays, and improved the course of treatment.

On the average, it took approximately 3 minutes of interaction with the decision support system to construct a differential diagnosis. Therefore, this type of screening could easily become part of the routine history-taking and physical exam of a patient. Furthermore, this study underlines the discriminatory power of the clinical history and physical signs and symptoms in suspecting the presence of a certain disease in a patient. Factors such as the patient's occupation, travel history, animal/insect exposure, and unusual dietary habits can be especially important in helping to diagnose many rare infectious and parasitic illnesses.

The results of this evaluation indicate that for two rare diseases (brucellosis and murine typhus), the decision support system appears to perform well (i.e., with high sensitivity) in suggesting the correct diagnosis in a patient. However, the specificity of the system has not been evaluated. A prospective double-blinded study in a general clinical or hospital setting would make that determination.

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Emergence of Epidemic O'nyong-nyong Fever in Southwestern Uganda, After an Absence of 35 Years

To the Editor: In July 1996, an uncommon disease suspected to be O'nyong-nyong fever was recognized in the Rakai district of southwestern Uganda. It was reported to have started in June 1996. The disease spread into the neighboring Mbarara and Masaka districts of Uganda and in the bordering Bukoba district of northern Tanzania.

The initial symptoms of O'nyong-nyong fever are high fever and generalized maculopapular skin rash with crippling arthritis, primarily in the big joints, in the absence of joint effusion. Other features are lymphadenitis, eye pain and reddening with no discharge, chest pain, and general malaise. The disease is self-limiting. All age groups and both sexes are equally affected. In areas where the disease is epidemic, 60% to 80% of the people are infected, and familial clustering is found in affected households. No deaths have been reported, but two miscarriages have been associated with infection.

The Ministry of Health (Uganda), in collaboration with the Uganda Virus Research Institute, began epidemiologic and clinical investigations of the epidemic in August 1996. Acute-phase serum samples were collected from patients, and adult mosquitoes were collected from within and around patients' homes. Virus isolates were made from acute-phase serum samples from several patients by intracranial inoculation and passage in baby mice. Attempted virus isolations from mosquito specimens are in progress. Serum samples and aliquots of the virus isolates were sent to the Centers for Disease Control and Prevention, Fort Collins, Colorado, USA, for reisolation and identification. A portion of the capside and NS4 genes of the virus isolates was sequenced and identified as O'nyong-nyong virus; the virus was isolated and sequenced directly from another serum sample. Two serum samples were positive for IgM antibody to O'nyong-nyong antigen.

O'nyong-nyong virus was responsible for a similar epidemic in 1959 to 1961, which started in northern Uganda and spread south and eastward into Kenya, Tanzania, and Zambia, and then northward from Tanzania into southwestern Uganda, where it subsided. The disease has reemerged in this area after 35 years of absence.

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Prostatitis and Benign Prostatic Hyperplasia: Emerging Infectious Diseases?

To the Editor: In their excellent article, Molecular Approaches to the Identification of Unculturable Infectious Agents, Gao and Moore (1) point out that molecular approaches should be unleashed on diseases such as sarcoidosis, Kawasaki disease, and type I diabetes mellitus, which are thought but not proven to be infectious. The authors, however, are overlooking the more common and most likely infectious disease of unknown etiology today—prostatitis.

According to the pathologist McNeal, the prostate gland is the most commonly diseased internal organ of the human body (2). Prostatitis is the most common prostate disease, resulting in more physician visits than either benign prostatic hyperplasia or prostate cancer, according to the National Institutes of Health (3). Despite its frequency, prostatitis as a disease and as a histologic lesion is understudied (4).

By the Meares and Stamey culture localization procedure, in which the first voided urine, a midstream urine, the expressed prostatic secretions, and a final voided urine are compared, more than 90% of cases in patients with chronic pelvic symptoms are labeled as "nonbacterial" prostatitis or prostatodynia, both of which are thought to be incurable diseases (5).

The University of Washington has documented white blood cell counts as high as 38,000 per mm^3, in "nonbacterial" prostatitis patients (6). According to urologist Thomas Stamey, up to 50% of all men experience symptoms of prostatitis during

their lifetimes (7). The prostatitis lesion was found in 40 (44%) of 91 men at random autopsy (8). In another study of 100 consecutive autopsies on men who died suddenly in automobile accidents and from other causes, the prevalence of histologic signs of prostatitis increased with age and was highest when benign prostatic hyperplasia was also present. Prostatitis was present in 22% of men under 40 years of age and in 60% of those over 40 years of age (9).

In fact, the line between benign prostatic hyperplasia and prostatitis is blurred. Prostatitis as a histologic lesion has been found in 98% of patients with benign prostatic hypertrophy (10). Microbial tests on benign prostatic hyperplasia tissue have found significant rates of infectivity. In another study, more than 70% of transurethral resection of the prostate specimens showed clinical or laboratory signs of infection (11). Benign prostatic hyperplasia and prostatitis cannot be distinguished by symptoms, and some believe that they may be the same disease.

In these days of prostate specific antigen testing, more than 50% of men who undergo biopsies for prostate cancer have a prostatitis lesion whether they have cancer or not (Gottesman et al., unpublished data; McNeal, personal communication, 1995). Prostatitis occurs at an early age, and prostate cancer decades later, in the same part of the prostate gland, the peripheral zone.

Why aren't DNA techniques being unleashed on what is apparently the most common and most purulent unknown inflammatory disease in men—an inflammatory lesion that is associated with benign prostatic hyperplasia and prostate cancer? Surely, DNA microbial testing has important implications for all three major prostate diseases—prostatitis, benign prostatic hyperplasia, and prostate cancer.

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Risk Factors for Severe Leptospirosis in the Parish of St. Andrew, Barbados

To the Editor: Leptospirosis, an important zoonosis in most warm-climate areas, is endemic in most Caribbean countries (1). The disease was first reported in Barbados 60 years ago (2), and since 1979 has been the subject of continual study as the result of the establishment of the Leptospira Laboratory by the governments of Barbados and the United Kingdom. The annual incidence of severe leptospirosis in Barbados over the past 17 years has been approximately 11.5 cases per 100,000 population with a death rate of 13%. However, the incidence rate varies in the parishes of Barbados. For the 12-year period from 1979 to 1991, the lowest incidence rates were in St. Peter (9.5 cases per 100,000 population) and St. Michael (9.9 cases per 100,000 population), while the highest was in St. Andrew (40 cases per 100,000 population). This greater than fourfold difference in incidence rates has been attributed to differences in rainfall (3). We performed a retrospective case-control study to determine what other factors were important.

We identified cases of leptospirosis from the records of the Leptospira Laboratory and included them in the study if they occurred from January 1980 to December 1993, if the home address was in the Parish of St. Andrew, and if laboratory evidence of leptospirosis was confirmed by one or more of the following: an IgM ELISA titer ≥160 in a single sample, a titer in the microscopic agglutination test (MAT) of ≥800 in a single sample, a fourfold or greater rise in antibody titer between two samples tested by the same method, or isolation of leptospires from blood or urine cultures (3).

Of the 36 cases of laboratory-confirmed leptospirosis and 41 controls (selected for residence close to the case-patient), 22 patients and 38 controls were included in the study. For case-patients, the mean age at onset of symptoms was 30.8 years (range 8 to 73 years); 28 (78%) of 36 cases occurred in males. The mean age of controls was 31.3 years (range 13 to 78 years); 15 (39.5%) of 38 controls were male. Controls were matched for age, but because St. Andrew is a sparsely populated parish, and because the survey was conducted during the day, it was difficult to recruit sufficient male controls. The participants were administered a questionnaire, and blood samples were taken and tested for leptospiral antibodies. Serologic results were compared with the results obtained for each of the patients during their acute illness and with the results of previous follow-up studies conducted over several years.

Gardening was a significant risk factor (odds ratio [OR] 4.57, 95% confidence level [CL] 1.09-20.36) and appeared to remain so whether gloves were worn or not, as was the presence of dogs around the home (OR 7.82, 95% CL 1.79-46.55). With few exceptions, the respondents kept dogs, and these animals are an important risk factor for leptospirosis in Barbados (6). A positive association was observed between illness and wearing boots in the garden or yard (OR 8.5, 95% CL 1.93-42.55), but this may be because case patients had changed their behavior since recovery, because they were working in wetter areas than the controls, or because the male/female ratio was lower among controls. We were unable to define the odds ratios for walking barefoot some or all of the time because none of the controls admitted to going barefoot. The most important risk factor we identified was walking through ponds or stagnant water (OR 25.62, 95% CL 2.89-1151.84). Flooding is common during the rainy season in Barbados, and people living in rural areas such as St. Andrew are often exposed in this way. These risk factors bear a striking resemblance to those identified in the outbreak in Nicaragua a few months after our study (7).

We conclude that almost all of the patients had multiple risk factors for leptospiral infection. Few indicated a change in lifestyle since recovering from leptospirosis. Serologic evidence of recent reexposure to leptospirosis was detected in two (17%) of 12 case-patients.

The relatively high rainfall in St. Andrew may have contributed to their risk for leptospirosis by enhancing the survival of leptospires in the soil and water. The incidence of leptospirosis in St. Andrew shows a close association with mean monthly rainfall, the highest incidence during the period studied being October and November. However, when individual cases were examined, a less strong correlation was observed between onset of symptoms and rainfall in the preceding month and with rainfall in the preceding 3-month period. No evidence was observed of clustering of cases in months or years with rainfall above the mean. Similar findings have been reported for the island as a whole (4,5). The incidence of leptospirosis appeared to lag behind the rainfall, since rainfall tended to increase from June to a peak in November, while leptospirosis incidence increased from August to November. There was a marked decrease in rainfall in December each year, with the dry season continuing until May. However, continuing low incidence of leptospirosis was seen throughout the less wet months, until during the months of May to July only one case occurred during the study period.

On the basis of these findings, we conclude that the ground remains sufficiently damp during the period from December through the early months of the year for leptospires to survive. As the middle months of the year are reached, the ground may become too dry for leptospires to survive. This would also account for the apparent lag between the onset of the rainy season and the rise in leptospirosis incidence, as the ground may take some weeks of consistent rainfall to become saturated.

No clustering of cases in time was observed, which confirms that leptospirosis in Barbados is endemic and that increases in incidence result from multiple sporadic cases rather than microepidemics (5). Cases were clustered geographically, but this may have been an artifact resulting from

variation in population density. Moreover, the place of residence is not necessarily the place of exposure to leptospirosis.

We emphasize the importance of public education regarding the relative risks, as a means of preventing exposure, and of continuing education of physicians and primary health-care workers to raise their awareness of the seasonal distribution and early symptoms of leptospirosis.

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Electronic Communication and the Rapid Dissemination of Public Health Information

To the Editor: In the United States, communicable disease surveillance, investigation, and control are the responsibility of the states. The Centers for Disease Control and Prevention (CDC) provides epidemiologic and laboratory support to the state and territorial epidemiologists (state epidemiologists) and state public health laboratory directors (state laboratory directors), who are located in each of the 50 states, Washington, D.C., the Virgin Islands, the Federated States of Micronesia, American Samoa, the Marianas Islands, and Puerto Rico. Historically, communication between CDC and these state representatives has been conducted by telephone, facsimile, or letter, and more recently by the WONDER (1) electronic mail (e-mail) system. We examined the timeliness and coverage of the WONDER system when used to contact state epidemiologists and laboratory directors during two recent foodborne outbreaks.

The first outbreak was reported to CDC on February 10, 1995, by the Communicable Disease Surveillance Centre (CDSC) in the United Kingdom. CDSC had linked an outbreak of salmonellosis in the United Kingdom to a snack food distributed to many countries including the United States (2). CDC decided to notify all state epidemiologists about the outbreak immediately so that they could take appropriate action to protect consumers and report suspected cases. This e-mail message was ready to be accessed by all state epidemiologists from 4:27 p.m. Eastern Standard Time (E.S.T.) on Friday, February 10, 1995.

The second outbreak involved *Salmonella* serotype Stanley infections associated with the consumption of alfalfa sprouts. In the United States, the outbreak was recognized when a larger than expected number of isolates of *Salmonella* Stanley for the first week of June 1995 was reported (3). CDC notified state epidemiologists and laboratory directors about the outbreak and requested that cases of *Salmonella* Stanley infection be reported and *Salmonella* Stanley isolates be sent to CDC. This e-mail message was ready to access from 9:41 a.m. E.S.T. on Friday, June 9, 1995.

These two e-mail messages were sent to two group codes maintained by the Council for State and Territorial Epidemiologists and the Association of State and Territorial Public Health Laboratory Directors on the CDC WONDER e-mail system. The subject heading for these messages indicated that they were urgent and from CDC. The messages were available for 22 days from the day of posting, at which time unaccessed messages were automatically returned to sender. Each message was sent with an automatic receipt acknowledgment function.

Because many of the territories are not regularly connected to WONDER, only the 50 states, the District of Columbia, and Puerto Rico were included in the study. The time to receipt was calculated on the basis of working days (Monday through Friday) only. E-mails accessed during a weekend were attributed to the following Monday.

In February, 48 of 50 states were on the state epidemiologists WONDER e-mail distribution list; 47 states, Puerto Rico, and the District of Columbia accessed the e-mail message within 22 days; one state did not access it within that period; 8 (16%) accessed the message the day it was sent; 28 (57%) accessed it within 1 working day—three of these accessed the message during the weekend; and 43 (88%) of 49 recipients accessed the message within 1 week. While no additional cases were reported, e-mail communication may have hastened product recall, thereby preventing further cases.

In June, 49 states were on the state epidemiologists WONDER e-mail distribution list; 48 states and Puerto Rico accessed the e-mail message within 22 days; two did not access the message within that period; 25 (51%) accessed the message the day it was sent; and 40 (82%) accessed the message by the second working day—two of these accessed the message on a weekend.

Thirty-eight states and Washington, D.C., were on the state laboratory directors WONDER distribution list in June; 25 (64%) accessed the message the day it was sent, and 32 (84%) of 38 accessed the message by the second working day—one of these accessed the message on a weekend. All 38 states and Washington D.C. accessed the e-mail message within the systems' 22-day limit. The pattern for state laboratory directors was almost identical to that for state epidemiologists.

Within 3 weeks of transmission of the June message (by June 30, 1995), state health department laboratories had forwarded 55 *Salmonella* Stanley isolates to CDC: 44 (80%) of these were the outbreak strain. These reports contributed to a traceback that implicated a single alfalfa seed distributor.

The use of e-mail to communicate health related messages to epidemiologists and laboratory directors was timely and highly successful in these incidents. By the second working day, more than half of the intended recipients had accessed the February message, and more than 80% had accessed the June message. However, not all state epidemiologists and laboratory directors access WONDER e-mail daily, and so other means of communication would be necessary if contact were required within 1 working day.

Because epidemiologists and laboratory directors have to dial into the WONDER mainframe by modem to find out if they have new messages and to receive them, retrieving WONDER e-mail messages can be less than timely; there is no mechanism to alert users to incoming WONDER e-mail messages. This delay is likely to be overcome as more epidemiologists and laboratory directors become connected to the Internet by local area networks that automatically check for incoming messages several times per hour. Some epidemiologists and laboratory directors have been slower to access their WONDER e-mail address because they also had an Internet address and thus accessed the WONDER system less often.

Perhaps more than one person in each state office should be on the distribution list to ensure message delivery when one representative is absent. We confirmed only that the message had been accessed by someone using the state epidemiologists' password; however, it is possible that someone other than the state epidemiologists accessed the message on their behalf adding to the delays.

Electronic communication by public health groups (e.g., Epi-net links public health agencies in the United Kingdom, Salm-net links agencies involved in foodborne disease surveillance and control in Europe) is rapidly increasing (4). However, there is a need for a global network that allows public health agencies of every country to rapidly communicate real or potential emergent disease threats.

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Emerging Infectious Diseases Conference

"The Hot Zone—1997: Conference on Emerging Diseases" will be held in Lexington, Kentucky, at the Hyatt Regency on June 27 and 28, 1997. The conference is hosted by the University of Kentucky and the University of Cincinnati. Topics include human immunodeficiency virus infection, vector-borne diseases, viral diseases, tick-borne diseases, prion diseases, and antibiotic resistance. Continuing medical education credit hours (10.5) will be offered. For complete information contact Continuing Medical Education, University of Kentucky, L007 Kentucky Clinic, Lexington, KY 40536-0284 or e-mail sbsize00@pop.uky.edu.

"Emergent Illness and Public Scholarship" Fellowships

Emory University's Center for the Study of Public Scholarship announces four semester-long fellowships for its program on Emergent Illness and Public Scholarship to be held during the 1997-1998 academic year. Emergent Illness and Public Scholarship is the first of a 3-year series of programs funded by the Rockefeller Foundation and organized by the Center for Public Scholarship. The center is directed by Dr. Ivan Karp and is housed in the Institute for Liberal Arts. Emergent Illnesses and Public Scholarship is being coorganized by Emory's Center for the Study of Health, Culture, and Society, which is directed by Dr. Randall M. Packard.

Emergent illnesses, from Legionnaires' disease to AIDS, not only challenge the scientific and medical communities to find new explanations and treatments, but prompt patients and their caretakers and families to challenge the authority and expertise of biomedical specialists. In various settings globally, the formal scholarship of health scientists coexists with, and is frequently challenged by, that of lay persons searching for knowledge and explanations of their own illnesses.

Emergent Illness and Public Scholarship will address the politics of knowledge as well as the mechanics by which information travels between professionals and lay communities that simultaneously produce and consume knowledge. Issues of education, technology, audiences, and the ownership of knowledge will stand alongside more traditionally focused biomedical and epidemiologic concerns as foci of the workshop.

Fellows in the program will spend part of the semester talking across disciplinary and institutional boundaries about emerging illness; help determine the agenda of the workshop series; plan one of its sessions; and interact on a limited basis with Emory graduate students.

Especially welcome to the program are applicants working on health issues outside academia, as the workshop's goal is to transcend the boundaries that often mute discussions between producers of knowledge with differing credentials.

For more information, contact: Michael McGovern, Center for the Study of Public Scholarship, S-412 Callaway Building, Emory University, Atlanta, GA 30322.

International Workshop on Molecular Epidemiology and Evolutionary Genetics

The 2nd International Workshop on Molecular Epidemiology and Evolutionary Genetics of Pathogenic Microorganisms, will be held at ORSTOM, Montpellier, France, May 26-28, 1997. The workshop is organized under the auspices of ORSTOM (French agency for scientific research in developing countries), CNRS (French agency for basic research) and the Centers for Disease Control and Prevention, Atlanta, Georgia, USA. The goal of the workshop is the integration of laboratory science and epidemiology, in using genetic information to study evolution, emergence, reemergence, and dispersal of microorganisms. The objectives of the workshop are to 1) integrate molecular biologic and evolutionary genetics approaches in areas of diagnosis, strain typing, species identification, pathogenesis, antigenic variation, drug and vaccine resistance, and host and vector specificity; 2) foster interaction between scientists working on parasites, yeasts and fungi, bacteria, and viruses; and 3) provide health care providers, public health professionals, and laboratory scientists an opportunity to discuss the joint use of genetic tools and methods needed to meet the challenges of diagnosis and management of emerging, reemerging, and endemic infectious diseases.

The deadline for submitting abstracts to Dr. Michel Tibayrenc by fax (33-467547800) or e-mail (tibayren@orstom.rio.net) is March 15, 1997. For more information about the workshop, please contact Dr. Michel Tibayrenc or Dr. Altaf Lal (aal1@.cdc.gov).

Editorial Policy and Call for Articles

Emerging Infectious Diseases (*EID*) is a peer-reviewed journal established expressly to promote the recognition of emerging and reemerging infectious diseases and improve the understanding of factors involved in disease emergence, prevention, and elimination.

Emerging infections are new or newly identified pathogens or syndromes that have been recognized in the past two decades. Reemerging infections are known pathogens or syndromes that are increasing in incidence, expanding into new geographic areas, affecting new populations, or threatening to increase in the near future.

EID has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the editor at 404-639-3967 (telephone), 404-639-3039 (fax), or eideditor@cdc.gov (e-mail).

EID is published in English and features three types of articles: Perspectives, Synopses, and Dispatches. The purpose and requirements of each type of article are described in detail below. A Spanish version of Vol. 1, No. 1–Vol 2, No. 1 is available electronically from the National University of La Plata, Faculty of Veterinary Science, Argentina (ftp://fvc.medvet.unlp.edu.ar/pub/EID).

Instructions to Authors

Manuscripts should be prepared according to the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (*JAMA* 1993:269[17]:2282-6).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, each table, figure legends, and figures. On the title page, give complete information about each author (full names and highest degree). Give current mailing address for correspondence (include fax number and e-mail address). Follow Uniform Requirements style for references. Consult *List of Journals Indexed in Index Medicus* for accepted journal abbreviations. Tables and figures should be numbered separately (each beginning with 1) in the order of mention in the text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Italicize scientific names of organisms from species names all the way up, except for vernacular names (viruses that have not really been speciated, such as coxsackievirus and hepatitis B; bacterial organisms, such as pseudomonads, salmonellae, and brucellae).

All articles are reviewed by independent reviewers. The Editor reserves the right to edit articles for clarity and to modify the format to fit the publication style of *Emerging Infectious Diseases*.

Documents sent in hardcopy should also be sent on diskette, or by e-mail. Acceptable electronic formats for text are ASCII, WordPerfect, AmiPro, DisplayWrite, MSWord, MultiMate, Office Writer, WordStar, or Xywrite. Send graphics documents in Corel Draw, Harvard Graphics, Freelance, or save as .TIF (TIFF), .GIF (CompuServe), .WMF (Windows Metafile), .EPS (Encapsulated Postscript), or .CGM (Computer Graphics Metafile). The preferred font for graphics files is Helvetica. If possible, convert Macintosh files into one of the suggested formats. Submit photographs as glossy, camera-ready photographic prints.

Send all manuscripts and correspondence to the Editor, Emerging Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop C-12, Atlanta, GA 30333, USA, or by e-mail to eideditor@cdc.gov.

Perspectives: Contributions to the Perspectives 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 infectious diseases, including microbial adaption 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. Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of additional 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. Provide a short abstract (150 words) and a brief biographical sketch.

Synopses: Submit concise reviews of infectious diseases or closely related topics. Preference will be given to reviews of emerging and reemerging infectious diseases; however, timely updates of other diseases or topics are also welcome. Synopses 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Dispatches: Provide brief updates on trends in infectious diseases or infectious disease research. Include descriptions of new methods for detecting, characterizing, or subtyping emerging 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 of text) should not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; and figures or illustrations, not to exceed two. To expedite publication of information of a more urgent nature, we post the journal's dispatches on the Internet as soon as they are cleared and edited. As soon as the full issue is completed, these dispatches become part of the issue.