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Emerging Infectious Diseases

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

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Emerging Infectious Diseases

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The goals of the *Emerging Infectious Diseases* (*EID*) journal are 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 whose study elucidates the factors influencing the emergence of infectious diseases. Inquiries about the suitability of proposed articles may be directed to the editor at 404-639-3967 (telephone), 404-727-8737 (fax), or eideditor@cidod1.em.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.

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 name 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.*

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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 optional. 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 optional. Provide a short abstract (150 words) and a brief biographical sketch.

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Molecular Approaches to the Identification of Unculturable Infectious Agents

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New molecular biologic techniques, particularly representational difference analysis, consensus sequence–based polymerase chain reaction, and complementary DNA library screening, have led to the identification of several previously unculturable infectious agents. New agents have been found in tissues from patients with Kaposi's sarcoma, non-A, non-B hepatitis, hantavirus pulmonary syndrome, bacillary angiomatosis, and Whipple's disease by using these techniques without direct culture. The new methods rely on identifying subgenomic fragments from the suspected agent. After a unique nucleic acid fragment belonging to an agent is isolated from diseased tissues, the fragment can be sequenced and used as a probe to identify additional infected tissues or obtain extended portions of the agent's genome. For agents that cannot be cultured by standard techniques, these approaches have proved invaluable for identification and characterization studies. Applying these techniques to other human diseases of suspected infectious etiology may rapidly elucidate novel candidate pathogens.

Identifying the causative agent of an infectious disease is the cornerstone for its eventual control. In recent years, a great deal of progress has been made in identifying new agents associated with both well-known and newly emerging infectious diseases. A number of syndromes exist, however, in which infectious etiology is likely, but the pathogen resists cultivation with standard microbiologic techniques. For emerging diseases, rapid identification and characterization of the responsible agent are crucial first steps for epidemic control.

The rapid identification of a hantavirus responsible for an outbreak of severe pulmonary distress syndrome in the southwestern United States (1, 2) demonstrated that applying molecular biologic approaches can accelerate the identification of an unknown agent. With extensive nucleic acid and protein databases readily available, isolating and sequencing genomic fragments from an unknown agent can provide important clues regarding its origin and biologic behavior. Once a new agent's phylogenetic relationship to other known organisms is established, appropriate culture conditions, serologic tests, and perhaps even therapeutic strategies can be rapidly developed.

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Although they have revolutionized our ability to identify new pathogens, innovative molecular biologic techniques must be applied in conjunction with traditional epidemiologic procedures. Beral, Jaffe and colleagues, for example, showed that AIDS-related Kaposi's sarcoma (KS) is likely to be caused by a transmissible agent other than human immunodeficiency virus (HIV), before any likely causative agent was isolated (3, 4). These findings focused the attention of investigators on AIDS-KS, which resulted in the isolation of viral DNA from AIDS-KS lesions and the description of a new human herpesvirus (5, 6). Spurious associations between infectious agents and diseases are common, however, and only through careful epidemiologic studies can a causal link between an organism and a disease be established. Epidemiologic criteria for causality (7-9), superseding Koch's postulates, have been used for 30 years, and a critical phase in the process of new pathogen discovery involves the unambiguous establishment that an agent is central to the disease process.

The various molecular biologic approaches to agent identification differ in technical detail, but all rely on isolating nucleic acid fragments belonging to the agent's genome from diseased tissue. The formidable tasks of identifying and separating small unique nucleic acid fragments from human genomic material have been approached by various means, each with its own particular strengths and weak-

nesses. The appropriate technique depends on the type of infectious agent involved (e.g., bacterial or viral), whether the disease occurs in a normally sterile site, and whether it can be passaged through animals.

Once a fragment from the agent's genome is isolated and sequenced, standard genomic walking techniques are used to extend the known sequence, and computer homology searches can be used to identify the likely phylogenetic relationship of the agent to other known organisms. In this article, we highlight recent successful situations when molecular approaches were used to identify and characterize unknown agents of infectious diseases.

Representational Difference Analysis

Representational difference analysis is one of the more robust methods of identifying new agents since it does not require prior knowledge of the agent's class (10). The technique is based on polymerase chain reaction (PCR) enrichment of DNA fragments present in diseased tissue but absent from healthy tissues of the same patient (Figure). Representational difference analysis is also an important tool for identifying polymorphic DNA sequences associated with noninfectious diseases (11).

Representational difference analysis depends first on digesting DNA from both healthy and diseased tissues by using a restriction enzyme and then on separately "simplifying" the resulting genomes to reduce their sequence complexity. This is done by ligating PCR primers to both sets of DNA and nonspecifically amplifying the mixtures. Since PCR most efficiently amplifies fragments of 150 to1500 bp, restriction fragments in this size range are enriched, and the fragments of the sequence represented outside this size range (90%) are reduced.

Unique strands of DNA from diseased tissue representing restriction fragments of an exogenous agent are isolated in a subtractive hybridization process coupled to PCR amplification. First, the priming sequences ligated on DNA restriction fragments of both normal and infected tissues are removed. New primer sequences are ligated only to the diseased tissue DNA fragments. These are then hybridized with an excess of the healthy tis-

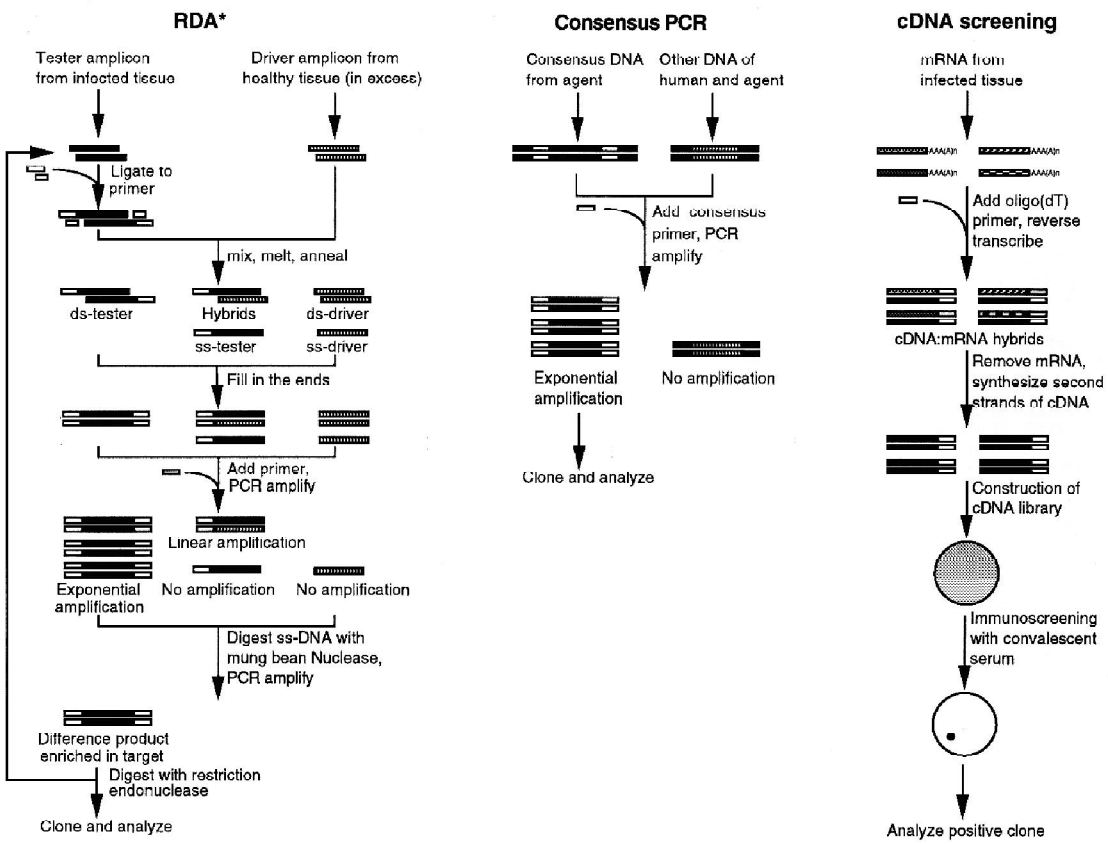

Figure. Schematic representation of different molecular approaches to the identification of unculturable infectious agents.

sue representation. Human DNA fragments common to both diseased and healthy tissues reanneal to each other and, since the healthy tissue fragments are in excess, any given human fragment derived from the diseased tissue will reanneal to a complementary strand from the healthy tissue representation. Thus, common human sequences found in both representations will only have one PCR priming sequence or none present on two complementary strands. However, DNA fragments from the infectious agent will not find complementary strands in the healthy tissue representation and will reanneal with each other. Only hybrids with both strands derived from the diseased tissue representation will have priming sites and be able to undergo subsequent exponential PCR amplification. Several rounds of representational difference analysis are performed, which successively enrich the mixture for unique DNA sequences present only in the diseased tissue representation.

KS-Associated Herpesvirus and KS

The power of representational difference analysis and the difficulties encountered in establishing the etiology of disease are illustrated by its application to KS (5), a vascular neoplasm that frequently occurrs in homosexual men with AIDS (3). Geographic clustering (12, 13) and association with specific sexual behavior (4, 14) suggest that the disease is caused by a sexually transmitted agent. Several agents have been investigated, including human cytomegalovirus (CMV), human papillomavirus, human herpesvirus 6 (HHV-6), and HIV (for review, see [15]), but no convincing etiologic link has been established.

Using representational difference analysis, Chang and colleagues isolated two unique DNA sequences (KS330Bam and KS631Bam) from a KS lesion in an AIDS patient (5). These DNA sequences are homologous to portions of minor capsid and tegument protein genes from Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), a New World monkey herpesvirus. Both EBV and HVS are gamma herpesviruses associated with neoplastic disorders in humans (EBV) (16) and nonhuman primates (HVS) (17). These results suggested that a new human herpesvirus, now called KS-associated herpesvirus (KSHV or HHV-8), could be the KS agent.

The strong association between KS and KSHV was demonstrated by using Southern hybridization and PCR to amplify a 233-bp DNA fragment ($KS330_{233}$) internal to KS330Bam from all 25 intact and amplifiable DNA samples from KS lesions that were examined (5). Ninety tissues from patients without AIDS or KS were examined, and none showed evidence of KSHV infection.

Although controversy remains over the role of KSHV in KS (18-20), epidemiologic and biologic studies strongly suggest that the agent is a causal factor in KS (21). KSHV has now been identified in 211 (94.2%) of 224 KS lesions examined by a number of groups around the world, and the virus is found in all forms of KS, AIDS-related and non-AIDS-related (22-29). Two independent studies have shown that KSHV is present in peripheral blood mononuclear cells of AIDS-KS patients before onset of KS (30, 31), indicating that the virus is unlikely to be a "passenger virus" in KS lesions. Further, the virus has been localized to KS tumor tissues by semiquantitative PCR (25) and by PCR in-situ hybridization (26). The virus has only been found in 8 (1.8%) of 449 solid tissues from control patients without KS (5, 22, 25, 27, 28, 32, 38) and thus appears to be specifically associated with KS. Although early reports suggested that the virus is present in skin tumors from transplant patients without KS (19), subsequent studies have not confirmed this finding (33, 34). One study has found evidence of viral DNA by nested PCR, but not unnested PCR, in semen from (23%) of healthy donors; these results suggest that the prevalence of infection in North America may be higher than that indicated by tissue or lymphocyte studies (35). This intriguing finding has not been reproduced by other groups (23, 36); however, it should be explored in large rigorous studies.

Lymphoproliferative disorders are common secondary malignancies in KS patients with and without AIDS (37). KSHV has also been found in a rare subset of AIDS-related, body cavity-based lymphomas, which are manifested as primary effusions (5, 38). In these AIDS-related lymphomas, tumor cells are coinfected with EBV (38, 39) but, unlike tumor cells in EBV-related Burkitt's lymphomas, these cells do not exhibit c-myc gene rearrangement. EBV-uninfected, KSHV-related body cavity-based lymphomas have also recently been identified (40, 43). Another lymphoproliferative syndrome associated with KS is Castleman's disease in both AIDS patients and HIV-seronegative patients. KSHV has been found in AIDS-related multicentric Castleman's disease lesions at a high copy number and less frequently in tissues from Castleman's disease patients without AIDS (41).

Identifying KSHV DNA sequences by representational difference analysis also led to the quick identification of an in vitro culture system for growing the virus. KS330Bam and KS631Bam probes were used to identify a B-cell line derived from a body

cavity-based lymphoma that was stably infected with both KSHV and EBV (42). Extended sequencing and transmission studies have been performed with body cavity-base lymphoma cell lines, which clearly define KSHV as a new human herpesvirus of the genus *Rhadinovirus* (6). This has recently been confirmed by detecting herpesvirus particles in a body cavity-based lymphoma cell line by electron microscopy (43).

Discovery of a continuously infected cell line has allowed the first generation of serologic assays for KSHV antibodies to be developed (6). Second-generation immunoblotting assays, which appear to be both sensitive and specific for detecting KSHV antibodies, indicate that KS patients are infected with the virus months to years before the disease develops and that few North American blood donors are infected (S-J Gao, P.S. Moore, unpublished observation). Thus, identifying a virus associated with KS by using molecular approaches has rapidly led to new assays that use traditional serologic techniques for detecting infection.

HHV-6 and Multiple Sclerosis

The difficulties of using representational difference analysis for new pathogen identification are also illustrated by the search for the agent that causes multiple sclerosis (MS) (44). An infectious cause for MS has been proposed (45-47), and geographic and household clustering of cases consistent with an infectious process have been shown (45, 48-50). Challoner and colleagues used DNA from sclerotic plaques in brain tissues from MS patients and performed representational difference analysis against pooled DNA from peripheral blood leukocytes of healthy donors. Use of pooled lymphocyte DNA in representational difference analysis opens the possibility for examining rare banked tissues for which healthy control tissues are not available. A 341-bp representational difference analysis band was isolated from MS tissues with near sequence identity to a region of the major DNA-binding protein gene of HHV-6 (44). Detecting exogenous DNA in diseased tissue by this technique, however, does not exclude the possibility that a commensal agent may be identified that is not the cause of the disease being examined. HHV-6 is a neurotrophic virus present in brain tissues from healthy control patients (51), and more than (70%) of both MS patients and controls were positive for the representational difference analysis sequence by PCR (44). Although case-patients and controls cannot be differentiated by the presence or absence of these HHV-6 sequences, only MS tissues showed nuclear staining of oligodendrocytes surrounding plaques when monoclonal antibodies against HHV-6 viral proteins were used (44). There may be a subtle difference in tissue distribution for the virus in MS patients not found in controls, or the HHV-6 variant B group 2 infecting MS plaques may be particularly prone to generating the autoimmune response seen in this disease.

GB Hepatitis Viruses

Since representational difference analysis relies on PCR amplification, the technique is suited for detecting agents with a DNA-based genome; sequences can be amplified by generating a cDNA intermediate, but detecting RNA viruses is problematic since mRNA expression patterns differ between tissues and are likely to give spurious representational difference analysis bands. Simons and colleagues overcame this problem by passing an agent associated with non-A, non-B (NANB) hepatitis through primates and using cell-free extracts of primate plasma for representational difference analysis and discovered two new human hepatitis viruses (52, 53).

A novel form of NANB hepatitis was first identified in a physician who became ill with hepatitis, and the disease was transmitted to primates by injecting serum from patients into the animals (54). Extensive studies demonstrated that the virus, named the GB agent, was different from all known human hepatitis viruses (hepatitis A [HAV], hepatitis B [HBV], hepatitis C [HCV], and hepatitis E) (55-57). Total RNA from the samples was reverse-transcribed to obtain cDNA by using cell-free preinfection and acute-phase plasma from infected monkeys. After cDNA synthesis, representational difference analysis was performed, and seven cDNA clones were found to be specifically associated with this form of hepatitis. Sequence analysis and comparison with other known hepatitis viruses identified two unique flaviviruses, GBV-A and GBV-B, as the GB agents (52, 53, 58). A third virus, GBV-C, was subsequently identified using the known GBV-A, GBV-B, and HVC consensus sequences (52b and see below).GBV-A and GBV-C are closely related phylogenetically. Cross-challenge experiments showed that GBV-C probably originated in human hepatitis patients, whereas

GBV-A and B may be tamarin monkey viruses that were inadvertently passaged along with the human virus (53, 58).

In addition to having difficulty in identifying RNA viruses, representational difference analysis has several other limitations. Polymorphic human DNA can be amplified through this technique (5), and not all bands generated by it belong to the suspected agent. Representational difference analysis may produce DNA fragments from an agent whose genome has not been sequenced (e.g., most bacteria and fungi), and sequence homology searches may be unable to distinguish the agent's genomic DNA fragments from unsequenced human DNA. Only normally sterile site tissues are appropriate for this technique because normal flora that differs between tissue sites could result in spurious amplification. Finally, for viruses with small genomes, multiple restriction digests may be required to identify a unique restriction fragment of the appropriate size that can be efficiently amplified. Although the technique has been successfully used by several groups to identify polymorphic DNA, the procedure is complex and not uniformly reproducible.

Consensus Sequence–Based PCR

Consensus sequence PCR relies on the use of highly conserved DNA sequences, such as ribosomal RNA (rRNA) gene sequences from known organisms, to amplify DNA from related organisms not yet discovered (59). This technique is simple and extremely successful in identifying new human pathogens. Subunit rRNA genes evolve in a relatively slow and uniform manner, which makes these sequences extremely useful for establishing phylogenetic relationships (59). By using conserved DNA sequences from bacterial 16S rRNA genes, at least two new bacteria associated with human diseases have been identified (60, 61), and PCR amplification of conserved hantaviral capsid DNA sequences resulted in the rapid identification of a new hantavirus associated with an outbreak of severe pulmonary disease (1).

Unlike using representational difference analysis, using consensus sequences to amplify DNA requires knowledge of the suspected agent's phylogenetic relationship to other organisms. The technique generally should be used on normally sterile site tissues if sequences from normal flora are also likely to be amplified. Although the lack of broadly amplifiable consensus primers limits use of this technique to prokaryotic and eukaryotic pathogens, consensus sequences are to likely exist among many of the classes of viruses that eventually could be used in screening panels of diseased tissues when a viral cause is suspected.

Bartonella: Bacillary Angiomatosis and Cat-Scratch Disease

Phylogenetic studies of bacteria have relied on comparisons of highly conserved rRNA gene sequences present in prokaryotes (62). rRNA genes are present in all living cells and contain regions of highly conserved sequences with intervening variable regions. Conserved sequences can be used to amplify and sequence the intervening variable regions by PCR. This technique was exploited by Relman and colleagues to identify the bacillus associated with bacillary angiomatosis (60).

Bacillary angiomatosis is an inflammatory vascular proliferative process that affects the skin, lymph nodes, and visceral organs of AIDS patients (63). Although bacilli can be identified by Warthin-Starry staining of lesions (64, 65), the suspected causal organism was resistant to cultivation by standard techniques. Consensus oligonucleotide primers complementary to the 16S rRNA genes of eubacteria were used to amplify 16S rRNA gene fragments directly from tissue samples (60). Phylogenetic analysis of the amplified DNA sequence showed that the organism belonged to the genus *Rochalimaea* (now renamed *Bartonella*) (60). *Bartonella* organisms were also cultured from bacillary angiomatosis lesions (66) and blood from bacteremic patients (67, 68), and serologic analyses have associated the organisms with cat-scratch disease (69). Current evidence suggests that bacillary angiomatosis in HIV-seropositive patients results from infection with either *B. quitana* or a newly described *Bartonella* species, *B. henselae* (68, 70), whereas cat-scratch disease is primarily caused by infection with *B. henselae* (71) (for review, see [72]).

Whipple's Disease

Consensus sequence PCR was also used to identify an organism associated with Whipple's disease, one of the most persistent mysteries in microbiology (61). Whipple's disease is a systemic illness, first described in 1907, characterized by arthralgias, diarrhea, abdominal pain, and weight loss (73). Rod-shaped bacilli were identified histologically in Whipple's disease lesions in the early 1960s (74), but the suspected bacteria were not culturable by standard techniques (73). The agent was found to be a gram-positive actinomycete (*Tropheryma whippelii* gen.nov.sp.nov.), unrelated to any char-

acterized species when a bacterial 165 rRNA sequence was amplified and sequenced directly from infected tissue (61, 75).

Hantavirus Pulmonary Syndrome

Consensus PCR primers have been successfully used to identify and classify bacteria, and similar techniques can be used to diagnose new viral agents. In May 1993, an outbreak of unexplained acute respiratory illness with a high death rate occurred in the southwestern United States (76). In the initial phases of the investigation, the cause of the syndrome was not clearly known to be infectious. Serologic tests quickly detected cross-reactive antibodies to known hantaviral antigens in the serum of patients; these results suggested that a previously unrecognized hantavirus was the cause of the disease (77). PCR primers, based on consensus sequences within the G2 protein coding region of the M segment of the genomes of known hantaviruses, were designed and used in nested reverse-transcription PCR to amplify a short segment of the viral genome from diseased tissues (1).

Sequence analysis of the PCR products showed that the amplicon differed from the other known hantaviral sequences by least 30%, and phylogenetic analysis demonstrated that the new virus is most closely related to Prospect Hill hantavirus (1), a zoonotic hantavirus endemic in North America (78). Hantavirus antigens have been detected in endothelial cells from patients (1, 79), and virus particles have been identified in infected pulmonary endothelial cells and macrophages (80). Deer mice (*Peromyscus maniculatus*) are the primary host for the virus (1), and the virus has been passaged through laboratory-bred deer mice and cultured in Vero E6 cells (2). This newly recognized virus was originally named Muerto Canyon virus (2) but has since been renamed Sin Nombre virus in light of nomenclatural disputes regarding the appropriateness of a descriptive name.

Complementary cDNA Library Screening

Identification of HCV and HGV

A third major approach to new organism identification relies on screening cDNA libraries made from diseased tissues by using hyperimmune serum from specimens. This method was successfully used to identify the cause of most cases of NANB hepatitis, HCV. When serologic tests for HAV and HBV became available, it became clear that most cases of transfusion-associated hepatitis in the United States were not caused by either virus (81, 82). Conventional techniques failed to identify the agent responsible for most cases of NANB hepatitis (83), despite evidence that the disease was caused by a bloodborne, small, enveloped virus readily transmissible to chimpanzees (84, 85).

Since the virus was presumed to be an RNA virus, Choo and colleagues made a cDNA expression library from RNA isolated from an infected chimpanzee's plasma (86). While the GBV-A, B, C were identified by direct detection of viral cDNA through representational difference analysis, HCV cDNA was identified by immunologic detection of cDNA that was encoding viral protein. Viral antigens expressed from the cDNA library were identified by immunoscreening with convalescent-phase human sera. A cDNA clone was isolated encoding an antigen that could be used to screen convalescent-phase sera from patients with NANB hepatitis. Identification of the clone rapidly led to the production of a recombinant antigen used for serologic screening to detect specific antibodies in infected chimpanzees and patients with hepatitis after transfusion. Extended sequence analysis demonstrated that the agent, now known as HCV, is closely related to the family *Flaviviridae* and is the major cause of NANB hepatitis throughout the world (86, 87). Recently, the same approach was successfully used to identify HGV, which has been found to be almost identical to GBV-C, the human hepatitis virus identified by representational difference analysis (88). The potential role of HGV and GBV-C in human disease still remains uncertain (88b).

Using convalescent-phase sera to screen cDNA libraries from diseased tissues is a novel method for pathogen identification. It is a potentially useful technique for diseases in which well-defined convalescent-phase sera are available, and it requires tissues with a high titers of the agent. On the other hand, constructing and screening DNA libraries are laborious, and cross-reactive antigens are likely to be detected, especially for diseases in which autoantibodies are common.

Future Directions

Nucleic acid database information is rapidly expanding for all classes of organisms, and a significant fraction of the human genome has already been sequenced. Even small laboratories can exploit new and relatively inexpensive molecular biologic technologies to search for new pathogens. Once a small, unique nucleic acid fragment from a pathogen has been identified, nucleic acid detection and serologic assays can often be rapidly developed to establish an etiologic link with disease.

New pathogens are likely to be identified by some of these molecular biologic approaches. A number of diseases have long

been suspected to have an infectious cause and are appropriate candidates for these techniques. The eventual identification of infectious etiologic agents of diseases such as sarcoidosis (89), Kawasaki's disease (90), and type I diabetes mellitus is likely. New techniques, such as arbitrarily primed PCR and phage display libraries (91), have not been used for pathogen discovery but show promise for expanding the repertoire of techniques available for identifying unculturable agents. Three years passed between the initial descriptions of AIDS and the identification of HIV (92). Use of molecular biologic approaches could lead to rapid identification and control of the next pandemic caused by a newly emergent infectious disease.

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Dr. Gao received his Ph.D. in Virology from the University of Bordeaux, Bordeaux, France. He is a research scientist in the School of Public Health, Columbia University, New York, where his work focuses on the serology and seroepidemiology of Kaposi's sarcoma-associated herpesvirus (KSHV). He also works on the induction of lytic phase replication of KSHV as well as genes that may be involved in the latent phase replication of the virus.

Dr. Moore is an associate professor of clinical public health at Columbia University. His current research is focused on delineating the relationship between Kaposi's sarcoma and KSHV (HHV8) and understanding the tumorigenic potential of this virus. Along with Dr. Yuan Chang, his laboratory co-director and wife, he is working on both epidemiologic and basic virologic studies of KSHV and other new potential pathogens.

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DNA Vaccines for Emerging Infectious Diseases: What If?

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A novel and powerful method for vaccine research, colloquially known as DNA vaccines, involves the deliberate introduction into tissues of a DNA plasmid carrying an antigen-coding gene that transfects cells in vivo and results in an immune response. DNA vaccines have several distinct advantages, which include ease of manipulation, use of a generic technology, simplicity of manufacture, and chemical and biological stability. In addition, DNA vaccines are a great leveler among researchers around the world because they provide unprecedented ease of experimentation. To facilitate diffusion of information, an Internet site has been established called THE DNA VACCINE WEB (URL:http://www.genweb.com/dnavax/dnavax.html). In this review, a brief survey is undertaken of the experimental models and preclinical work on DNA vaccines to contribute to a greater awareness of the possibilities for emerging infectious diseases.

"But there is no single problem that is more pressing than our fast-deteriorating relations with the microbial world" wrote Barbara Culliton (1), the editor-in-chief of *Nature Medicine*, at the end of 1995. This stark statement concluded her comments on, among other threats, the reemergence of cholera and plague, the growing number of Lyme disease cases, and humanity's occasional but frenetic duels with Ebola virus. What if one day an Ebola-infected traveler makes it to the boarding lounge and embarks on an airplane? As pointed out by David Heymann, director of the World Health Organi-zation's new Division of Emerging Diseases, the virus would spread to far corners of the world (2) with dramatic consequences. This scenario has already been played out with human immunodeficiency virus (HIV). Vaccines have traditionally been used as weapons against health threats. In the case of HIV infection, the basis for one has not yet been clearly delineated. For scientific, commercial, and practical reasons vaccines cannot solve all the problems posed by emerging infectious organisms. However, novel and powerful methods for vaccine research, and possibly for vaccines themselves, hold some promise in our efforts to curb emerging disease threats. These methods involve the deliberate introduction of a DNA plasmid carrying a protein-coding gene that transfects cells in vivo (albeit at a low efficiency) and expresses an antigen causing an immune response (3). This procedure, known as a DNA vaccine, is perhaps better described as DNA-mediated or DNA- based immunization, with the understanding that the objective is not to raise an immune response to the DNA itself.

This method is conceptually sound and experimentally straightforward; however, its most novel aspect is that it works at all! It was not expected that pure plasmid DNA could be taken up by cells, after parenteral introduction in a simple saline solution (4), to levels allowing expression of enough protein to induce an immune response. A more radical method of introducing DNA involves the bombardment of DNA-coated gold particles. When applied to the skin, these particles produce good immune responses with much less DNA than is required by other routes, such as intramuscular or intradermal needle injection (5). More esoteric still is the application of pure DNA solution (as nose drops) to the nasal membranes, which has been reported to work (5) but is perhaps too inefficient for further consideration.

DNA vaccines have distinct advantages: They can be manufactured far more easily than vaccines composed of an inactivated pathogen, subcellular fraction, or recombinant protein. Since almost all plasmids can be manufactured in essentially the same way, substantial economies of scale can be achieved. DNA is very stable and resists temperature extremes; consequently, the storage, transport, and distribution of DNA-based vaccines are more practical and less expensive. In addition to the commercial, there are vaccine research and development considerations. It is now possible to change the sequence of an antigenic protein, or to add heterologous epitopes, by simply introducing mutations to the plasmid DNA. The immunogenicity of the modified protein can be directly

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assessed after injecting the plasmid DNA. This simple method could increase considerably our understanding of the immune response to antigens.

In addition, both in vaccine research and in actual use, DNA-mediated immunization is the great leveler among researchers around the world. It is easy to use because once the protein coding sequences are cloned into a suitable expression vector, the direct introduction of the plasmid vector (into mice for example) allows experimental assessment of the immune response and its consequences, without further experimental steps such as the preparation of a recombinant protein as antigen.

Because it is so straightforward and requires only simple molecular biologic techniques, the method should be practical in many laboratories around the world. It would be unrealistic to deny that certain diseases are not of great interest to large pharmaceutical firms. Moreover, even when triumphs over disease are achieved, as in the case of hepatitis B virus (HBV) infection (6), marketing strategies can exclude the vaccine from regions where it would do the most good. DNA-mediated immunization can be used in countries that cannot implement more complicated and expensive strategies.

What if DNA vaccine research could be carried out worldwide by a generic technology, where human creativity adds substantial value to the work? This short review outlines the possibilities offered by DNA-mediated immunization.[1] I will review experimental models and preclinical work on DNA vaccines and discuss new developments that are based on the idea that DNA delivery can be used to induce immune response to proteins.

Animal Models of DNA Vaccines

Various experimental models of DNA vaccination have been reported (Table 1). Most of the pathogens studied have been viruses, which is consistent with the method used: since the genes transferred by the plasmids require the host cellular machinery to be expressed, DNA-based immunization most resembles a virus infection. However, genes from other microorganisms have also been used with success. The types of polypeptides expressed are often the envelope proteins of viruses, but various proteins have been used. Indeed, it is not obvious what aspects of a protein produce an effective immune response by this unusual method of antigen delivery.

The immune responses obtained are clearly broad-based when they have been well characterized. In several models, antibodies are reproducibly induced, and the antibodies ultimately are of the immunoglobulin G (IgG) type, indicating a T-dependent class switch. In the case of immunization for HBV surface antigen, the fine specificity of the humoral response in mice mimics, to a certain extent, that observed during infection in humans (12). T-cell proliferation and cytokine secretion have been studied in several models, and the cytokine profile indicates a Th1 type response, characterized by the secretion of interleukin-2 and γ-interferon. The immune responses can be remarkably long lasting; however, the duration of the immune response does not appear to have any deleterious effects on the animals because they are protected against challenge long after immunization (41). Also, in some cases, as in the DNA-mediated immunization against HBV surface antigen, antibody levels reach a plateau at titers of about 10^4 and remain stable for at least 18 months. If the mice are boosted with a second DNA injection at 7 months, a further 10-fold increase in titer can be obtained (14).

Cytotoxic T lymphocytes (CTL) are invariably induced to class I epitopes of the proteins encoded by the transferred genes. These CTL responses can be quite strong (10), and DNA-mediated immunization can circumvent haplotype-linked nonrespon- siveness (13). Cytotoxic immune responses are thought to be important in clearing viral infections because this type of T-cell response allows the immune system to recognize virally infected cells and destroy them, thus removing the virus.

What is surprising about the induction of CTL with DNA-mediated immunization is that either professional antigen-presenting cells have been successfully transfected, or the free protein can be processed more effectively with CTL induction than with a classical immunization protocol. A large body of immunologic evidence suggests that only professional antigen-presenting cells can prime T lymph- ocytes in the first stages of the immune response. Thus, the transfection of muscle fibers, for example, would not normally be expected to lead to such efficient immune responses. Solution of this enigma should provide insight into some fundamental immune processes. In the meantime, DNA-based immunization is an excellent and

[1] An Internet site, THE DNA VACCINE WEB (URL http://www.genweb.com/Dnavax/dnavax.html), provides journal references, abstracts, experimental protocols, available plasmids, and other information about DNA vaccines.)

Table 1. Animal models of DNA vaccines

Pathogen	Antigen	Animal species	Reference
Bovine herpesvirus	Glycoprotein	Cattle,| mouse	(7)
Hepatitis B virus	Capsid protein (core antigen)	Mouse	(48)
Hepatitis B virus	Envelope protein (surface antigen)	Mouse Rabbit Rat Chimpanzee*	(3, 8-14, 53)
Hepatitis C virus	Core/nucleocapsid	Mouse	(12, 15)
Herpes simplex virus	Glycoprotein B Glycoprotein D ICP27	Mouse|*	(16-18)
Human immuno-deficiency virus-1	Envelope glycoprotein gp160 Noninfectious particles	Mouse Nonhuman primates	(19-22)
House dust mite	Allergen	Rat*	(52)
Influenza virus	Hemagglutinin Matrix protein Nucleoprotein	Chicken* Ferrets* Mouse* Nonhuman primates*	(23-29)
Leishmania major	Major surface glycoprotein	Mouse*	(50)
Lymphocytic chorio-meningitis virus	Glycoprotein Nucleoprotein	Mouse|*@	(30-32)
Mycobacterium tuberculosis	*M. leprae* hsp65	Mouse|	(33)
Mycoplasma pulmonis	*M. pulmonis* DNA *M. pulmonis* DNA expression library	Mouse*	(34, 35)
Papillomavirus	Major capsid protein L1	Rabbit*	(51)
Plasmodium yoelii	Circumsporozoite protein	Mouse|	(36-38)
Rabies virus	Glycoprotein	Mouse*	(39-41)
Simian immuno-deficiency virus	Env, gag	Monkey	(49)
Schistosoma japonicum	Paramyosin (Sj97)	Mouse	(42)

The symbols | and * refer to reports of partial or complete protection, respectively, to challenge by the infectious agent. In one case, enhanced immunopathology (@) after intracranial viral challenge has been reported (32).

Updated versions of this table can be found on THE DNA VACCINE WEB site (URL http://www.genweb.com/Dnavax/dnavax.html) where links are provided to the Medline abstracts of most references. See also a compendium of recent articles on DNA vaccines in a special issue of the Annals of the New York Academy of Sciences, Volume 772 (New York Academy of Sciences Conference: "DNA Vaccines: A Novel Approach to Vaccination," Arlington, VA, April 7-9, 1995). The Medline abstracts of the articles in this issue can be found on THE DNA VACCINE WEB.

simple tool to achieve CTL responses for which efficient methods have been needed for years.

One of the first uses of DNA-mediated immunization was to induce such cytotoxic cellular immunity to a conserved protein of influenza A virus to determine if overcoming its seasonal variation was possible. Ulmer et al. (26), obtained cytotoxic T lymphocytes directed against the conserved influenza nucleoprotein, which protected mice against the disease, even when they were challenged with a heterologous virus carrying a different hemagglutinin but the same nucleoprotein sequence.

The crucial point, however, is the ability of DNA-based immunization to protect animals from infection upon challenge, and this has been demonstrated in several model systems, particularly with influenza and rabies viruses, as well as *Mycoplasma pulmonis* and *Plasmodium yoelii*. Donnelly et al. (29) have shown that a mixture of plasmids can be used to induce antibody to the influenza hemagglutinin surface protein and cytotoxic immunity to the viral nucleoprotein and matrix protein. This DNA vaccine protected ferrets and African green monkeys against viral challenge by using an antigenically distinct human influenza virus more effectively than the contemporary commercial vaccine. The influenza model has now been the subject of much preclinical work, and human trials are in progress.

One human trial uses plasmid vectors expressing HIV-1 genes delivered to HIV-seropositive persons by intramuscular injection. This protocol uses Marcaine to facilitate DNA uptake, although the mechanism of this effect has not been clearly delineated (20). More clinical trials will likely be initiated since plasmid DNA is now considered an innocuous substance compared with other genetic vectors used in therapy.

Further Questions on Methods

Most studies listed in Table 1 have used intramuscular needle injection to deliver DNA; however, intradermal particle bombardment works very well (provided that one has access to biolistic apparatus). Intradermal particle bombardment may ultimately work better than intramuscular injection in primates, including humans, since extensive connective tissue in muscle may impede DNA diffusion and transfection in larger mammals that do not lead sedentary lives in cages.

In most of our own experimental work (8-12), we have used a protocol that induces muscle regeneration and increases the number of transfected muscle fibers (43), presumably because of improved diffusion of the DNA and better transfection efficiency of immature fibers. The actual protocol is probably not suitable for routine prophylactic vaccination in humans, although it could be considered for therapeutic immunization. Nonetheless, this model system shows that muscle fibers can take up DNA better under some circumstances, and further understanding of this process may improve formulations for DNA uptake in muscle. Such improvements will almost certainly be a prerequisite for intramuscular DNA vaccination in humans. The improved uptake of DNA in regenerating muscles composed of immature fibers also suggests that newborn mammalian muscle may also take up DNA more effectively than adult muscle. Since improving childhood vaccination programs is a goal, this characteristic may be an important consideration in the use of DNA vaccines, if they can be used for children.

The regeneration protocol is particularly useful for scientists who are beginning research with DNA-mediated immunization. The use of available control plasmids to inject DNA for validating the procedure is also an important consideration. The various steps that can be taken to achieve reproducible immunization with plasmid DNA transfer have been discussed (44). Careful attention to the details of the intramuscular injection protocol (43,45) can make a substantial difference in the outcome.

Vaccine Development and the Limits of the Protective Immune Response

A successful vaccine must confer protective response to the recipient, and therefore, the limits of the immune response must be known. This knowledge can be purely empirical, as in the case of the first polio vaccines for which the precise protective epitopes were not known. Such knowledge is not required when using a killed or attenuated viral preparation. In contrast, for recombinant vaccines a single protein should induce an immune response that will provide sterilizing immunity; this is also true for DNA vaccines and represents a major conceptual limitation in the use of this approach for vaccination. Although mixtures of individual proteins or their genes can be envisioned, in the case of recombinant protein vaccines this would be pro- hibitively complicated and expensive. For DNA vaccines this is far easier to imagine since the injected material is always DNA, and no matter what genes are carried by the plasmid, the production process is the same.

The only human vaccine that uses a recombinant protein as its basis is that against HBV, which has been used for nearly 10 years. Such a high-tech vaccine was possible because empty viral particles, from the plasma of persons chronically infected with HBV, could be purified and used to induce humoral immunity against the so-called surface antigen that would protect against infection. The plasma-derived particles are still used as a vaccine in many countries, in part because of the cost of the recombinant product (6). The envelope proteins of viruses are always good targets for inducing protective immunity.

However, this rational clearly breaks down in the case of a virus, such as HIV, which mutates so rapidly, apparently in response to immune pressure. The fundamental knowledge required to determine what would comprise a potential HIV vaccine is still lacking, and therefore, it is unlikely that a vaccine can be developed until further basic research provides more insight. Thus HBV and HIV illustrate two extremes in vaccine development: with HBV a simple antibody response to a single antigen neutralizes the virus, whereas with HIV some form of cytotoxic immune response is probably necessary. DNA-mediated immunization has a role to play in further research.

Expression Library Immunization

An attempt has been made to use DNA-mediated immunization to develop a systematic method for producing a vaccine. Barry et al. (34) took advantage of the very small amounts of DNA required with the particle bombardment method (5,46). Since a single nanogram of DNA coated on the gold particles can induce an immune response, one microgram can potentially introduce a thousand different genes. On this basis, a library of gene fragments was prepared from *Mycoplasma pulmonis* by cloning the genomic DNA into a plasmid expression vector. Since this organism has a relatively small genome (about 10^6 base pairs), enough of the total DNA protein-coding sequences might be expressed to induce immunity to the pathogen. Since only a small part of the genetic complement of the organism is expressed and expression is mostly from only a fragment of genes and not entire functional proteins, pathogenic effects would be avoided, while all the advantages of broad-based immunity produced by a DNA vaccine would be present.

Protection against *M. pulmonis* has been achieved after immunization with different expression libraries (34). The next steps could consist of using the method to screen for the gene or genes responsible for protective immunity. Seen in this light, this approach solves the problems of having a more general way to determine the limits of protection, which is vital in establishing what the protein or DNA composition of a vaccine would be. The search for a single gene, or a small number of genes, should be facilitated by the use of the DNA-based immunization method. However, if many gene products are required to confer protection, defining the correct mixture may not be straightforward.

This discussion raises another consideration. From several points of view (at least regulatory and manufacturing), a vaccine must contain defined components. Therefore, the expression library immuni- zation approach cannot be used with simple mixtures of uncharacterized gene fragments. Although in principle it is possible, would anyone be prepared to have a human vaccine composed of an undefined mixture of HIV or Ebola virus gene fragments that seemed to confer protection in animal models? Once again, the simple method means that further creative research may rapidly provide insight into vaccine design.

What If an Ebola Outbreak Threatens the World?

Let us take the most provocative scenario to illustrate what can perhaps be done, in principle and in practice, in the face of a rampantly infectious viral disease. When preventive measures against an agent like Ebola virus are needed on an emergency basis, speed is imperative. The filoviruses Ebola and Marburg are extremely pathogenic, causing a fulminating febrile hemorrhagic disease; they grow fast, kill most cells, and the infected person bleeds to death, usually within 48 hours of infection.

These viruses are enveloped filamentous particles with a nonsegmented negative-strand RNA. The genes of both members of the filovirus genus, the Marburg virus group and the Ebola group, have already been cloned and sequenced. There does not, as yet, seem to be any great variation among the proteins sequences for a given virus; however, Ebola and Marburg are not highly related serologically. The filoviruses are similar to paramyxoviruses, such as the respiratory syncytial virus.

This basic molecular information is enough for testing the ability of one or more of the genes to induce an antibody and CTL response that might help protect against disease. One would choose genes whose proteins are not responsible for the

pathology of the virus, but this should be relatively easy, since the growth potential of the virus largely accounts for its extreme toxicity. One option is the gene encoding the glycoprotein, a viral membrane protein requiring glycosylation for its native structure. Such glycosylation might be important for the antigenicity of the protein if it is to mimic the filamentous envelope of the virus. Here, the fact that DNA vaccination involves synthesis of the protein in the cells of the mammalian recipient of the DNA vaccine means that the appropriate glycosylation pattern can be produced.

If Ebola or Marburg, or a related filovirus, escaped a restricted ecologic niche, the following scenario might unfold. If the emerged virus is found (by serologic testing) to be one of the existing, characterized ones, existing cloned genes could be used. Otherwise, a virus isolate would need to be obtained, and the genes would need to be cloned. Cloning can be accomplished easily since related genes are available for probes in molecular cloning experiments.

Since one strategy for prophylactic vaccination is to induce a humoral response to the outer coat of the virus and block entry to the cells, cloning would allow an attempt to quickly counter the virus infection or at least to slow down its spread in the organism or within the population. A full-length envelope glycoprotein gene could be isolated from the cloned virus genome (perhaps by polymerase chain reaction) and cloned directly into a suitable DNA vaccine plasmid vector. A GMP production run could be carried out, perhaps within a week, and DNA vaccine could begin to be distributed to areas most critically at risk.

The above scenario assumes that DNA vaccines will be accepted for use in humans, which seems likely for high-risk situations, and that the basic method for preparing GMP-grade plasmid DNA is available, which is currently the case. Since August 1995, a collaboration between Qiagen (Hilden, Germany) and Pharos (Seraing, Belgium) has offered full-GMP production of plasmid DNA to companies and the scientific community in general. The molecular method needed to rapidly go from a virus isolate to the vaccinating plasmid vector should represent only a relatively minor bottleneck to the development of an urgently needed vaccine, at least compared with any other approach used before. It is not surprising that Bernard Dixon, writing in Bio/Technology (47), has called DNA vaccines "the third vaccine revolution."

This review poses questions about DNA vaccines and suggests that the answers lie in new methods of research and development. If DNA-mediated immunization were used in all countries that have expertise in molecular biology, novel vaccines would be developed. Ultimately, a major goal of the DNA vaccine approach for public health might well be to bring vaccine development within the reach of researchers working on infectious disease problems in which there is no great commercial interest. If DNA vaccines come into widespread use for public health applications, vaccines for many diseases could be produced rapidly since, in the end, the product is simply a DNA plasmid.

What if such a method were used for human vaccination? The cost of production and delivery of vaccines would be reduced, thus allowing vaccines to reach areas of the world somewhat deprived of preventive public health measures, particularly the recent biotechnologic methods. If new infectious diseases appear in the future, as they surely will, perhaps these new tools will be used to combat them more effectively.

Today's research method can be tomorrow's vaccine. DNA vaccines will be within the means of many more populations and countries since full GMP production technology will be both simpler than technology for other products and far more available to research scientists. A little more than 2 years stood between the first published description of a DNA vaccine and the beginning of the first clinical trial, which indicates that the necessary infrastructure for producing DNA vaccines was rapidly put in place. But more importantly, this short time span bodes well for the ability of public health agencies all over the world to bring scientific research to bear on diseases relevant to their own situation and to disease prevention.

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Conjugate Vaccines and the Carriage of *Haemophilus influenzae* Type b

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Pharyngeal carriage of *Haemophilus influenzae* type b (Hib) is important in the transmission of Hib organisms, the pathogenesis of Hib disease, and the development of immunity to the bacterium. The remarkable success of current vaccination programs against Hib has been due in part to the effect of conjugate Hib vaccines in decreasing carriage of Hib. This review explores evidence for this effect, and discusses the possible mechanisms of the mucosal influence of Hib conjugate vaccines.

Many junior doctors today have not had occasion to treat a child for *Haemophilus influenzae* type b (Hib) disease. The remarkable absence of cases of this disease is due to the use of conjugate vaccines. In countries with established Hib vaccination programs the incidence of disease has declined sharply (1). In fact, in some countries protection provided by Hib conjugate vaccines appears to extend to unvaccinated infants in the population (2,3). This phenomenon has been attributed to the conjugate vaccines' effect of decreasing Hib colonization; however, few controlled studies have been conducted in this area and many aspects of the Hib conjugate vaccines' influence on carriage remain speculative. This review explores Hib carriage in the context of current efforts at elucidating the effect of conjugate vaccines on Hib within human mucosae.

What Is Carriage?

A carrier is a person who harbors a specific infectious agent in the absence of clinical illness with or without a detectable immune response (4). The carrier state may reflect carriage of the organism in the incubation period before clinical symptoms appear, during an illness, or after recovery from illness. The carrier state may be short or long, and it may be intermittent or continuous. Carriers may spread infection to others. Latency should be distinguished from the carrier state, in that a latent organism is not transmissible. Hib carriage can be synonymous with Hib colonization and is defined as the presence of viable Hib organisms in the human pharyngeal mucosa. This definition depends on the sensitivity and specificity of the process used to identify viable bacteria in a healthy host.

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The Detection of Carriage

Because Hib carrier status is usually determined by culture techniques, the efficiency of every step in the microbiologic investigation must be maximized. Potential problems in the microbiologic detection of Hib include consistency of swabbing technique, which is difficult to maintain; survival of the organism during transport on the swab to the culture medium; and the morphologic similarity of *Haemophilus* species on solid media, their fastidious growth requirements, and the abundance of other bacteria in the specimens.

The development of an antiserum agar culture method by Michaels et al. (5) has overcome some of the problems in isolating Hib from a sample containing mixed flora. This method has been successfully modified for use in large scale studies (6). Pharyngeal swabs are plated onto a transparent solid medium impregnated with high-titer antiserum for the serotype b capsular polysaccharide. Isolated Hib colonies in a mixed culture are readily recognized on this medium because they are surrounded by halos of antigen-antibody precipitate.

Most surveys of Hib carriage use Michaels' antiserum agar method to establish carrier status. Before this method became available, the diversity of culture methods used to isolate Hib from pharyngeal swabs was a major factor complicating the interpretation of data from different studies. In most investigations, difficulties in isolating Hib may have contributed to an underestimate of Hib carriage rates.

The Epidemiology of Hib Carriage

The most important factors contributing to the epidemiology of Hib carriage are social and demographic. The probability of Hib carriage in a young

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child seems closely related to the likelihood of exposure to the organism.

Most surveys agree that nasopharyngeal or throat cultures recover Hib in 3% to 5% of young children (7,8), with age being a prominent determinant of Hib carriage rates (9). Carriage rates are low in the first 6 months of life, reach a maximum between the ages of 3 and 5 years, and gradually decline in adulthood. Under circumstances of crowding or Hib disease within a closed population, the carriage rate may be substantially higher (7,10,11). Hib carriage rates among children increase with the number of siblings in a family (9), and in the United States, especially after the occurrence of Hib disease in a child care center, carriage rates of 50% have been found (12). The influence of concurrent upper respiratory symptoms on Hib carriage rates remains controversial, and studies differ in their findings on the influence of season, sex, and race on carriage rates. Most studies find no association of Hib carriage with these factors. Antimicrobial therapy affects Hib carriage; in particular, if given appropriately, rifampicin may eliminate throat carriage of Hib and reduce the risk for secondary disease among contacts (13).

Longitudinal studies of children further characterize Hib carriage. Although many children may be transient carriers of Hib (14), carriage tends to persist for many weeks or months (10,12,13). It seems that close contact and generous exchange of respiratory secretions is required for the transmission of Hib between hosts. Even when the contact between a known carrier and a susceptible child is intimate, spread of Hib occurs slowly over weeks or months (7,8). Bijlmer has suggested that village population dynamics and living conditions in the Gambia contribute to different kinetics of colonization and transmission of Hib, as persistence of carriage in Gambian children is short-lived (15).

Hib Carriage and the Transmission of Disease

Since most patients with Hib disease have not had contact with a person who had invasive disease, and the organism has no known reservoir outside humans, asymptomatic carriers have been recognized as the major source of infection. The relationship between carriage rates and the risk for disease is not simple. The spread of infection in the presence of low carriage rates has been described (16), and no overt disease has been reported despite high carriage rates (7,17). Furthermore, organisms isolated from the pharynx appear to lack certain virulence attributes found in organisms isolated from patients with invasive disease (18).

Like surveys of Hib carriage, epidemiologic surveys of Hib disease have found that rapid dissemination of Hib strains has not been the rule, although the temporal clustering of several episodes of systemic disease in child care centers has occurred. The secondary attack rate for household contacts is approximately 500 times higher than the endemic-disease risk for the general population (1). The high frequency of Hib carriage in homes and child care centers of patients who have Hib disease suggests either that a high concentration of carriers precedes and increases the probability of cases, or that affected children are potent sources of infection for others in close contact.

Although encapsulation enhances the ability to survive the dehydrating stress that occurs during transfer between hosts (20), a great deal remains unknown about the transmission of Hib. What are the modes of transmission between hosts? How long can organisms remain viable between hosts? Is there a threshold colonizing population or dose of organisms to ensure successful transmission? Is there any correlation between the size of the infective dose or the number of carried organisms and the likelihood of invasive disease?

Hib Carriage and the Pathogenesis of Disease

The accepted pathogenesis of invasive Hib disease begins with the pharynx as the portal of entry. The infant rat model of *H. influenzae* meningitis has been used to study the early pathogenic events of Hib disease. Infant rats contracted meningitis after intranasal challenge with Hib (21). There was an age-dependent susceptibility to meningeal invasion (22), and bacteremia followed the nasopharyngeal inoculation of organisms. The incidence of bacterial meningitis, irrespective of rat host age, was directly related to the intensity of bacteremia (22). To reach the bloodstream, this nonmotile bacterium must pass through or between epithelial cells, penetrate the basement membrane and subepithelial tissue, and enter the endothelium of a blood vessel. The manner by which this occurs remains under investigation.

Untreated, Hib meningitis and epiglottitis are fatal in most cases. The death of the host is disadvantageous to the infecting bacterium since death terminates transmission and propagation of organisms. Vascular invasion by Hib may be circumstantial or accidental rather than the result of evolutionary advantage.

Hib Carriage and the Development of Immunity

The capsular polysaccharide of Hib is a linear polymer of ribose, ribitol, and phosphate (23) and is called polyribosylribitol phosphate (PRP). PRP is the principal determinant of virulence of Hib (24,25) and a target for antibody-mediated immunity. Considerable evidence indicates that antibody to PRP is a principal protective host factor (26).

At birth, maternal anti-PRP IgG confers protection; however, as the level of maternal antibody in the infant declines, the risk for and incidence of Hib disease rise. As children approach 2 years of age, their own antibody to the capsular polysaccharide begins to appear. The antigenic stimulus for this age-dependent development of bactericidal activity may be through mucosal exposure to Hib or to other cross-reacting antigens (27).

High serum anti-capsular antibody concentrations are associated with the Hib carrier state in children older than 18 months (17,28), but how colonization increases serum anti-PRP antibody concentrations remains unclear. Hib antigen may be absorbed across the mucosa, or the whole organism may traverse this barrier during colonization, causing low grade asymptomatic bacteremia and a systemic antibody response. It is possible that Hib organisms are phagocytosed by lymphoid cells, in the pharyngeal mucosa, that may act as antigen-presenting cells (29), leading to the production of specific serum or mucosal antibodies, with or without direct vascular invasion by the organism. Other mucosal surfaces, such as the gastrointestinal mucosa, may play a role in anti-PRP immunogenesis. The incidence of bacteremia and meningitis in infant rats was significantly lower for rats fed with *Escherichia coli* that possessed K100 capsular antigen (cross-reactive with type b capsular antigen) than for rats fed with *E. coli* K92 or saline (30).

Many have argued that the high prevalence of anti-PRP antibodies and the low rates of Hib carriage in children point to sources other than Hib that give rise to serum anti-PRP antibodies. However, the technical difficulties in identifying Hib carriage may have led to an underestimate of carriage rates. With more sensitive culture techniques, it now seems more plausible that Hib carriage or infection could account for the acquisition of natural immunity.

Vaccination and Hib Disease

The initial development of vaccines against Hib in the late 1960s was prompted by three major concerns: the high case-fatality rate of Hib disease, the high incidence of central nervous system sequelae in children surviving Hib meningitis, and the gradual emergence of strains resistant to preferred antibiotics. Because serum anti-PRP antibodies were known to be a principal protective factor in the host, efforts were made to increase the antibody concentration by active immunization with a vaccine composed of purified capsular polysaccharide. While the PRP vaccine was effective in protecting healthy children 18 months of age and older against invasive Hib disease (with an estimated efficacy of 90%), it did not protect younger children who have the highest incidence of Hib disease (31).

The explanation for the vaccine's inability to protect younger children lies in the chemistry of the capsular material. PRP, a heteropolymer of pentose sugars, does not elicit a T-cell–dependent immune response and is not an efficient immunogen, especially in young children. Carbohydrate antigens, such as these capsular polysaccharides of encapsulated bacteria, are characterized as T-cell-independent type 2 (TI-2) antigens. However, for many antigens the classification T-cell–independent or T-cell–dependent refers more to the pattern of antibody response than to the involvement of T-cells in eliciting that response. In the very young, the antibody response to T-cell–independent antigens is low, consists of a high proportion of IgM antibody, and there is no booster response to repeated doses of antigen.

Converting polysaccharide into a T-cell–dependent antigen, to which infants can respond, requires the covalent linkage to protein molecules, thus producing a conjugate vaccine. Studies of the different conjugate vaccines have confirmed that conjugation results in a T-cell–dependent response (32). Even though the protein carriers and covalent linkages are biochemically and structurally different, they appear to be more immunogenic than pure PRP vaccines. Conjugate vaccines can confer protection against Hib in infants under 6 months of age (33).

Four conjugate vaccines have undergone clinical evaluation: PRP-D (Connaught Laboratories), PRP-T (Pasteur-Merieux), PRP-OMPC (Merck Sharp and Dohme), and HbOC (Praxis Biologicals). Each of these vaccines is distinguished by its carrier molecule, the size of the hapten saccharide, the type of linkage between hapten and carrier, and the ratio of polysaccharide to protein. These physicochemical differences between the vaccines influence their immunogenicity. Additionally, dose variation, such as the age at which the primary series of two or three immunizations is given and the interval between each dose of vaccine, may affect the level of anti-

body response and/or the protection provided.

The concentration of vaccine-induced serum antibody against Hib needed for protection is not precisely known. The issue is complicated as similar concentrations of antibodies may vary in functional activity (34). The maintenance of a threshold concentration of serum anti-PRP antibody may not be necessary if immunized children are primed for an effective booster response on exposure to Hib.

Efficacy studies have shown that PRP-D, HbOC, PRP-OMP, and PRP-T can prevent more than 90% of Hib disease (1,33,35,37). However, PRP-D proved to be ineffective in preventing Hib disease in an efficacy trial among Alaskan children (38). Thus, the choice between the conjugate vaccines may be critical only in populations in which infection pressure is very high, and the age of greatest incidence of disease is low. In these populations, conjugate vaccines with high immunogenicity at the first dose may be the best to use (39), so that the youngest children at the highest risk are afforded some protection at the earliest opportunity. In most populations, the choice of vaccine has to be based on other factors, the most important of which is cost.

Conjugate Vaccination and Hib Carriage

After a 4-year national immunization program for infants in Finland, Takala et al. (40) reported that Hib colonization was less prevalent among 327 children vaccinated with PRP-D vaccine than among 398, previously studied, unvaccinated children (0% vs 3.5%). The children studied were healthy 3-year-olds, from whom throat swabs were taken during a well-child visit to their local health center. However, the control cultures were obtained before the nationwide vaccination study; thus temporal factors other than vaccination may have influenced the results. Geographic factors necessitated sending swabs by mail to the center of sample processing, which may have limited the sensitivity of the microbiologic assay. Additionally, possible exposure to the bacterium from contacts was not measured.

In child care centers in Dallas, a prospective study was done to determine the prevalence of Hib colonization between 1987 and 1989 (41). During this period, conjugate vaccination was introduced in the United States, so both vaccinated and unvaccinated children attended the centers. Of 283 children studied, 59 had received unconjugated polysaccharide vaccine, and 89 had received conjugate vaccine (of which 94% received PRP-D). The Hib acquisition rates over the surveillance period were 21% and 9%, respectively. Among children exposed to at least one child with a positive culture result, the efficacy of conjugate vaccination to prevent Hib colonization in an unmatched analysis was 64%. No effect on colonization was found with PRP vaccination.

A clinic-based study in metropolitan Atlanta found a decreased Hib carriage rate in a population of 2- to 5-year-old children, 75% of whom were vaccinated with an Hib conjugate vaccine (42), and in Apache and Navajo Indian children who had received the Hib-OMPC vaccine appropriate for their age (43). These studies were performed after vaccination programs had begun, when adequate control populations were not available, and logistic factors often necessitated suboptimal microbiologic procedures.

The district by district implementation of a conjugate vaccine (PRP-T) in the Oxford Region in England enabled examination of a conjugate vaccine's effects over time in contemporary groups of vaccinated and unvaccinated infants (44). The children were recruited at birth so as to minimize selection bias concerning Hib carriage. Sampling error was limited, as one person took all the throat swabs, and laboratory analysis was standardized and took place in a single center. Other factors that could have influenced exposure to Hib or susceptibility to carriage were subjected to statistical control. Infants who had received Hib conjugate vaccine at the ages of 2, 3, and 4 months had significantly lower Hib acquisition rates than controls. In accordance with this, the point prevalence of Hib carriage was consistently lower in vaccines than in controls at 6, 9, and 12 months of age. The rates of acquisition and the period prevalence of *H. influenzae* serotypes e and f did not differ between vaccines and controls.

How Does Conjugate Vaccine Against Hib Affect Carriage?

The unconjugated Hib polysaccharide vaccine raises the IgA antibody concentration in nasal secretions and saliva of both adults and children (45). However, it does not affect the oropharyngeal carriage of Hib (31). The same lack of effect on pharyngeal carriage has been seen with other parenterally administered polysaccharide vaccines: meningococcal group A and C and several serotypes in the *Streptococcus pneumoniae* polysaccharide vaccine (47,48).

Conjugate vaccines induce higher concentrations of serum anti-PRP antibodies in young children than polysaccharide vaccines. It has been suggested that high serum concentrations might lead to passive transudation of IgG antibodies to mucosal surfaces (49). In an infant rat model, the serum IgG antibody concentration needed for an effect on Hib

colonization was 7 µg/mL (50). More recent research has found that the presence and concentration of IgG anti-PRP antibodies in saliva correlated with the concentration in serum after conjugate vaccination (51). Secretory IgA anti-PRP antibody was found in the saliva of children who had no similar, detectable serum IgA. This suggests that the anti-PRP IgG in saliva was derived from serum, whereas the IgA antibodies were locally produced. It seems that conjugate vaccines can induce a mucosal IgA response as well as a systemic IgG response, and while there is some evidence that high serum levels of IgG are relevant to the prevention of acquisition of carriage, the role of IgA remains unclear.

A decline in the serum, and thus perhaps mucosal, anti-PRP antibody concentration after primary immunization offers a simple explanation for the time-dependent effect of conjugate vaccines on acquisition of Hib. The geometric mean titer (GMT) of serum anti-PRP antibody in children in Oxfordshire, at 5 months of age, soon after completing a course of Hib immunization, was 5 µg/mL (52). The GMT in these children at 12 months of age was 0.83 µg/mL (53). A concentration of 1µg/mL after immunization with Hib polysaccharide vaccine has been accepted as the concentration associated with long term protection against invasive Hib disease (54). Acquisition of Hib and prolonged Hib carriage may occur only below a threshold concentration of serum or mucosal antibody.

How specific antibody contributes to the demise, or inhibits the attachment of Hib in the pharyngeal mucosa remains unresolved. Possible mechanisms include antibody-mediated opsonophagocytosis, direct bactericidal activity, or stereotactic inhibition. However, simple antibody-mediated bactericidal mechanisms may not completely explain the modulation of colonization in vaccinated persons. Carriage was not rapidly curtailed when conjugate vaccine was administered to current Hib carriers (44). This result is not easily explained, although the intracellular sequestration of Hib (55) may render a source of organisms inaccessible to antibodies in secretions. It is also possible that conjugate vaccine does not add significant antigenic stimulus in a child carrying Hib, with Hib antigen present in the mucosa.

Vaccines against Hib intervene in the normal relationship between Hib and host by increasing serum and/or mucosal anti-PRP antibody concentrations in the host before the host has any exposure to the bacterium. One could speculate that the primary role of vaccines in limiting Hib colonization is to prevent the acquisition of organisms. Secondarily, a large boost in the antibody concentrations, caused by mucosal contact with Hib in an immunized host, could lead to the more rapid elimination of Hib from the mucosa. There is also tenuous evidence of decreased Hib colony counts in cultures from colonized, vaccinated children (28).

In ecologic terms, Hib may precariously occupy the pharynx. A small influence that upsets the ecology in vaccinated persons may have a profound effect on the population kinetics of Hib. A rise in the concentration of serum and mucosal anti-PRP antibodies in infants could be enough to dramatically affect the pattern of Hib transmission. It seems likely that this effect would initially be greatest in places of close contact between children: families and child care institutions. Subsequently, the effect would become evident in a more widespread population.

The paucity of data on the ecologic behavior of Hib in the pharynx, the influence of conjugate vaccines on this behavior, and the importance of this influence, have prompted attempts at mathematical modeling to predict the population kinetics of Hib carriage and disease after conjugate vaccination. These models remain in their infancy but are awaited with interest.

Because they differ in biochemical composition and immunogenicity, the conjugate vaccines may vary in their long-term protective efficacy and effect on Hib carriage and transmission. Additionally, differing immunization regimens in populations may affect any generation of herd immunity. For example, administering a booster dose of conjugate Hib vaccine after the age of 12 months may prolong the presence of high concentrations of anti-PRP antibody in children, thereby enhancing the potential to limit transmission of Hib in the population.

In their relationship with the human host, *Neisseria meninigitidis*, *S. pneumoniae*, and Hib have numerous and close parallels. With the use of principles similar to those applied to Hib vaccines, conjugate vaccines against these other encapsulated pathogenic bacteria are now being developed and tested. The findings in studies of Hib may be a paradigm for the effect of conjugate vaccines on colonization by *N. meninigitidis* and *S. pneumoniae.* It will be important to examine this possibility closely as the vaccines become available.

The effect of conjugate vaccines on Hib carriage has been established in epidemiologic terms, but molecular knowledge about vaccination and mucosal immunity is limited. Much remains to be learned about the interaction between host and bacterium at the mucosal surface and about the contribution of conjugate vaccines to this complex relationship.

Dr. Barbour, a Rhodes Scholar from Western Australia, received a D.Phil. at Oxford University with work on the influence of conjugated Hib vaccine on the oropharyngeal carriage of Hib. She is currently training in clinical pediatrics at the John Radcliffe Hospital in Oxford.

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Application of Molecular Techniques to the Diagnosis of Microsporidial Infection

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Microsporidia are now recognized as important pathogens of AIDS patients; the ability of these parasites to cause disease in immunocompetent persons is still being elucidated. Improved diagnostic tests for microsporidial infection are continually being sought for establishing diagnosis in order to avoid laborious electron microscopy studies that require invasively acquired biopsy specimens. Modified trichrome or chemofluorescent stains are useful for detecting microsporidia in bodily fluids and stool specimens, but they do not allow for speciation of microsporidia. Polymerase chain reaction with specific primers will allow the detection and speciation of microsporidia in biopsy tissue, bodily fluids, and stool specimens.

Microsporidiosis is truly an emerging infectious disease. Although microsporidia were discovered more than 100 years ago (1), the first well-documented case of human microsporidiosis was not reported until 1959 (2). Human microsporidiosis remained an uncommon infection until the human immunodeficiency virus (HIV) pandemic; the first cases of microsporidial infection in HIV-infected patients were reported in 1985 (3,4). Since then, more than 400 HIV-associated microsporidial infections have been reported, which indicates that microsporidia are common opportunistic pathogens in patients with AIDS (5). Infections caused by three new species, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *E. hellem*, have been described in patients with acquired immunodeficiency syndrome (AIDS). Originally thought to be an opportunistic pathogen of AIDS patients exclusively, *E. bieneusi* was reported in 1994 to be the cause of acute, self-limited traveler's diarrhea in an immunocompetent person who was not infected with HIV (6).

As microsporidia have been increasingly recognized as pathogens of both immunosuppressed and immunocompetent persons, the need for rapid and specific diagnosis of microsporidial infection has arisen. For instance, albendazole therapy can cure *E. intestinalis* infections but is reported to be of little benefit to patients infected with *E. bieneusi* (5); therefore, identifying microsporidia to species level could have important implications in the clinical management of patients. Current diagnostic methods for microsporidia infection, though continually being improved, have shortcomings that molecular diagnostic techniques may be able to overcome.

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Biologic Properties of Microsporidia

Microsporidia are obligate intracellular parasites that infect most invertebrates and all classes of vertebrates. They belong to the phylum *Microspora* in the subkingdom *Protozoa*. They are considered ancient eukaryotes that multiply by binary fission and have a membrane bound nucleus; they lack mitochondria and Golgi membranes (5). In addition to causing disease in humans, these parasites cause disease in insects, mammals, and fish; therefore, they may create economic headaches for industries such as fisheries and silk production. They may also be beneficial to humans as biologic control agents for such pests as grasshoppers and locusts (5).

In host cells, microsporidia replicate either in a parasitophorous vacuole, like the members of the genus *Encephalitozoon*, or directly in the cytoplasm like *E. bieneusi*. Vegetative and spore stages of the organisms can be found in the host cell as the parasite undergoes merogony and sporogony, resulting in the production of the infective spore stage of the parasite (7) (Figure 1). The spores of microsporidia contain the uniquely characteristic coiled polar tubule (Figure 2). Under appropriate conditions within a suitable host, the polar tubule of the spore is extruded (Figures 3 and 4). Contact of the end of the tubule with a host cell membrane allows the spore to transfer its contents (sporoplasm) to initiate infection within the new host cell. An influx of calcium into the spore coincides with the extrusion of the polar tubule, and this mechanism may provide a target for therapy for microsporidiosis since calcium

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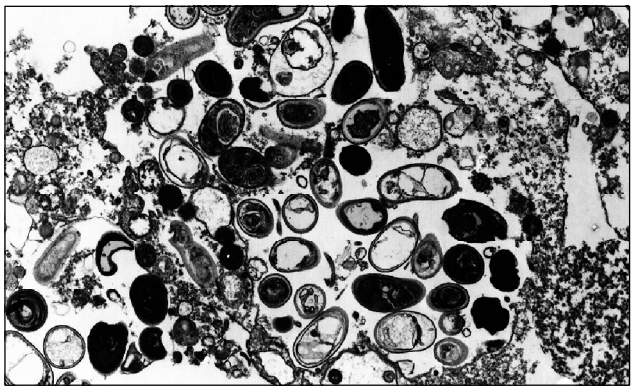

Figure 1. Transmission electron micrograph of a host cell from cell culture parasitized by *Encephalitozoon intestinalis*. Both vegetative forms and spores can be observed inside the parasitophorous vacuole. Original magnification, x5,200.

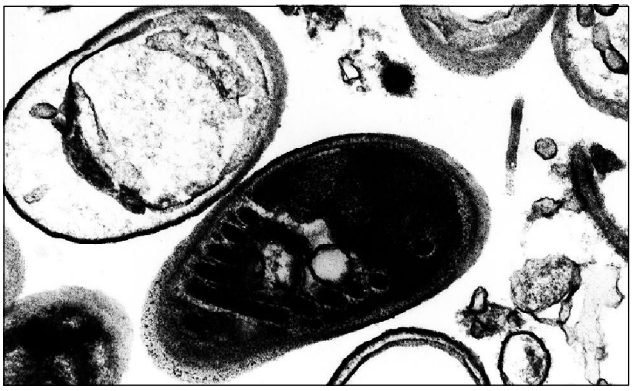

Figure 2. Transmission electron micrograph showing the coiled tubule within an *Encephalitozoon intestinalis* spore from cell culture. This unique structure is diagnostic of microsporidial spores. Original magnification, x28,500.

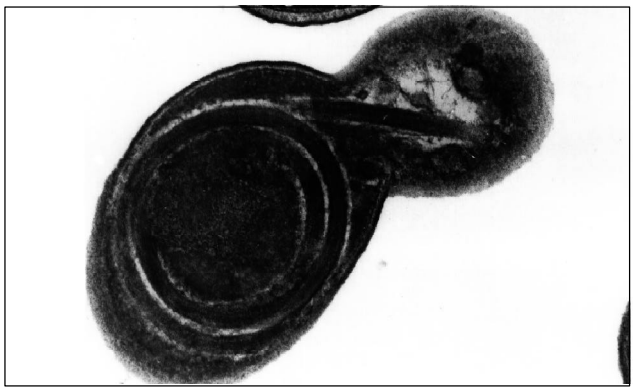

Figure 3. Transmission electron micrograph of a cell culture-derived *Encephalitozoon intestinalis* spore showing the polar tubule in the process of being extruded. The coiled arrangement of the tubule within the spore is clearly demonstrated. Original magnification, x39,000.

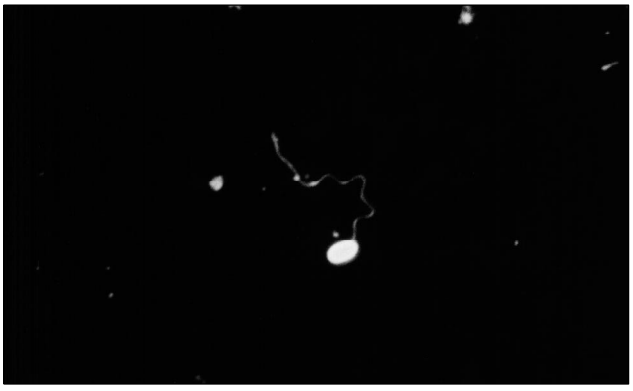

Figure 4. *Encephalitozoon hellem* spore from cell culture showing the extruded polar tubule stained by indirect fluorescence antibody by using rabbit polyclonal antibody and FITC-labeled anti-rabbit antibody. Original magnification, x1,000.

target for therapy for microsporidiosis since calcium channel blockers have been shown to inhibit extrusion and the infection of host cells in vitro (8).

The human microsporidial pathogens and their clinical manifestations are shown in Table 1. The most common microsporidial disease is prolonged diarrhea with wasting caused by *E. bieneusi* or *E. intestinalis* in AIDS patients with CD4 T-cell counts below 50 cells/ul. Microsporidia have been reported in up to 39% of AIDS patients with diarrhea (9). Microsporidia may disseminate to cause systemic infection (Table 1); these organisms have been observed in urine, bile, and duodinal aspirates, as well as in ocular, sinus, bronchial, renal, hepatic, and other tissue (5). *E. hellem*, which primarily causes eye infections, has been exclusively found in AIDS patients. Ingestion and inhalation of spores have been suggested as likely modes of transmission for microsporidia (5,10). Biochemical, immunologic, and molecular studies performed on *E. cuniculi* isolates from mice, rabbits, and dogs indicate that the parasite can be classified into at least three strains (11). An evaluation of the immunologic and molecular characteristics of *E. cuniculi* isolated from humans and rabbits indicated that the isolates were an identical strain, thus suggesting that this microsporidion is a zoonotic parasite (12).

Traditional Diagnostic Methods

Transmission Electron Microscopy

Definitive diagnosis of microsporidial infection relies on observating microsporidia in biopsy tissue, bodily fluid specimens (e.g. urine, sinus aspirates, bile, cerebral spinal fluid), or stool examined by transmission electron microscopy (TEM). Microsporidia can be identified to genus or even species level on the basis of morphologic character-

Table1. Named human microsporidial pathogens and their clinical syndromes

Microsporidia genus and species	Clinical syndromes
Pleistophora species	Myositis
Nosema connori	Disseminated infection
N. ocularum	Keratitis
Vittaforma corneae	Keratitis
Encephalitozoon cuniculi	Peritonitis, fulminant hepatitis, seizures, rhinosinusitis
E. hellem	Conjuctivitis, keratoconjunctivitis, bronchiolitis, pneumonia, rhinosinusitis, disseminated infection
E. intestinalis	Diarrhea, disseminated infection
Enterocytozoon bieneusi	Diarrhea, wasting syndrome, cholecystitis, cholangitis, bronchitis, pneumonia

Source: ref. 5.

istics. All stages of the parasite's life cycle can be found in infected tissue, but only the spore stage can be observed in bodily fluids and stool. The size and ultrastructure of spores, particularly the configuration of the coiled tubule, distinguish *E. bieneusi* from the *Encephalitozoon* species (Figures 5 and 6). Limited success was reported when TEM was used to detect spores in bodily fluids and stool specimens to avoid obtaining specimens by invasive procedures (5). Tissue specimens are required for speciation within the *Encephalitozoon* genus because the meronts and sporonts, nuclear configuration, and location of replication in the host cell must be carefully studied (7,10). Problems with the use of TEM include its lack of sensitivity when performed on bodily fluids and stool, the requirement for invasive procedures to acquire biopsy specimens, and the laborious procedures for specimen preparation and examination.

Light and Fluorescence Microscopy

Histologic examination of biopsy specimens allows diagnosis of microsporidial infection but not genus or species identification of the parasites. Tissue stains used to detect microsporidia include hematoxylin and eosin, Gram, Giemsa, Warthin-Starry, and chromotrope 2R modified trichrome stains (13,14). The small size of the organisms and the lack of a noticeable tissue inflammatory response make microsporidia detection difficult (5). Once again, the invasive procedure required for obtaining specimens and the length of time needed for processing them are major drawbacks.

Diagnosing microsporidial infections by light microscopy examination of noninvasively acquired specimens has been a challenge for laboratorians. Microsporidial spores are not observed in traditional ova and parasite examinations, and the parasites are generally overlooked in Gram-stained preparations of stool samples because their size, shape, and staining characteristics are similar to those of many enteric bacteria. Weber's modification of the trichrome stain has allowed more definitive identification of microsporidia by light microscopy (15). With this stain, microsporidial spores appear bright pinkish-red, and most have a distinctive diagonal or equatorial line that allows microsporidia and bacteria to be easily differentiated (Figure 7). The

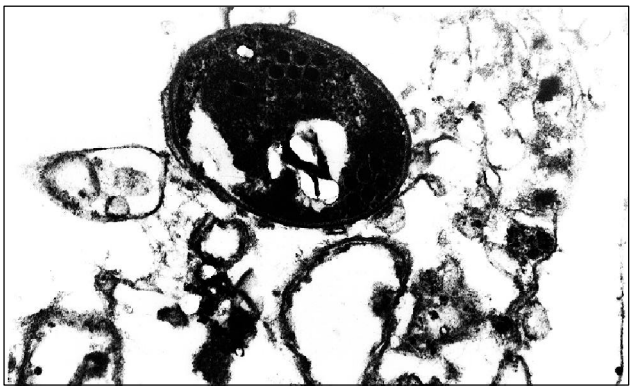

Figure 5. Transmission electron micrograph of an *Enterocytozoon bieneusi* spore in a stool specimen showing the characteristic arrangement of the polar tubule in six turns with two tiers. Original magnification, x28,500.

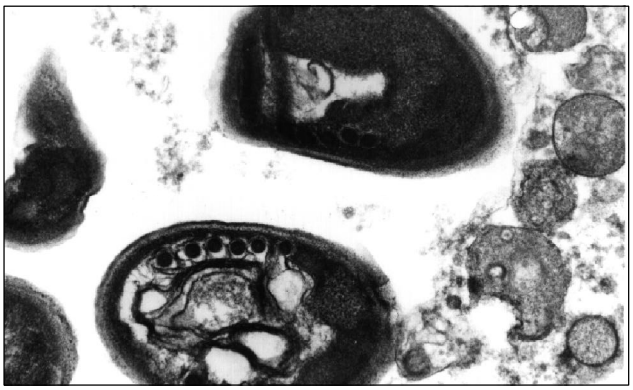

Figure 6. Transmission electron micrograph of an *Encephalitozoon intestinalis* spore from cell culture showing the polar tubule with four to seven coils in single rows, which is typical of the genus *Encephalitozoon*. Original magnification, x28,500.

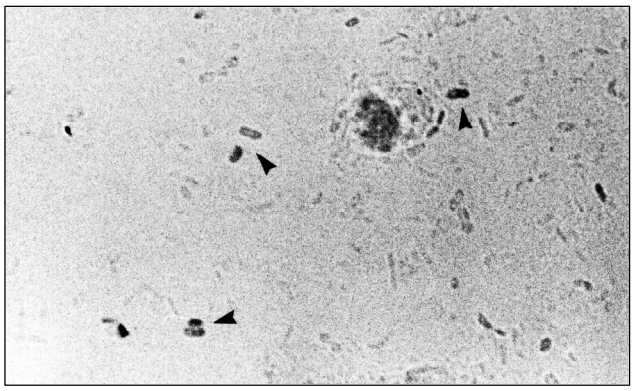

Figure 7. Spores of *Encephalitozoon intestinalis* in a stool specimen stained with modified trichrome stain. Note the characteristic equatorial line in one of the spores. Original magnification, x1,000.

sensitivity and specificity of the modified trichrome stain have not been well established. When results of the modified trichrome stain performed on stool specimens and a TEM examination of duodenal biopsy specimens from HIV-infected patients were compared, *E. bieneusi* spores were detected in stool specimens in 9 of 9 patients with moderate or abundant parasites in tissue and in 8(57%) of 14 stool specimens from patients whose tissue contained few parasites (16). This illustrates that microsporidial spores may not be observed in the stool of some infected patients with the modified trichrome stain.

Various chemofluorescent brighteners (such as calcofluor white and Uvitex 2B) bind to the endospore layer of microsporidia and allow spores to be detected quickly and easily in smears of specimens examined with a fluroescence microscope (5). Fluorescein-labeled polyclonal and monoclonal antibodies are being developed for the detection and speciation of microsporidia (5,17). Didier et al. (17) recently compared modified trichrome, calcofluor white, and a fouorescent polyclonal antibody stain and found that the polyclonal antibody stain was the least sensitive method for detecting microsporidia in stool, urine, and duodenal lavage specimens. They proposed screening specimens with calcofluor white and confirming positive smears by using a modified trichrome stain. However, modified trichrome and calcofluor white stains do not allow for speciation of microsporidia; therefore, species-specific antibodies should be used to provide definitive identifications. *E.intestinalis*-specific monoclonal antibodies have been used in an immunofluorescence assay for detecting spores in urine, stool, bronchial brush biopsy specimens, bronchoalvolar lavage fluid, and samples obtained from nasal swabs (18). *E. bieneusi*-specific antibodies may not be developed until the organism is successfully cultivated in long-term culture.

Cell Culture

Microsporidia have been isolated from a variety of specimen types and in a variety of cell lines (5). *E. hellem*, *E. intestinalis*, and *Vittaforma corneae* have been isolated from human specimens and maintained in continuous culture. Recently, *E. bieneusi* has been cultivated on a short-term (6 months) basis in vitro (19). Detecting microsporidia in infected cell cultures may take 3 to 10 weeks (19,20). Isolating microsporidia in cell culture as a means of diagnosing infection is laborious and lengthy and is prone to failure with specimens from nonsterile sites. Therefore, cell culture is not recommended as a routine laboratory technique for diagnosing microsporidiosis.

Serology

Serologic assays used to detect antibodies to microsporidia in human sera include immunofluorescence, immunoperoxidase, enzyme-linked immunosorbent assay (ELISA), and Western blot (21-23). The sensitivity and specificity of these methods for detecting antimicrosporidial antibody are not known because no comparative evaluations have been published. Studies have demonstrated increased rates of seropositivity to *E. cuniculi* in persons who live in tropical regions and have tropical diseases (21,24). Most notable were *E. cuniculi* seropositivity rates of 4.7% in patients with malaria and 9.1% in patients with schistosomiasis (24). A study of homosexual men in Sweden found that 10 (33%) of 30 were seropositive for antibodies to *E. cuniculi*, and all seropositive patients plus half of the seronegative patients had sometimes visited tropical countries (25). Studies to detect microsporidial antibodies in HIV-infected and non-HIV-infected patients have demonstrated that AIDS patients can mount an immune response to microsporidial infection; however, serologic methods are not useful as diagnostic tools because these studies have found that at least half of the serum specimens from persons without a history of microsporidial infection had positive titers (22,23). Some of the problems with serologic testing include the poor response to antigen challenge in immunosuppressed persons (26), the probability that different (both pathogenic and nonpathogenic) microsporidia contain common cross-reactive antigens (23,26), and the lack of species-specific reagents in part because *E. bieneusi* cannot be grown in continuous culture.

Molecular Techniques

Characterization of the microsporidial genome has focused on the small subunit ribosomal RNA

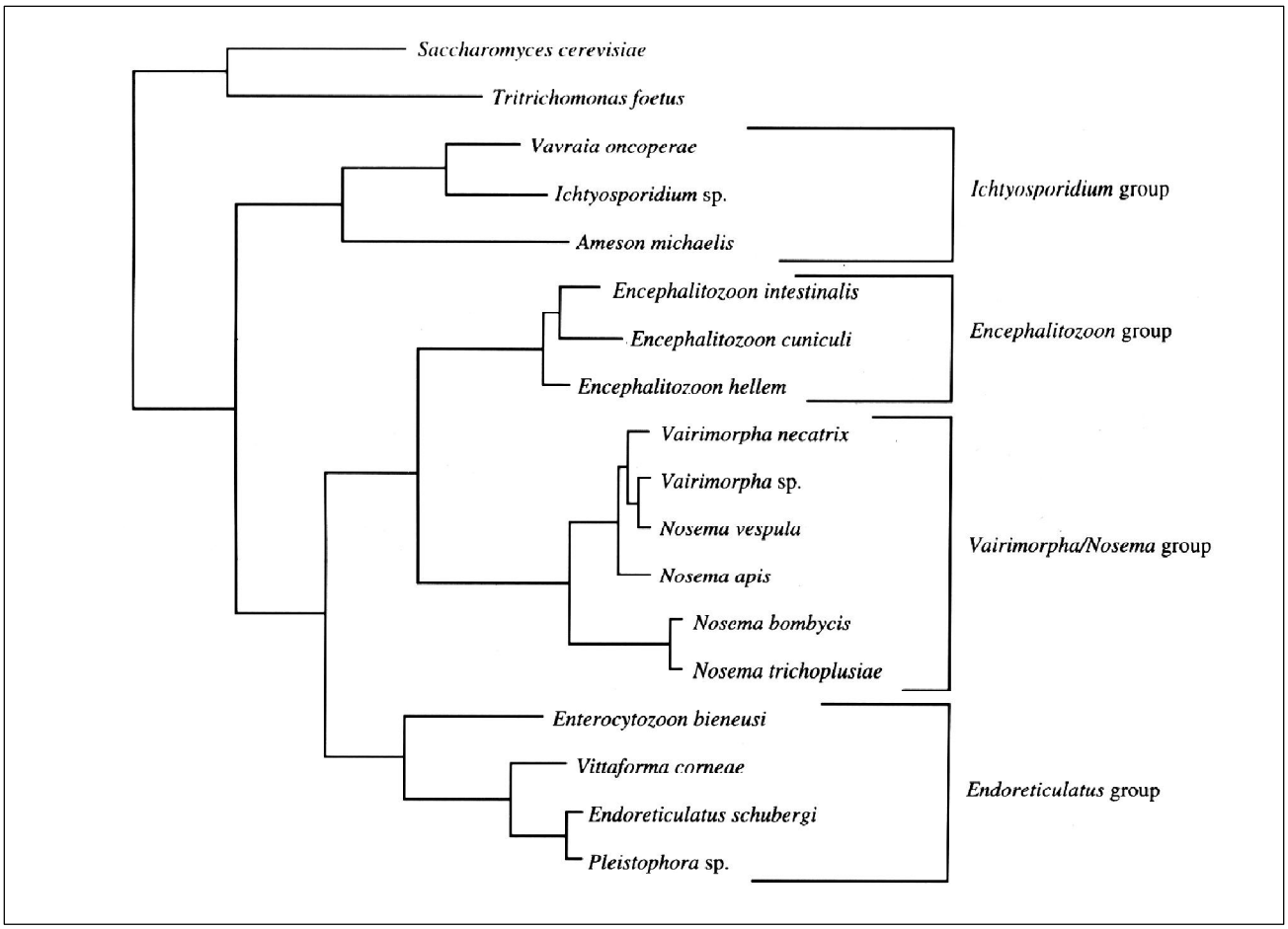

Figure 8. Cladogram representing the phylogenic relationship of several microsporidial genera as determined by small subunit ribosomal DNA sequence similarity. The human pathogens can be found in the *Encephalitozoon* group and the *Endoreticulatus* group. (Reprinted with permission from ref. 35.)

(SSU-rRNA) gene. The sequence of the SSU-rRNA gene of the microsporidium *Vairimorpha necatrix* was published by Vossbrinck et al. in 1987 (27). The *V. necatrix* SSU-rRNA gene was far shorter than a typical eukaryotic SSU-rRNA gene and lacked several universal (and eukaryotic-specific) sequences. PCR amplification with primers complementary with conserved sequences of the *V. necatrix* SSU-rRNA gene has been used to generate sequence information from microsporidia that infect humans. SSU-rRNA gene sequences have been published for *E. cuniculi* (28,29,30,31), *E. hellem* (30,31), *E. intestinalis* (31,32), and *E. bieneusi* (31,33), and an unpublished sequence for *V. corneae* has been deposited into GenBank National Center for Biotechnology Information, National Institutes of Health, accession 0.5 U11046.

Taxonomic decisions concerning the microsporidia have been based historically on morphologic characteristics as established by TEM. These criteria are still valid for organisms with unique morphology and ultrastructure and were used to justify the reclassification of *Nosema corneum* to *Vittaforma corneae* (34). This reclassification was confirmed by a study of microsporidial phylogeny based on evaluation of SSU-rRNA gene sequences that indicated that *N. corneum* was more closely related to the insect parasite *Endoreticulatus schubergi* than to the other *Nosema* species (35) (Figure 8). Phylogenetic analysis of the sequences of the SSU-rRNA genes of microsporidia is often inconsistent with traditional classifications that are based on morphologic characteristics observed by TEM; this was demonstrated by the use of recent sequence data to determine the correct taxonomic placement of *E. intestinalis* (32,35). This organism was named *Septata intestinalis* because unique septations between spores within the parasitophorous vacuole were observable by TEM (36). Although the various stages of the microorganism throughout its life cycle are indistinguishable from those of *E. cuniculi*, the observation of septations

allowed the organism to be identified. The *E. intestinalis* SSU-rRNA gene contained sufficient unique sequences to establish this organism as an independent species but shared about 90% sequence homology with the other two characterized *Encephalitozoon* species, *E. cuniculi* and *E. hellem*.

Molecular techniques have confirmed the existence of different strains of *E. cuniculi* that have been isolated from mice, rabbits, and dogs (11). Three antigenically different strains were detected by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Western blot. Double-stranded DNA heteroduplex mobility, restriction fragment length polymorphism with the restriction endonuclease *Fok* I, and DNA sequencing were performed on the PCR products generated by using a set of primers to amplify the entire microsporidial SSU-rRNA gene and a second set of primers specific for *Encephalitozoon* species. These methods allowed three strains of *E. cuniculi* to be clearly separated. The existence of different strains of *E. bieneusi* was suggested after SSU-rRNA gene sequences of organisms from maxillary sinus mucosa had been compared with those from intestinal enterocytes (37). Sequencing errors in this study were minimized by analyzing multiple recombinant DNA clones. SSU-rRNA sequences from *E. bieneusi* and *Encephalitozoon* species derived from a variety of clinical specimens must be compared to establish the existence of intraspecific genetic variation in these organisms.

Molecular Diagnosis

Primer pairs that amplify the entire microsporidial SSU-rRNA gene sequence produce amplicons of approximately 1,550 base pairs in length from *Encephalitozoon* species and *E. bieneusi* (28,33). Although these primer pairs have proven useful for sequencing and taxonomic studies, the targets are too long to be efficiently amplified from clinical specimens in a diagnostic assay. Targets for diagnostic PCR that can be amplified efficiently usually range from 100 to 400 base pairs for formalin-fixed tissue or up to 700 to 1,000 base pairs in fresh specimens (38). Several primer pairs designed to amplify short regions (250 to 607 base pairs) of the SSU-rRNA gene, and their application in the diagnosis of microsporidial infection by PCR have been published (Table 2). Primers specific for *E. hellem* and *E. cuniculi*-specific- primers have been used to identify microsporidia cultured from patient specimens (39,40), but only *E. bieneusi* and *E. intestinalis* SSU-rRNA DNA have been amplified directly from patient specimens (31,33,41,42). A pair of *E. bieneusi*-specific primers amplified cloned *E. bieneusi* SSU-rRNA gene sequences but did not reliably amplify DNA from infected tissue (33). Not unexpectedly, therefore, some primer sets appear to be adequate for amplification from cultured organisms or cloned sequences but may not reliably amplify microsporidial DNA in patient specimens. The primer pair V1 and EB450 (Table 2) amplifies

Table 2. SSU-rRNA PCR primer pairs for the diagnosis of microsporidial infection

Primer pair (no. nucleotides)		Primer designation	Organisms amplified	Amplicon size in base pairs	Source of Target	References
5'-CACCAGGTTGATTCTGCCTGAC-3'	(22)	V1	*Enterocytozoon bieneusi*	348	Biopsied tissue	33, 41
					Duodenal aspirates	31
5'-ACTCAGGTGTTATACTCACGTC-3'	(22)	EB450	*Encephalitozoon hellem*		Cultured organisms	33
5'-GAAACTTGTCCACTCCTTACG-3'	(21)	EBIEF1	*E.bieneusi*	607	Bile	43
5'-CCATGCACCACTCCTGCCATT-3'	(21)	EBIER1			Duodenal aspirates	
5'-TGAGAAGTAAGATGTTTAGCA-3'	(21)		*E.hellem*	547	Cultured organisms	39
5'-GTAAAAACACTCTCACACTCA-3'	(21)					
5'-ATGAGAAGTGATGTGTGTGCG-3'	(21)		*Encephalitozoon cuniculi*	549	Cultured organisms	39, 40
5'-TGCCATGCACTCACAGGCATC-3'	(21)					
5'-CACCAGGTTGATTCTGCCTGAC-3'	(22)	V1	*Encephalitozoon intestinalis*	370	Biopsied tissue	31
5'-CTCGCTCCTTTACACTCGAA-3'	(20)	SI500				
5'-CACCAGGTTGATTCTGCCTGAC-3'	(22)	PMP1*	*E.bieneusi*	250	Stool	42
5'-CCTCTCCGGAACCAAACCTG-3'	(21)	PMP2	*E.cuniculli*	268	Cultured organisms	
			E.intestinalis	270	Stool	
			E.hellem	279	Cultured organisms	

*The nucleotide sequence of primer PMP1 is identical to that of primer V1.

E. bieneusi from TEM- confirmed infected tissue and *E. hellem* from cell culture (33). This primer pair has not, however, been evaluated for its ability to amplify *E. hellem* from patient specimens. Primer pair V1 and EB450 was extensively tested by da Silva, et al. (43), who found that the primer pair did amplify *E. bieneusi* DNA from some patient specimens that had evidence of the parasite by electron microscopy. In addition, the primer pair did not amplify DNA of *E. bieneusi* derived from a short-term culture. da Silva et al. (43) described a pair of highly specific PCR primers for amplifing *E. bieneusi*; these are called EBIEF1 and EBIER1 (Table 2), and are based on SSU-rRNA sequences they generated. This primer pair amplified *E. bieneusi* DNA from cultured organisms, cloned regions of the SSU-rRNA, and patient specimens, but did not amplify SSU-rRNA-coding regions of 13 other genera and species of microsporidia. The primer pair V1 and SI500 amplifies *E. intestinalis* from intestinal biopsy material confirmed by TEM as infected, but does not simplify *E. bieneusi*-infected tissue samples or *E. cuniculi* from cell culture (31). This primer pair has not been evaluated for its ability to amplify other microsporidia.

To avoid acquiring specimens by invasive procedures, PCR was used to detect microsporidia in stool specimens (42). Primer pair PMP1 and PMP2 (Table 2) allows *E. bieneusi* and the *Encephalitozoon* species (42) to be amplified and will also amplify *V. corneae* from culture (D.P. Fedorko, unpublished data). DNA is easily extracted from cultured organisms and biopsied tissue specimens, but extracting DNA from spores requires harsh conditions employing both mechanical and chemical disruption. A major problem with performing PCR on stool specimens is the presence of PCR inhibitors. Treating the stool specimens with sodium hypochlorite or 10% formalin before DNA extraction inactivates microorganisms in the stool and has the beneficial effect of inactivating Taq polymerase inhibitors.

Both DNA probes and restriction enzyme digestion have been used to confirm the identity of PCR amplicons (33,41,42). An internal 30 meroligonucleotideEB150 (5'TGTTGCGGTAATTTG-GTCTCTGTGTGTAAA-3'), complementary to a region of the amplicon produced by primer pair V1 and EB450, has been used to detect *E. bieneusi* by Southern blot (31,41). Probe EB150 has been reported, however, to hybridize with *E. hellem*, amplified by V1 and EB450, albeit at a lower intensity than *E. bieneusi* (31). PCR products amplified from stool specimens by using primers PMP1 and PMP2 have been digested with restriction endonucleases *Hae*III and *Pst*I to distinguish between infection with *E. bieneusi* and *E. intestinalis* (42). These restriction enzymes do not allow *E. intestinalis* to be differentiated from *E. cuniculi*, thus limiting their use to diagnosing gastrointestinal infection.

An efficient approach for the molecular detection of microsporidia in patient specimens would involve using universal or "pan-microsporidian" primers for amplification. The species of microsporidia detected could be determined by using restriction endonuclease digestion or DNA probe assays of the amplified DNA or repeat PCR with species-specific primers. Negative specimens would require no further evaluation. Primers PMP1 and PMP2 appear to be panmicrosporidian (42), but this primer pair needs to be evaluated for its ability to amplify microsporidian DNA from a wide range of clinical specimens. Didier et al. (44) have used pan-*Encephalitozoon* primers that amplified a product approximately 1,000 base pairs in length, which included a large portion of the SSU-rRNA gene and a small portion of the large subunit rRNA gene. They successfully amplified *E. hellem* DNA from urine and conjunctival specimens from a patient with AIDS. Southern blotting and a species-specific probe were used to identify the organism to species.

The application of molecular diagnostic techniques for microsporidiosis is in its infancy. There have been no published reports of comparisons of PCR to other methods to determine sensitivity and specificity. Careful selection of primers and probes coupled with highly stringent conditions will be required to detect and speciate microsporidia in patient specimens. The potential for PCR to identify species of microsporidia from non-invasively acquired specimens makes this technique an extremely attractive diagnostic option. Although PCR can be used for detection and speciation of microsporidia in patient specimens, screening for microsporidia by using chemofluorescent stains and modified trichrome stains followed by confirmation and speciation with PCR may become the paradigm for the laboratory diagnosis of microsporidiosis.

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Coccidioidomycosis: A Reemerging Infectious Disease

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Coccidioides immitis, the primary pathogenic fungus that causes coccidioidomycosis, is most commonly found in the deserts of the southwestern United States and Central and South America. During the early 1990s, the incidence of coccidioidomycosis in California increased dramatically. Even though most infections are subclinical or self-limited, the outbreak is estimated to have cost more than $66 million in direct medical expenses and time lost from work in Kern County, California, alone. In addition to the financial loss, this pathogen causes serious and life-threatening disseminated infections, especially among the immunosuppressed, including AIDS patients. This article discusses factors that may be responsible for the increased incidence of coccidioidomycosis (e.g., climatic and demographic changes and the clinical problems of coccidioidomycosis in the immunocompromised) and new approaches to therapy and prevention.

Emerging infectious diseases have been defined as "infections that have newly existed in a population or have existed but are rapidly increasing in incidence or geographic range" (1). In what sense is coccidioidomycosis an emerging infectious disease? Coccidioidomycosis is not a new disease; it was first recognized and reported slightly more than 100 years ago by a medical student in Argentina (2). In fact, coccidioidomycosis has affected inhabitants of the desert Southwest for thousands of years (3). However, in the past several years, the number of cases of coccidioidomycosis has increased dramatically, and the clinical symptoms of this illness have changed in patients with acquired immunodefficiency syndrome (AIDS). In this article, we explore some of the reasons for the increased incidence of coccidioidomycosis, review the new clinical data, and discuss current approaches to therapy and prevention.

Etiology

Coccidioidomycosis is caused by *Coccidioides immitis*, a dimorphic fungus that grows as a mold in the soil. The mold forms arthroconidia within the hypha, a type of conidia formation known as enteroarthric development (Figure 1) (4). *C. immitis* is the only species within the primary pathogenic fungi that has this type of conidia development. Alternate conidia undergo autolysis, leaving empty spaces between viable arthroconidia. The arthroconidia are released into the atmosphere when the wind ruptures the hypha. *C. immitis* infects humans and animals almost exclusively by the respiratory route (5). Once inhaled, the arthroconidia cluster in the lungs and undergo a dramatic morphologic change. The round cells, which develop into spherules, undergo repeated internal divisions until they are filled with hundreds to thousands of offspring, termed endospores. This

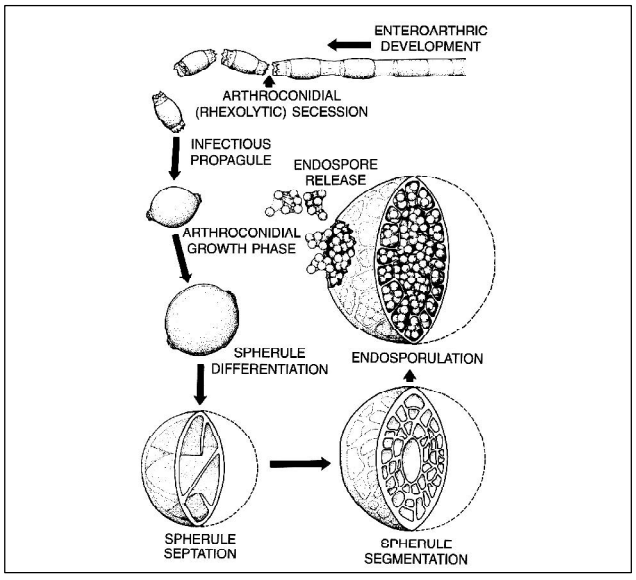

Figure 1. The dimorphic life cycle of *Coccidioides immitis*.

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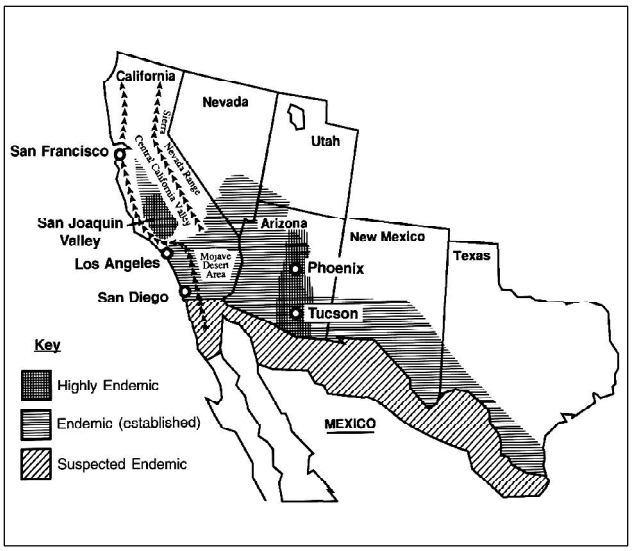

Figure 2. The geographic distribution of coccidioidomycosis. Cross-hatching indicates the heavily disease- endemic area, single hatching, the moderately disease- endemic area.

process occurs over 48 to 72 hours (6). When the spherule ruptures, each released endospore has the capacity to develop into a mature spherule.

Epidemiology

C. immitis is primarily found in desert soil. It is present in highest numbers in the San Joaquin Valley in California, southern Arizona, southern New Mexico, west Texas, and the desert areas of northern Mexico (Figure 2). The organism is also found in scattered foci in coastal southern California, southern Nevada, and Utah (7) and is endemic in a few areas in Central and South America, especially in Venezuela (7). *C. immitis* is distributed unevenly in the soil and seems to be concentrated around animal burrows and ancient Indian burial sites (8,9); it is usually found 4 to 12 inches below the surface of the soil (7).

Since *C. immitis* infects humans by the respiratory route, exposure to dust is one critical factor determining the risk for infection (10). Coccidioidomycosis is not spread from person to person, except in extraordinary circumstances. Coccidioidomycosis probably had its most profound effect on the population of the United States during World War II when several training airfields were built in the San Joaquin Valley. The rate of new infections in military personnel was 8% to 25% per year (10). Coccidioidomycosis was the most common cause of hospitalization at many airbases in the Southwest. Though the death rate was very low, many soldiers were sick for weeks to months, and their training was completely disrupted. At least in part because of efforts to minimize dust, the infection rate declined as the war went on (10).

The incidence of coccidioidomycosis varies with the season; it is highest in late summer and early fall when the soil is dry and the crops are harvested (10). If it rains at this time of the year (which is unusual in southern California), disease incidence declines as the amount of dust decreases. Dust storms are frequently followed by outbreaks of coccidioidomycosis. One particularly severe dust storm in 1977 carried dust from the San Joaquin Valley up to the San Francisco Bay area and resulted in hundreds of cases of nonendemic coccidioidomycosis in areas north of the San Joaquin Valley (11). More recently, an earthquake centered in Northridge, California, was associated with 170 cases of acute coccidioidomycosis in Ventura County, which normally has a low incidence of this disease. The airborne dust associated with landslides triggered by the earthquake was implicated in the increase in the number of cases (12).

Occupational or recreational exposure to dust is also an important consideration. Agricultural workers, construction workers, or others (such as archeologists) who dig in the soil in the disease-endemic area are at increased risk for the disease (13,14). During World War II, C. E. Smith, one of the most perceptive and influential epidemiologists to study coccidioidomycosis, recommended dust control as a primary measure to reduce risk for exposure (10). However, because the desert is inherently dusty, many cases of coccidioidomycosis are acquired just by driving through the disease-endemic area.

Clinical Illness

C. immitis is transmitted by the respiratory route. Smith et al., in a prospective study of cases of coccidioidomycosis acquired during World War II by soldiers at three San Joaquin Valley airbases, skin-tested the airmen periodically and questioned them about illnesses in the interval. They found that most infections (60%) were asymptomatic and resolved spontaneously; 15% were not severe enough to require medical care, and 25% were clinically important and required a substantial amount of time off work (15). In symptomatic patients, the pulmonary illness ranges from a self-limited flulike illness to pneumonia (16). Approximately 5% of primary infections result in erythema nodosum or erythema marginatum with associated noninfectious arthritis; most of those patients have a self-limited infection (17). Particularly in persons with diabe-

tes, multiple thin-walled chronic cavities tend to develop as a residual effect of pulmonary coccidioidomycosis (18). Unlike in tuberculosis, in coccidioidomycosis, dissemination almost always becomes evident within a few weeks of the primary pneumonia, although in cases of limited dissemination it may not become clinically evident until months later (15,19). Coccidioidomycosis can disseminate and cause miliary disease, bone and joint infection, skin disease, soft tissue abscesses, and meningitis (15,16). These extrapulmonary complications are uncommon (<5% of infections).

The risk for disseminated coccidioidomycosis is much higher among some ethnic groups, particularly African-Americans and Filipinos. In these ethnic groups, the risk for disseminated coccidioidomycosis is tenfold that of the general population (5,20). Presumably, a gene (or genes) that increases susceptibility to infection is more prevalent in these ethnic groups than in the general population. Such a resistance gene has been identified in mice (21-23), but not yet in humans. The mechanism by which the resistance genes affect the course of the disease in mice is not clear. Pregnant women and the immunosuppressed are also at high risk for developing disseminated disease (Figure 3) (24). One study demonstrated that the growth rate of spherules was influenced by human sex hormones, which may partially account for the increased risk of disseminated disease in pregnancy (25). Pregnancy also redirects the immune response toward humeral (T_H2) immunity and away from delayed hypersensitivity (T_H1) (26), which may influence resolution of coccidioidomycosis. Generalized suppression of cell mediated immunity also increases the risk of disseminated disease (27). Coccidioidomycosis is particularly severe in patients with organ transplants or AIDS.

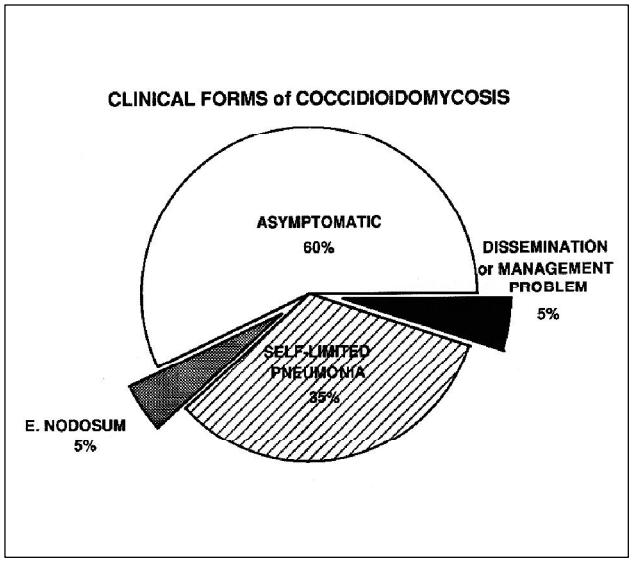

Figure 3. The most common clinical presentations of coccidioidomycosis in immunocompetent patients (16).

Though disseminated coccidioidomycosis is uncommon, and symptomatic coccidioidal pneumonia usually resolves without therapy, many of these patients are very ill for weeks to months. Galgiani reported that a group of college students in Tucson who had coccidioidomycosis required an average of six clinic visits before the disease resolved (16). Therefore, this can be an expensive illness in terms of medical costs and time lost from work or school, even when the infection resolves spontaneously.

Coccidioidomycosis Epidemic in California

Kern County, in the San Joaquin Valley, California, is one of the most highly coccidioidomycosis-endemic regions. The number of new cases of coccidioidomycosis in the area has varied widely from year to year; a low incidence of coccidioidomycosis from 1987 to 1990 (<500 reported cases a year in Kern County), was followed by a high incidence from 1991 to 1994 (28-30). The number of reported cases, which were identified by serologic testing at the Kern County Health Department (the reference serology laboratory for the county), probably represent approximately 10% of the total number of infected persons in that county (Figure 4) (28). The medical costs for infected persons in Kern County are estimated at $66 million (29). In 1992, 4,500 new cases were reported to the California State Department of Health Services (30), most from Kern County; the number of new cases also increased in almost all counties in central and southern California (30). The increase in reported cases in California in 1991-92 was dramatic but certainly an underestimate of the magnitude of the problem (31).

The epidemic seems to be waning, for reasons that are not clear, but the marked increase in incidence from the 1980s to 1991 through 1993 is indisputable. What factors may account for this increase? One major consideration is the weather. C. E. Smith observed years ago that the number of cases of coccidioidomycosis was higher in the summer after a rainy winter than after a dry winter (10). In March 1991, a 5-year drought in California ended with a heavy rainfall. Rainfall was also heavy in the winters of 1992 and 1993. Though the relationship between the weather and the density of *C. immitis* in the soil may never be understood in detail, the following scenario seems plausible. During drought years, the number of organisms competing with *C. immitis* decreases. *C. immitis* does not thrive, but it remains viable though dormant. After

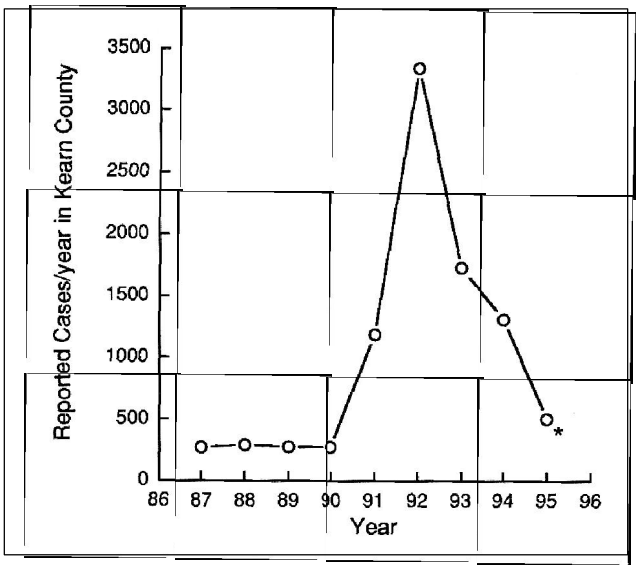

Figure 4. The number of new cases of coccidioidomycosis identified by serologic testing at the Kern County Public Health Laboratory (source of data: Dr. Ron Talbot). The asterisk indicates a projected number.

heavy rain, the arthrocondia germinate and multiply to a higher density than usual because of the lack of competing organisms. Once the soil dries in the late summer and fall, the arthroconidia become airborne and potentially infectious (29).

Another reason for the sudden increase in disease incidence might have been the number of susceptible persons in the disease-endemic area. The number may have been the result of both increased migration of susceptible persons and decreased immunity in the indigenous population. Immunity comes from prior infection and is manifest as a positive coccidioidin skin test. In almost all cases, coccidioidomycosis confers lifelong immunity. As a result of years of low incidence, the number of nonimmune persons may have increased, as evidenced by the decrease in prevalence of positive coccidioidin skin tests among local high school students. In 1939, 50% to 60% of high school students in the San Joaquin Valley had positive skin tests (17), but in the 1980s only 3% to 5% of high school students had positive skin tests (T. Larwood, pers. comm.). Given the historical data, this estimate seems low, but another study also found a low prevalence. In 1985, workers in Tucson estimated that 30% of a random sample of persons in a Hispanic neighborhood had positive skin tests (32). In addition to the drought, irrigation of fields, the increasing amount of land under cultivation, and a decrease in indoor dust due to the widespread use of air conditioning may also have played a role in the relatively low incidence of infections in the 1980s.

Coccidioidomycosis in the Immunosuppressed

C. immitis is a primary pathogen that can cause disease in immunologically healthy persons. In the population as a whole, fewer than 5% of infected persons have persistent pulmonary infection or extrapulmonary dissemination of the disease (16). The incidence of clinically significant disease in immunosuppressed patients is much higher. In one study symptomatic coccidioidomycosis developed in 18 (7%) of 260 renal transplant patients in Arizona over a 10-year period, primarily in the first year after transplantation (33). This rate was substantially higher than the rate of infection in patients who were undergoing hemodialysis. Approximately 12 (67%) of infections in the patients with renal transplants were disseminated; the remainder were confined to the lung. Of patients with disseminated disease, 10 (83%) died, despite intensive therapy with amphotericin B. In another study from Tucson, all confirmed cases of coccidioidomycosis during a 4-year period were reviewed. The dissemination rate was 8 (73%) of 11 of patients who were receiving immunosuppressive therapies, compared with only 15 (14%) of 110 healthy controls (34). As more patients in the disease-endemic area receive liver, lung, and heart transplants, this problem will increase.

Pregnant women, especially those in the third trimester, are at high risk for developing disseminated coccidioidomycosis if they become infected (24). In the first and second trimesters, the risk is much lower. The reason is not entirely clear, but two factors may play a role: 1) the high sex hormone levels found in late pregnancy enhance the growth of *C. immitis* in vitro (25), and 2) the shift in the T-cell immune response late in pregnancy toward T_H2 cytokines (26) interferes with resolving the infection. In experimental animals, pregnancy increases the severity of leishmaniasis, another infection that is controlled by a T_H1 T-cell response (35).

Coccidioidomycosis in AIDS patients is also very likely to be life-threatening. The first cases of coccidioidomycosis described in AIDS patients were atypical, with a reticulonodular chest x-ray pattern, positive blood cultures, and infection of multiple organs (36). As we have gained more experience with coccidioidomycosis in HIV-infected persons, we have learned that the clinical spectrum is broader than originally reported. Fish and his colleagues collected data from 77 AIDS patients with coccidioidomycosis who were treated by physicians in Arizona and California (37). They grouped the patients according to their clinical symptoms (Figure 5).

Although the largest group of patients had dif-

fuse pulmonary infiltrates, a significant fraction had focal pulmonary disease, meningitis, or other extrapulmonary disease. Six patients had only a positive serologic test with no other evidence of infection. Excluding the patients who had only a positive serologic test, 81% of the patients in this series had a positive serologic test for coccidioidomycosis, either for IgM or IgG antibodies. However, only 69% of patients with diffuse pulmonary disease had a positive serologic test, and the death rate in this group was also the highest (70%). In all clinical groups, death was correlated with the number of circulating CD4 T-cells at the time of diagnosis. For clinicians, however, the most important message from this study is that coccidioidomycosis is not a uniformly fatal complication in patients with AIDS, and that many forms of this disease, including meningitis, respond to therapy. Patients with <200 CD4 T-cells/µl are more likely to have severe, disseminated infections.

A more recent prospective study of 170 HIV-infected persons in an area of Arizona where coccidioidomycosis is endemic showed a cumulative incidence of coccidioidomycosis of 25% over 41 months (38). The most important risk factors were the level of CD4 T-cells and the diagnosis of AIDS

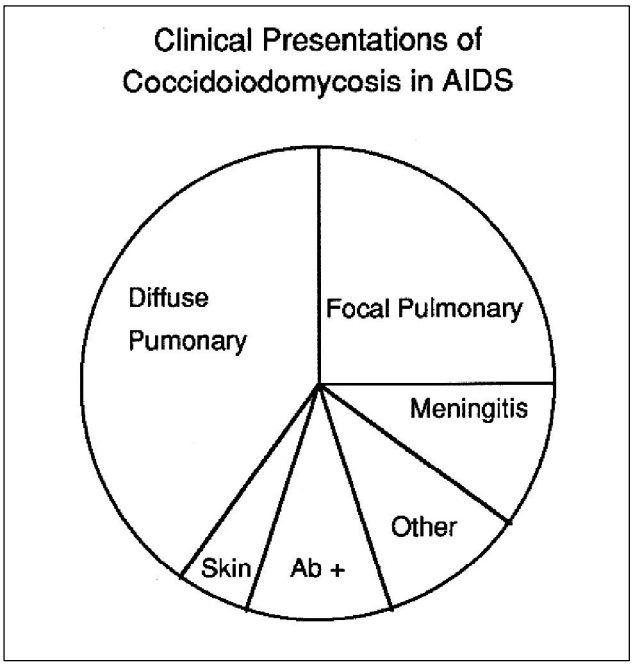

Figure 5. The most common clinical presentations of coccidioidomycosis in AIDS patients. The group "Others" includes dissemination to the lymph nodes, liver, spleen, and bone marrow. The antibody only group includes patients with serologic evidence of infection but no evident focus of infection. Since these were passively collected cases, a protocol to search for inapparent sites of infection had not been agreed upon (37).

(as opposed to HIV infection). HIV-infected patients with AIDS or <250 CD4 T-cells/µl were 8 to 35 times more likely to get coccidioidomycosis. History of coccidioidomycosis, a history of a positive skin test for coccidioidomycosis, or a prolonged stay in the disease-endemic area were not associated with an increased risk for infection. These data suggest that most cases were primary infections in severely immunosuppressed patients. Since patients with AIDS were not more likely to be exposed to the spores of *C. immitis* and all patients were seen prospectively at 4-month intervals and tested for antibody to *C. immitis*, severe immunosuppression appeared to increase their risk for infection, as well as disease. As in the retrospective study reviewed above, the clinical symptoms varied widely, ranging from mild to extremely severe. Only one patient had antibody titers to *C. immitis* by complement fixation test without any other evidence of disease.

Treatment

Various drugs are now available for treating coccidioidomycosis. In addition to amphotericin B, which must be given intravenously and is considerably toxic, triazole compounds have been found to be active agents for treating most manifestations of coccidioidomycosis. Fluconazole, in an uncontrolled study, was reported to be effective primary therapy for coccidioidal meningitis; since untreated coccidioidal meningitis is uniformly fatal, robust conclusions could be drawn from this trial (39). Forty-seven consecutive patients were treated with 400 mg/day of fluconazole; during the first 6 months of therapy, 33 (70%) of the patients responded to therapy. (A response was defined as a 40% reduction in a score, on the basis of clinical measurements and cerebrospinal fluid findings.) Two patients who did not respond to therapy died of coccidioidomycosis; both were HIV-positive. Because of previous experience with high relapse rates when azole therapy is stopped, the authors recommended lifelong treatment with fluconazole. In a small study, four of five patients treated for meningitis with itraconazole as sole therapy responded favorably (40). A recent article emphasized the high relapse rate after azole therapy is stopped (41). The alternative treatment to the azoles is amphotericin B. If amphotericin B is used to treat meningitis, however, it must be given intrathecally as well as intravenously, and this greatly increases the risk for a toxic reaction to that drug.

Clearly, fluconazole and itraconazole can be used to treat patients with nonmeningeal coccidioidomycosis (42-44). Whether one of these drugs is superior

to the other, or how either one compares to amphotericin B is not known. It seems prudent to treat extremely ill patients with amphotericin B, at least until their clinical situation stabilizes, although no published studies support that point of view. However, few (if any) patients with the acute miliary form of coccidioidomycosis have been included in any of the reported studies of any of the azole drugs. New agents that are more active against coccidioidomycosis are still sorely needed.

Prevention

Simple environmental measures, such as planting grass or paving roads in highly populated areas, decrease the amount of airborne dust and lower the risk for coccidioidomycosis (10). These measures do not necessarily eradicate *C. immitis* from the soil but lower the risk for airborne dispersion of the organism. At present, no practical method exists for eliminating *C. immitis* from the soil.

Vaccine Development

An alternative approach is to vaccinate persons at risk. A vaccine is feasible because natural infection almost always confers lifelong immunity from reinfection. Furthermore, good animal models exist to test vaccine candidates (21). Finally, genetically susceptible mice can be successfully immunized, which suggests that the genetically susceptible human population would also benefit from vaccination (21).

One vaccine that has been tested is a killed spherule vaccine developed by Pappagianis and Levine. It protected mice and other animals from experimental infection with *C. immitis* (45). Between 1980 and 1985, a double-blinded human study compared results of a formalin-killed spherule vaccine with results obtained from a placebo. In this study, which involved almost 3,000 people, only a minority of the vaccinated persons had positive skin test results to *C. immitis*. Although the incidence of coccidioidomycosis was low while this study was conducted, no difference was found in the number of cases of coccidioidomycosis or the severity of the disease in the vaccinated group compared with that for the placebo-receiving control group (46). One explanation for the ineffectiveness of this vaccine may be that relatively small numbers of killed organisms could be injected into human without unacceptable local side effects of pain and swelling. Nevertheless, the vaccine trial made it clear that immunization with tolerable numbers of whole killed-spherules does not provide immunoprotection against coccidioidomycosis in humans.

Since the cell wall of *C. immitis* is made up primarily of nonprotein macromolecules, it contains a large amount of material presumably nonantigenic for T lymphocytes. Therefore, the whole organism is not the ideal vaccine candidate. Ideally, one would like to vaccinate patients selectively only with antigens that stimulate a protective T-cell-mediated immune response. These antigens have been difficult to identify, and a consensus on what they are does not exist. Various approaches have been used to obtain antigenic proteins. In one, a lysate of arthroconidia (coccidioidin) or spherules (spherulin) was made (47). Alkali treatment has also been used to extract antigens from arthroconidia and spherules (48). Another approach has been to use *C. immitis* antigens obtained without extraction or autolysis. The advantage of this method is that one should obtain reproducible preparations of intact proteins. Cole and co-workers (49) found that when the outer conidial wall was removed from arthroconidia, the organism released various proteins (called the soluble conidial wall fraction). This mixture of proteins was extraordinarily effective in stimulating the proliferation of *C. immitis*-immune T cells in mice. Another antigenic mixture is a membranous material consisting primarily of proteins and lipids that the spherule phase of the organism spontaneously releases (the spherule outer wall). This spherule wall fraction has been shown to be an active antigen in T-cell-mediated immune responses in mice (50).

All of these mixtures are heterogeneous and difficult to fractionate biochemically. This is probably due, at least in part, to differences in glycosylation, which makes physically separating the proteins difficult. To resolve this problem, Galgiani and his colleagues deglycosylated the proteins from a toluene spherule lysate by using hydrogen fluoride (51,52). Although this treatment does remove all sugars, it is extraordinarily harsh and yields less than 10% of the initial protein, with most of the protein forming an insoluble precipitate. Nevertheless, the resulting product reacts with reference antiserum to *C. immitis* in immunoelectrophoresis. This antigen also stimulated a proliferative T-cell response in patient lymphocytes but not in those of the control group (noninfected donors).

Another way to attack the problem of generating pure *C. immitis* antigens is to use molecular biologic techniques. The advantage to this approach is that once antigens are molecularly cloned, and the protein is expressed, an essentially unlimited source of completely defined antigen is available. Therefore, one would not have to repeatedly grow *C. immitis*, extract the antigen, and purify it from a complex mixture. In addition, with the molecular

approach, antigens could be delivered as part of a living vaccine system, should that be required to effectively immunize people against coccidioidomycosis. We believe that systematically identifying and evaluating *C. immitis* T-cell reactive antigens in experimental animals is a rational approach to the ultimate development of a vaccine. Our laboratories, in collaboration with Garry Cole, have used a murine T-cell line that is specific for soluble conidial wall fraction antigens to identify one cloned fragment of a *C. immitis* protein (53). Recently, genomic DNA clones coding for this protein have been identified and sequenced. Significant homology exists between this *C. immitis* antigen and the human enzyme 4 hydroxyphenylpyruvate dioxygenase (54). This protein has been expressed in bacteria and was found to elicit T-lymphocyte proliferative responses in mice immune to *C. immitis*. We are testing its efficacy as an experimental vaccine.

With the exception of alkali extracted spherules (55) and whole killed spherules (45), none of the T-cell reactive antigens have been shown to be immunoprotective in experimental models. However, it is reasonable to expect that some antigen, or mixture of antigens, will be found that can confer protective immunity in experimental animals. Molecular strategies are available to accomplish this task and are an important area of future research. Once a vaccine has been successfully tested in animals, another human vaccine trial would be feasible.

Dr. Kirkland is associate professor of pathology and medicine; Dr. Fierer is professor of medicine and pathology and head of the Division of Infectious Diseases, University of California, San Diego School of Medicine. Drs. Kirkland and Fierer have worked together for the past 15 years. Currently, they are focusing on the genetic determinants for resistance to infection and on identifying candidates for a coccidioidomycosis vaccine.

Acknowledgments

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Antibody-Based Therapies for Emerging Infectious Diseases

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In the 19th century, it was discovered that immune sera were useful in treating infectious diseases. Serum therapy was largely abandoned in the 1940s because of the toxicity associated with the administration of heterologous sera and the introduction of effective antimicrobial chemotherapy. Recent advances in the technology of monoclonal antibody production provide the means to generate human antibody reagents and reintroduce antibody therapies, while avoiding the toxicities associated with serum therapy. Because of the versatility of antibodies, antibody-based therapies could, in theory, be developed against any existing pathogen. The advantages of antibody-based therapies include versatility, low toxicity, pathogen specificity, enhancement of immune function, and favorable pharmacokinetics; the disadvantages include high cost, limited usefulness against mixed infections, and the need for early and precise microbiologic diagnosis. The potential of antibodies as antiinfective agents has not been fully tapped. Antibody-based therapies constitute a potentially useful option against newly emergent pathogens.

In the mid-1990s, successful implementation of antiinfective therapy has become increasingly difficult because of widespread antimicrobial resistance, the emergence of new pathogens, and the occurrence of many infections in immunocompromised patients in whom antimicrobial drugs are less effective. Infections caused by some new pathogens (e.g., human immunodefficiency virus [HIV] and *Cryptosporidium parvum*) cannot be cured with existing antimicrobial drugs. Regaining the upper hand in the struggle against microbes requires multidisciplinary efforts which include developing new antimicrobial agents (1), improving surveillance for emerging microbial threats (2), teaching the correct use of antimicrobial therapy (3), expanding the use of vaccines to prevent infection (4), developing adjunctive immunotherapies (5), and conducting new basic research on the mechanisms of pathogenesis and drug resistance. In this article, the potential of antibody therapy in confronting the threat of emergent infections will be explored.

Antibody-Based Therapies: Then and Now

Antibody-based (serum) therapies were first used to treat human infections in the 1890s (6,7). In the early 20th century, serum therapy was used to treat a variety of bacterial infections, including those cased by *Corynebacterium diphtheriae, Strep-*

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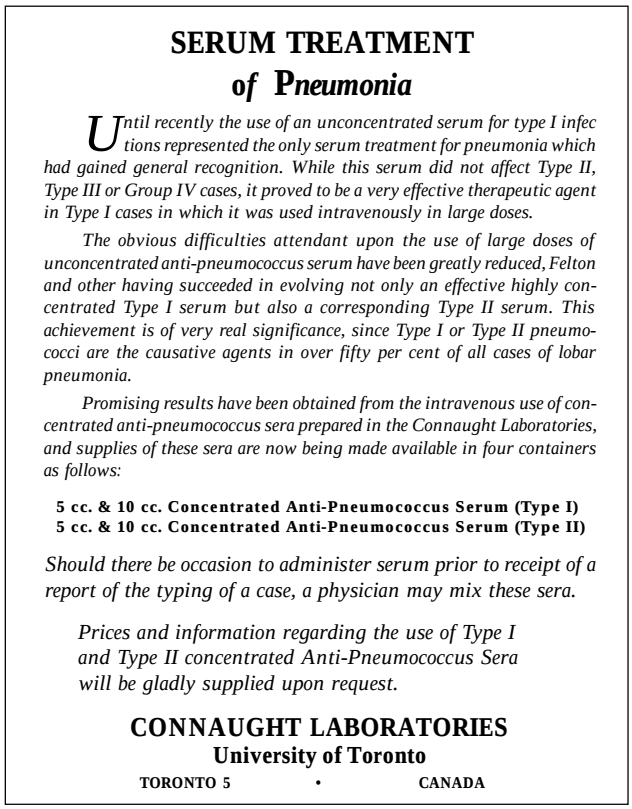

SERUM TREATMENT
of *Pneumonia*

Until recently the use of an unconcentrated serum for type I infections represented the only serum treatment for pneumonia which had gained general recognition. While this serum did not affect Type II, Type III or Group IV cases, it proved to be a very effective therapeutic agent in Type I cases in which it was used intravenously in large doses.

The obvious difficulties attendant upon the use of large doses of unconcentrated anti-pneumococcus serum have been greatly reduced, Felton and other having succeeded in evolving not only an effective highly concentrated Type I serum but also a corresponding Type II serum. This achievement is of very real significance, since Type I or Type II pneumococci are the causative agents in over fifty per cent of all cases of lobar pneumonia.

Promising results have been obtained from the intravenous use of concentrated anti-pneumococcus sera prepared in the Connaught Laboratories, and supplies of these sera are now being made available in four containers as follows:

5 cc. & 10 cc. Concentrated Anti-Pneumococcus Serum (Type I)
5 cc. & 10 cc. Concentrated Anti-Pneumococcus Serum (Type II)

Should there be occasion to administer serum prior to receipt of a report of the typing of a case, a physician may mix these sera.

Prices and information regarding the use of Type I and Type II concentrated Anti-Pneumococcus Sera will be gladly supplied upon request.

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Figure 1. Advertisement for type-specific anti-pneumococcal sera from the March 1931 issue of the *Canadian Medical Association Journal*. The text in this advertisement describes advancements in the preparation of antibody solutions and emphasizes the need for using type-specific serum in the therapy of pneumococcal pneumonia. Note the suggestion that type-specific serum can be mixed for empiric therapy of pneumonia. (Reprinted with permission.)

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Table 1. Serum therapy, human MAbs, and antimicrobial chemotherpy

	Antibody therapy		Chemotherapy	Comment
	Immune serum	Human MAb		
Specificity	Narrow	Narrow	Broad	Narrow specificity avoids selection of resistant organisms among nontargeted microbes. Narrow specificity requires a precise diagnosis before use.
Source	Animals Humans	Tissue culture Bioreactor Fermentation	Fermentation Chemical synthesis	Before antibiotics, most serum preparations were from horses and rabbits. MAbs are produced by tissue culture techniques. Industrial production of MAbs may utilize immunoglobulin synthesis in yeast, bacteria, or plants.
Toxicity	High	Low	Low	Toxicity of serum was due to allergic reactions to animal protein. Human immunoglobulin preparations are well tolerated (42). Antiidiotypic responses remain a problem for humanized MAb therapy (54).
Cost	High	High	Low	Serum therapy for pneumococcal pneumonia in the 1930s was costly(6) Immunoglobulin therapy remains very expensive.
Administration	Difficult	Easy	Easy	Serum therapy required considerable expertise, and because of life-threatening allergic reactions, dosage was often based on clinical experience.
Pharmacokinetics	Variable	Consistent	Consistent	Pharmacokinetics of heterologous polyclonal antibody depends on multiple variables, e.g.,animal source, isotype composition,and immune status of the recipient. Human MAbs are homogeneous reagents and can be expected to have more consistent pharmacokinetics.
Mechanism of action	Antimicrobial Immune enhancement Toxin neutralization	Antimicrobial Immune enhancement Toxin neutralization	Antimicrobial	Conventional antimicrobial chemotherapy kills or inhibits the replication of microorganisms. Antibodies function through a variety of mechanisms, e.g., promoting complement-mediated lysis, enhancing antimicrobial efficacy of host effector cells, efficacy of host effector cells, promoting phagocytosis,preventing attachment, and neutralizing toxins.

tococcus pneumoniae, *Neisseria meningitides*, *Haemophilus influenzae*, group A streptococcus, and *Clostridium tetani* (6,7). By the 1930s, serum therapy was standard treatment for lobar pneumonia (Figure 1). Several large controlled trials showed that administering type-specific serum reduced the death rate in patients with pneumococcal pneumonia by approximately 50% (6). However, when antimicrobial chemotherapy was discovered in the mid-1930s, serum therapy for bacterial infections was rapidly abandoned. Antimicrobial chemotherapy had important advantages over serum therapy: it was more effective and less toxic. The immediate side effects of serum therapy included fevers, chills, and allergic reactions (8,9). A delayed toxic reaction of serum therapy was "serum sickness," a syndrome characterized by rash, proteinuria, and arthralgia; this occurred in 10% to 50% of patients who received heterologous serum and was probably caused by immune complexes. Other disadvantages of serum therapy included the need to establish a precise diagnosis before selecting serum, lot-to-lot variation of serum, and the need for considerable physician expertise (Table 1). Serum therapy could fail because of inadequate dosage, delayed treatment, mis-labeling of serum, and because the infection was mixed or complicated (i.e., empyema) (10). Producing therapeutic sera was very expensive because of the costs of animal husbandry, antibody purification, refrigeration, and standardization by the mouse protection tests. When antimicrobial chemotherapy was first introduced, enthusiasm was expressed for combining serum therapy and antimicrobial chemotherapy. Support for combined therapy came from animal studies, which suggested that combination therapy was more effective than either therapy alone against several pathogens, including group A streptococcus (11), pneumococcus (12), and meningococcus (13), and some authorities recommended combined therapy for serious infections (14,15). However, several studies showed that combined therapy was not more effective than antimicrobial chemotherapy alone and that it caused significantly more side effects (16-18). Therefore, serum therapy was abandoned because it offered no measurable advantage in efficacy over chemotherapy and had substantial disadvantages in implementation, cost, and toxicity.

Today antibody therapy is indicated in infectious diseases in relatively few situations, including replacement therapy in immunoglobulin-deficient patients, post-exposure prophylaxis against several viruses (e.g., rabies, measles, hepatitis A and B, varicella), and toxin neutralization (diphtheria, tetanus, and botulism). Ironically, the general abandonment of antibodies as antimicrobial agents was followed by major advances in the technology of antibody production. In 1975, hybridoma technology provided the means to generate unlimited amounts of monoclonal antibodies (MAbs) (19). In recent years, major advances have been made in the techniques used to generate human antibodies and humanize murine MAbs (20).

The juxtaposition of three recent developments makes the reintroduction of antibody-based therapies an option for serious consideration. First, because of advances in technology, human antibody reagents can be synthesized; thus the toxicities traditionally associated with serum therapy can be avoided. Second, the emergence of new pathogens, the reemergence of old pathogens, and the increased prevalence of drug-resistant microorganisms have caused the effectiveness of existing therapeutic options to decline. Third, the difficulties involved in treating infections in immunocompromised patients suggest the need for adjunctive immunotherapy.

Polyclonal Sera Versus Monoclonal Antibodies for Therapy

Immune sera contain antibodies of multiple specificities and isotypes. Problems with immune sera include lot-to-lot variation (21), low content of specific antibodies (22), and some hazards in the transmission of infectious diseases (23). Commercially available intravenous immunoglobulin preparations obtained from human donors differ in their opsonic activity for common pathogens such as *Staphylococcus epidermidis*, *H. influenzae* type b, *S. pneumoniae*, group B streptococcus, and *Escherichia coli*, reflecting the characteristics of the donor pool (22). In contrast MAbs are generated in vitro by either hybridoma technology or recombinant DNA techniques. MAbs are homogenous immunoglobulins that, by definition, recognize one epitope and have markedly higher specific activity than polyclonal preparations. For example, 0.7 mg of two human MAbs to tetanus toxin have the same activity as 100 to 170 mg of immune globulin (24). The higher specific activity of MAbs may also translate into greater therapeutic efficacy. MAbs formulations are superior to polyclonal sera in homogeneity, constancy, specific activity, and (possibly) safety. For some infections, polyclonal preparations may be superior to MAbs because MAbs contain antibodies to multiple epitopes (i.e., they are polyvalent). However, different therapeutic MAbs can be combined to generate polyvalent preparations composed of antibodies with multiple specificities and isotypes. Given the advantages of MAb preparations over

immunesera, antibody-based therapies for emergent infections, if used, will likely rely primarily on MAb technology.

Advantages of Antibody-Based Therapies

Humans can produce antibodies to practically all existing pathogens. Antibody molecules are assembled from combinations of variable gene elements, and the possibilities resulting from combining the many variable gene elements in the germline enable the host to synthesize antibodies to an extraordinarily large number of antigens. During the generation of the antibody response, somatic mutations are introduced into immunoglobulin genes, which result in higher affinity antibodies and more diversity in specificity (25). Thus, antibodies are, as a class, broad-spectrum antimicrobial agents with activity against all classes of pathogens. However, individual antibodies are usually pathogen-specific. Pathogen-specific antimicrobial agents have the theoretical advantage that they do not select for resistant organisms among nontargeted microbes and are unlikely to produce great disturbances in the normal host flora.

Antibody-based therapies could, in theory, be developed against any pathogen. Although the level of antibody immunity differs among pathogens, it may be possible to develop useful antibody therapies even if natural antibody immunity plays little or no role in protection. Two fungi, *Candida albicans* and *Cryptococcus neoformans*, are pathogens for which protective antibodies can be generated despite uncertainty about the role of natural antibody immunity (26). The MAbs to *C. neoformans* enhance the therapeutic efficacy of amphotericin b (27,28), fluconazole (29), and 5-flucytosine (30) in murine models of cryptococcosis. Therefore, uncertainty regarding the role of natural antibody immunity in protecting against a given pathogen does not rule out the existence of antibodies that may be useful in therapy.

Microbial targets for therapeutic antibody development are not necessarily limited to extracellular pathogens. Although intracellular pathogens are commonly believed to be outside the reach of antibody immunity, several reports have suggested that some MAbs are active against some intracellular microorganisms. Some IgA MAbs can neutralize intracellular viruses (31), and an MAb to *Toxoplasma gondii* has been reported to interfere with intracellular replication of the parasite (32). It has been proposed that intracellular virus neutralization by IgA occurs by antibodies binding to viral proteins and interfering with viral assembly (31). Additional evidence for intracellular antibody activity comes from the observation that IgG anti-DNA autoantibodies can enter the cytoplasm and nucleus of living cells (33).

Antibodies mediate antimicrobial function through a variety of mechanisms, including inhibition of microbial attachment, agglutination, viral neutralization, toxin neutralization, antibody-directed cellular cytotoxicity, complement activation, and opsonization (34). Antibodies are extremely versatile antimicrobial molecules: some are active directly against the pathogen, some neutralize the toxic products of infection, and others enhance the efficacy of host effector cells. Some MAbs to poliovirus are neutralizing only at fever temperatures (35), which demonstrates their ability to function at physiologic extremes. The versatility of antibody-based therapies is illustrated by the ability of digoxin-binding antibodies to reverse digoxin toxicity (36) and recent attempts to treat septic shock by employing MAbs that bind cytokines (37).

Human IgG has favorable pharmacokinetics for use as an antimicrobial agent, including good tissue penetration (38) and a half-life of about 20 days (39). Murine MAbs have much shorter half-lives in humans, and these usually elicit human antibody responses (40). Human-mouse chimeric antibodies and humanized MAbs are synthetic molecules composed primarily of human antibody protein sequences that retain the antigen-binding site of the heterologous antibody (20). Human-mouse chimeric antibodies and humanized MAbs have longer half-lives than the murine precursor, but their half-lives are still much shorter than that of native human IgG (41). This area is being intensively investigated, and genetic engineering of antibody molecules may be used to synthesize MAbs with longer half-lives.

Immunoglobulin therapy with human reagents is generally well tolerated (42). Serious adverse reactions, including renal failure (43), aseptic meningitis (44), and thromboembolic events (45) can occur with high-dose (0.5 to 2 g/kg) antibody therapy. However, antiinfective immunoglobulin therapy with MAb preparations is unlikely to require the high doses of immunoglobulin used to treat rheumatic disorders and other conditions. For example, the heterologous immune sera used in the preantibiotic era were effective, although they contained small amounts of specific antibody. The higher activity of MAb preparations should permit a smaller amount of immunoglobulin proteins to be used and thus avoid the occasional toxicity reported with high-dose antibody therapy.

Problems with Antibody-Based Therapies

Most antibody therapies are pathogen-specific. This is a disadvantage in dealing with mixed infections. Mixed infection with multiple *S. pneumoniae* serotypes was recognized as a cause for the failure of serum therapy (46). For pathogens that are antigenically variable, one solution is to use antibody cocktails of agents active against the most common antigenic types. Antibody cocktails may also be designed to include antibodies of different isotypes to enhance antibody effector function. The successful implementation of antibody-based therapies would also require improvements in diagnostic microbiology. In the preantibiotic era, for lobar pneumonia, rapid protocols were developed for the isolation and typing of pneumococci from sputum (47). Recent advances in diagnostic microbiology, including polymerase chain reaction and nucleic acid hybridization, could substantially shorten the time required to establish a microbiologic diagnosis. The narrow spectrum of antimicrobial activity that characterizes antibody-based therapies is a drawback for commercial development, however. Pathogen-specific drugs, have smaller potential markets than broad-spectrum antimicrobial agents, and this makes them less attractive to the pharmaceutical industry. Conversely, the emergence of multidrug-resistant microorganisms and new pathogens for which no drugs exist could make pathogen-specific drugs attractive for commercial development.

Widespread use of antibody-based therapies could produce selective pressure on microbial populations for the emergence of antibody-resistant variants. Antibody-resistant mutants of *Borrelia burdorferi* have been produced in the laboratory (48), and may be selected in patients who undergo antibody-based therapies. Microorganisms may become resistant to antibodies by acquiring mutations that change the antigenic site recognized by the anti-bodies or by producing proteases that destroy immunoglobulins. The IgA protease genes of *N. gonorrhoeae* can be transferred between strains, and the widespread use of antibody therapies may select for protease-producing strains (49). However, the versatility of antibody technologies provides alternatives for countering antibody-resistant strains. For instance, new antibodies directed toward the mutated epitope could be developed, or antibodies that bind other antigenic targets could be introduced. Protease-producing strains could be countered with protease-resistant immunoglobulin molecules generated by introducing amino acid changes at protease cleavage sites. Alternatively, MAbs that neutralize proteases could be incorporated into therapeutic antibody cocktails in a manner analogous to the present practice of using beta-lactamase inhibitors to increase the effectiveness of beta-lactam antibiotics. Recognizing that the introduction of new agents has been inevitably followed by the emergence of resistance, researchers could attempt to minimize the emergence of antibody-resistant organisms from the outset by using cocktails of MAbs directed at multiple antigenic targets.Combining antibody therapy with chemotherapy also could reduce the probability of selecting for organisms resistant to either therapeutic modality.

Antibodies are more effective in preventing infection than in treating established infection. Antibody-based therapies have been most useful when administered early in the course of disease: serum therapy for pneumococcal pneumonia was most effective if serum was administered within 3 days of the onset of clinical symptoms (6). Because antibodies are proteins, therapy for invasive infections is likely to require systemic administration. This is a serious disadvantage in developing countries where access to medical care is limited. For enteric pathogens, oral antibody administration may be most effective (50,51). Efforts to develop MAbs for cancer therapy have run into unexpected pharmacologic problems (38,40). The finding that MAb uptake by tumors is partially dependent on antigen expression in the tumor (40) suggests that MAb uptake by infected tissues could depend on microbial antigen expression at the site. The same problems may be found when antibody penetrates tumors.

Antibodies can, in principle, elicit neutralizing antibody, allergic responses, or both. Administering rodent MAbs to patients elicits human antibodies to the rodent MAb (52). Antibody therapies against emergent pathogens, if attempted, are likely to use human, human-animal chimeric, or humanized antibodies. Mouse-human chimerics and humanized antibodies are less immunogenic than heterologous antibodies (41,53,54); therefore, the likelihood that the patient will mount a neutralizing antibody response to the therapeutic antibody may be reduced. Nevertheless, antiidiotypic responses have been observed in patients receiving humanized antibody therapy (54). The clinical importance of such antiidiotypic responses is uncertain. Many infections are single life-threatening episodes in the life of a person, and the occurrence of antiidiotypic antibodies following therapy may require repeated or long-term antibody administration.

Antibodies are largely excluded from the central

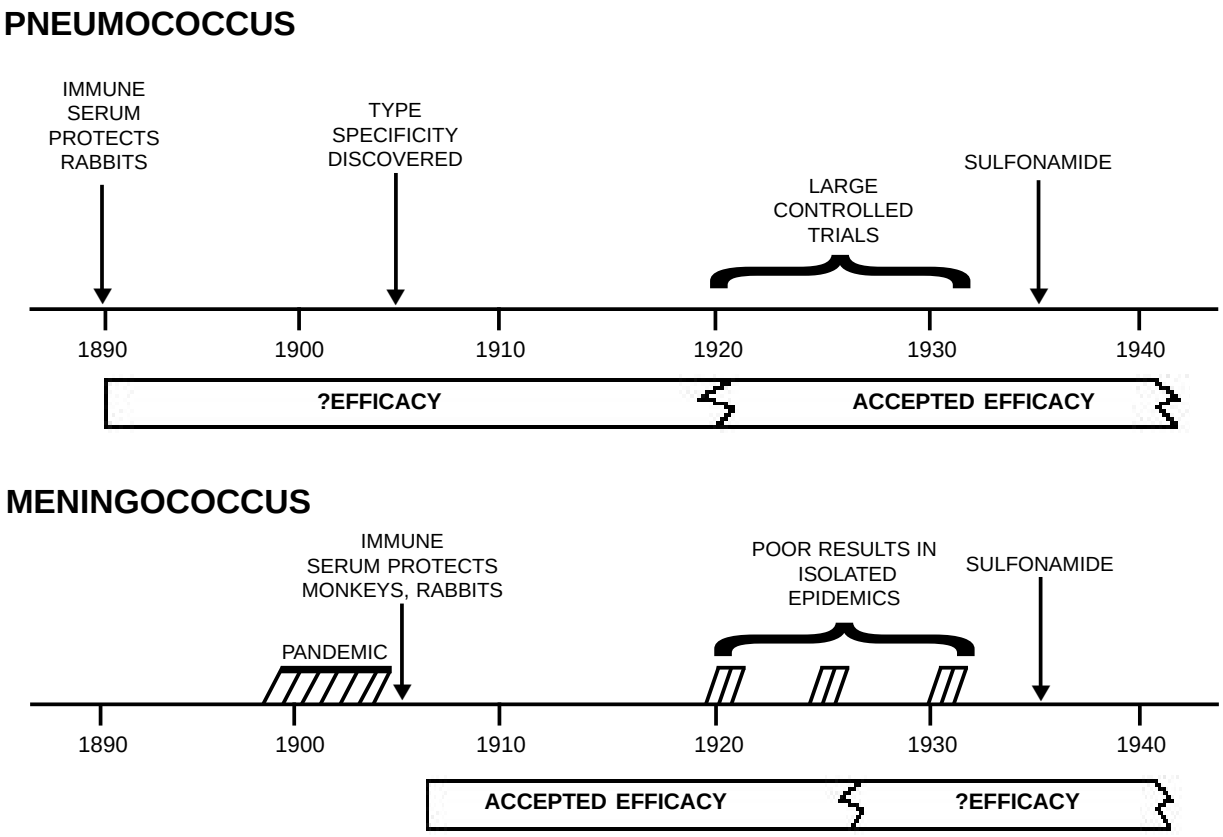

Figure 2. Schematic illustration of the major events in the development of serum therapy for pneumococcal pneumonia and meningococcal meningitis. For pneumococcal pneumonia, considerable uncertainty existed regarding the usefulness of serum therapy in the decades following the demonstration that immune sera could transfer protection to animals. However, the discovery that type-specific serum necessary for efficacy, followed by extensive clinical trials, led to the general acceptance of serum therapy for pneumococcal pneumonia in the late 1920s. For meningococcal meningitis, the antisera generated against the strains prevalent in the early 1900s proved to be effective in therapy. However, the efficacy of serum therapy in later epidemics of meningococcal meningitis was significantly lower, leading to uncertainty about the value of serum therapy for this infection (16).

nervous system by the blood-brain barrier. Nevertheless, antibody treatment of brain infections is feasible. In some brain infections, the blood-brain barrier is more permeable to serum components because of inflammation, and systemic antibody therapy was used successfully to meningococcal meningitis (55). If antibody penetration to brain tissue is a problem, two alternatives exist. First, antibodies can be administered directly into the subarachnoid space (as was done for the treatment of meningococcal meningitis in the preantibiotic era) (15,56). Second, antibody molecules can be engineered for enhanced brain penetration by altering their charge (57) or by linking them to proteins that cross the blood-brain barrier (58).

Antibody therapies are also costly to develop and expensive for the patient. For example, antibody prophylaxis for cytomegalovirus infections can cost several thousand dollars per patient (59). To be cost-effective, antibody-based therapies would have to provide a clear benefit over existing therapy. For emerging pathogens for which no therapy is available, the cost of antibody therapies may be justifiable, depending on the potential for death, illness, and long-term consequences of the infection. In the long run, advances in antibody production and improvements in technology may greatly lower costs and make antibody-based therapy more competitive with antimicrobial chemotherapy.

Historical Perspective on the Development of Antibody Therapies

In recent years, considerable interest has been expressed in using antibody-based therapies to treat septic shock (60). Unfortunately, MAbs to endotoxin have not been as effective in clinical trials as anticipated (61,62), and this has dampened some of the

enthusiasm for MAb therapies. The development of serum therapy for *S. pneumoniae* also encountered considerable difficulties in the preantibiotic era.

The Klemperers demonstrated that immune serum protected rabbits in 1891 (63), but reliable antibody therapies for pneumococcal pneumonia were not available until the 1920s (Figure 2). Translating the laboratory finding that immune sera protected rabbits against experimental pneumococcal infection to the successful use of serum therapy for lobar pneumonia in humans required extensive basic and clinical research. At the laboratory bench, developing antibody therapy for pneumococcal pneumonia required the discovery that antigenic variation existed among pneumococcal strains, that only type-specific sera provided protection, that certain vaccination schedules were necessary to elicit good antibody responses, the ability to standardize the serum potency (by the mouse protection test), and improved antibody purification techniques (6). At the bedside, implementing successful serum therapy required learning when and how to give serum, managing the side effects of serum therapy, and developing rapid protocols for recovering pneumococci from sputum for serum typing (64). The mouse protection test reduced but did not eliminate the problems of lot-to-lot variation in serum efficacy (21). The development and perfection of antibody therapies for pneumococcus contributed important research findings, which led to major discoveries in immunology (65). The high death rate for meningococcal meningitis also led to the rapid development of serum therapy (6). In the early 1900s, serum therapy markedly reduced the death rate of meningococcal epidemics, possibly because of antigenic changes in the pathogen (6). The lengthy time required for the development of serum therapy for pneumococcus, the variable efficacy of antimeningococcal sera (depending on the epidemic), and the more recent difficulties encountered in developing MAb therapy for septic shock suggest that developing antibody-based therapies for emergent pathogens will require extensive preclinical and clinical testing.

Other Antibody-Based Strategies Against Emerging Pathogens

Vaccines that elicit protective antibody immunity could be used to protect against emergent pathogens. A polysaccharide-protein conjugate vaccine is being developed (66) against *C. neoformans*. This vaccine elicits protective antibodies in mice (67), and it is hoped that vaccination will result in the production of effective anti-cryptococcal antibodies to prevent disease in patients at risk. The conjugate vaccine against *C. neoformans* is intended to elicit protective antibody immunity, even though the role of natural antibody immunity in protection against cryptococcosis is uncertain (26). Newer vaccines against common pathogens could help limit the spread of drug-resistant microorganisms. Dissemination of penicillin-resistant pneumococci has been associated with infection and carriage by young children among whom the current 23-valent pneumococcal polysaccharide vaccine is ineffective in inducing protective immunity (4). However, the effectiveness of polysaccharide-protein conjugate vaccine to *H. influenzae* type b suggests that a similar conjugate vaccine to *S. pneumoniae*, if available, could effectively abort childhood infection with antibiotic-resistant pneumococci and thereby limit the spread of these strains (4).

Future Directions

Immunoglobulins are an extremely versatile class of antimicrobial proteins that can be used to prevent and treat emerging infectious diseases. Antibody therapy has been effective against a variety of diverse microorganisms. The historical record clearly documents both the usefulness and the difficulties in developing and implementing passive antibody therapies. The experience with serum therapy for pneumococcal pneumonia and meningococcal meningitis suggests that extensive basic and clinical research is essential for the successful implementation of antibody therapy. Given the multitude of pathogens, the pathogen-specific nature of antibody therapies, and the costs of developing and using antibody therapies, the development of such therapies for most pathogens at present, would be impractical. However, for selected pathogens, antibody-based therapies could provide new therapeutic options. Opportunities for the development of antibody-based strategies include 1) pathogens for which there is no available antimicrobial therapy (e.g., *C. parvum* and vancomycin-resistant enterococcus); 2) pathogens that affect primarily immuno-compromised patients in whom antimicrobial therapy is not very effective (e.g., invasive fungal infections); 3) pathogens for which drug-resistant variants are rapidly spreading (e.g., *Pseudomonas aeruginosa* [68]); and 4) highly virulent pathogens for which few effective antimicrobial agents are available (e.g., methicillin-resistant *S. aureus*).

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HIV-1 Group O Virus Identified for the First Time in the United States

In 1994, seven unusual cases of HIV infection were identified in France (1). These cases occurred in foreign nationals whose specimens had atypical Western blot patterns and weak or no seroreactivity in HIV-1 peptide or recombinant-based enzyme immunoassays (EIA). In previous cases, such isolates had been loosely grouped as phylogenetic outliers, termed Group O strains, highly divergent from the Group M strains that gave rise to the HIV-1 pandemic (2). The 1994 cases were soon recognized as representing the global emergence of genetically distinct viruses known to be circulating in Cameroon and Gabon. This recognition prompted an immediate reassessment of HIV screening in Europe and North America. Subtype B strains of HIV-1 predominate in Europe and North America, and there has been increasing evidence for the entry of non-B strains into these populations through persons who were infected while abroad. However, there was no evidence of indigenous spread of the strains in North America (3,4).

We report here the laboratory findings related to the first documented HIV-1 Group O infection in the United States. The case was identified in April of this year through the CDC sentinel surveillance program for unusual HIV variants and involves a West-Central African woman (residing in the United States), with a 2-year clinical history of lymphadenopathy, thrombocytopenia, and progressive CD4 lymphocyte loss. In February 1995, the patient was tested for HIV-1-specific antibodies and was determined to be seronegative (Abbott HIV-1/2; signal/cutoff value = 0.7) by recombinant-antigen based EIA. The patient's serostatus was reevaluated the following October and November and again in April of 1996 with inconclusive results (Table 1). On these occasions, whole virus lysate-based EIAs yielded negative or weakly positive results (signal/cutoff ratios were 0.68 to 4.20); however, confirmatory Western blot assays remained indeterminate. The November 1995 sample was negative in both Genetic Systems formats; however, a signal/cutoff ratio of 0.89 was noted. The Western blot pattern remained remarkably stable through the year, and the patient's sera remained HIV-1 antigen–negative on each sampling occasion. Attempts by commercial reference laboratories to amplify proviral sequences from the patient's peripheral blood mononuclear cells by standard Group M gag-specific primers for polymerase chain reaction (PCR) or reverse transcriptase (RT)-PCR did not show HIV sequences.

To reconcile the serologic findings, a panel of HIV subtype–specific, peptide-based EIAs was performed at CDC (6) (Table 2). As points of reference, examples of results with sera representing the Group M subtypes A, B, C, D, E, and F are shown. A high degree of cross-reactivity exists among the Group M sera, with the strongest response usually directed against the V3 peptide from the subtype that infected the source patient. The exception to this (Table 2) is seen in the Brazilian subtype F sera, which react strongly with the subtype B peptide but not with the subtype F peptide, which was derived against subtype F isolates from Romania. The distinction between Brazilian and Romanian sera is reflected in the genetic diversity between isolates from the two regions (7). This broad seroreactivity among group M sera is also reflected in their response to group-specific peptides from the gp41. However, the patient's sera were only reactive to the Group O–specific V3 and gp41 peptides (OD = 1.704 and 2.467, respectively). This provided the first direct evidence that this patient was infected with a Group O variant.

The virus CDC7755 was isolated by cocultivating the patient's peripheral blood mononuclear cells with phytohemagglutinin A, which stimulated normal human peripheral blood mononuclear cells. Viral replication was monitored by antigen capture (Coulter SIV Core Antigen Assay, Hialeah, FL) and PCR amplification of the viral protease gene (8). Virus growth was rapid, highly cytolytic, and characterized by punctate syncytial formation throughout the primary culture. All PCR amplifications were performed with hotstart technology and nested primer sets to facilitate direct sequencing. A

Table 1. Results of enzyme immunoassay for antibodies to HIV

Sample date	Genetic Systems HIV-1 EIA[a]	Genetic Systems HIV-1/2 EIA[a]	OTC HIV-1 EIA[a]	Abbott HIV-1 EIA[a]	Abbott HIV-1/2 EIA[a]
February 1995	ND	ND	ND	ND	0.70
October 1995[a]	0.51	1.35	ND	ND	ND
November 1995[b]	0.41	0.89	ND	ND	ND
April 1996[c]	4.20	3.40	0.68	3.10	1.15

[a]HIV-1 WB = indeterminate; detected p17, p24, p31, p50, p66, and equivocal gp41; HIV-1 antigen = negative (0.40).
[b]HIV-1 WB = same as above; HIV-1 antigen = negative (0.34).
[c]HIV-1 WB = same as above; HIV-1 IFA = positive (2.5+).
ND = not done.

Table 2. Typical peptide reactivity patterns.

Subtype	V3-A	V3-B	V3-C	V3-D	V3-E	V3-F	V3-O	GP41-M	GP41-O
A	2.742	1.144	1.628	0.065	0.083	0.724	0.055	2.683	2.627
B	0.071	1.271	0.051	0.030	0.033	0.038	0.028	2.570	2.414
C	2.320	0.084	2.515	0.079	1.647	1.637	0.061	2.746	2.858
D	0.210	0.079	0.056	1.203	0.048	0.051	0.040	1.380	0.279
E	0.055	0.043	0.385	0.042	1.291	0.038	0.025	0.619	0.340
F	0.062	0.657	0.041	0.037	0.039	0.033	0.037	2.615	2.670
O	0.089	0.088	0.089	0.188	0.088	0.081	1.704	0.070	2.457

GP41-M and GP41-O refer to peptides derived from the consesus gp41 sequences for Group M and Group O variants.

consensus set of nested primers, which is generic for Group M viruses, was first used to attempt amplification and detection of the viral protease. This set did not detect proviral sequences during direct evaluation of the patient's peripheral blood mononuclear cells; however, a comparably derived consensus primer set for the Group O proteases readily amplified proviral sequences from the patient's cells and cocultures.

For sequence comparison and phylogenetic analysis, proviral sequences spanning the viral protease gene and portions of the p24 and gp120 gene regions were amplified from the patient's peripheral blood mononuclear cells and cocultures.[1] Amplification of a 738-bp fragment spanning the junction of the p24 and p17 genes of the gag region was accomplished through a nested set of generic primers (9). Group O consensus primer sets were derived to amplify the 297-bp viral protease gene and a 381-bp fragment spanning the C2V3 portion of gp120 within the env gene. Sequence identity was demonstrated between repeated patient samplings and successive days in cocultivation for each of the gag, pol, and env elements, thus confirming a common but unique source for the isolate. For phylogenetic analysis, these sequences were further edited to obtain the relevant portions of the protease gene (254 bp), p24 (419 bp), and C2V3 region(233 bp) of gp120. Phylogenetic analysis was performed by neighbor-joining and maximum-likelihood methods (10,11).

In the phylogenetic analysis of the protease gene, we included the two available Group O prototypes (ANT70 and MVP5180), reference strains for the HIV-1 subtypes representing Group M variants, and HIV-2 strains (Figure 1A). The fidelity of the phylogenetic results was verified by boot strap analysis (100 data sets) and pruning, i.e., the sequential removal of representative strains followed by reanalysis. The segregation of CDC7755 and Group O strains was absolute and is consistent with a divergence independent of the rooting of the Group M clades (Figure 1A). For each gene region, all resulting trees were consistent with the exclusion of the CDC7755 isolate from the Group M family and its strong inclusion with the Group O strains.

To better understand the evolutionary relationship between the patient s strain and other Group O isolates, we analyzed the C2V3 region of gp120 from multiple Group O strains. This gene region exhibits a high degree of divergence and has been sequenced for the largest number of Group O variants (Figure 1B). The chimpanzee isolate SIV-CPZ was used as an outgroup. We took this approach to avoid possible errors introduced through an alignment of very distant HIV-1 and HIV-2 strains. Our findings indicate that strain CDC7755 retains a distant evolutionary relationship to the MVP5180 isolate (Figure 1B, MVP51) yet represents a distinct evolutionary lineage within Group O. The nucleotide sequence divergence among the represented strains is 19% to 26%, values characteristic of Group O viruses and typical of intersubtype rather than intrasubtype genetic distances among the Group M variants. This level of diversity among the Group O members emphasizes the potential that the limited number of "O" variants yet recognized may represent a set of genetic lineages as distinct as the Group M subtypes.

An alignment of the deduced C2V3 amino acid sequence of strain CDC7755 with those of three Cameroonian Group O reference strains is presented in Figure 2; the V3 loop is bound by the two cysteine residues (residues 45 and 83). Unlike Group M viruses, which show greater heterogeneity in the regions flanking the V3 loop, the Group O viruses, including the CDC7755 strain, show substantial divergence within the loop itself. The pattern of potential N-glycosylation sites over the C2V3 region of the CDC7755 strain is consistent with that found

[1]Primers: gag/outer/forward - AGTACATGTTAAAACATGTAGTATGGGC; gag/outer/ reversed - CCTACTCCCTGACAGGCCGT CAGCATTTCTTC; gag/inner/forward - AGTACATGTTAAAACATGTAGTATGGGC; gag/inner/reverse - CCTTAAGCTTTTGT AGAATCTATCTACATA; protease/outer/forward - TTTGCCTCCCTCAAATC; protease/outer/reverse - TTACTGGCACTG GGGCTATGG; protease/inner/ forward - CCTCAAATCCCTCTTTG; protease/inner/reverse - TATAGGGAAGTTTAGTGTACA; env/outer/forward - CACAGAATTTAATGGAACAGGC; env/outer/reverse - TGTGTT ACAATAAAAGAATTCTCC; env/inner/forward - GTTACTTGTACACATGGCAT; and env/inner/reverse - AGAATTCTCCATGACAGTTAAA.

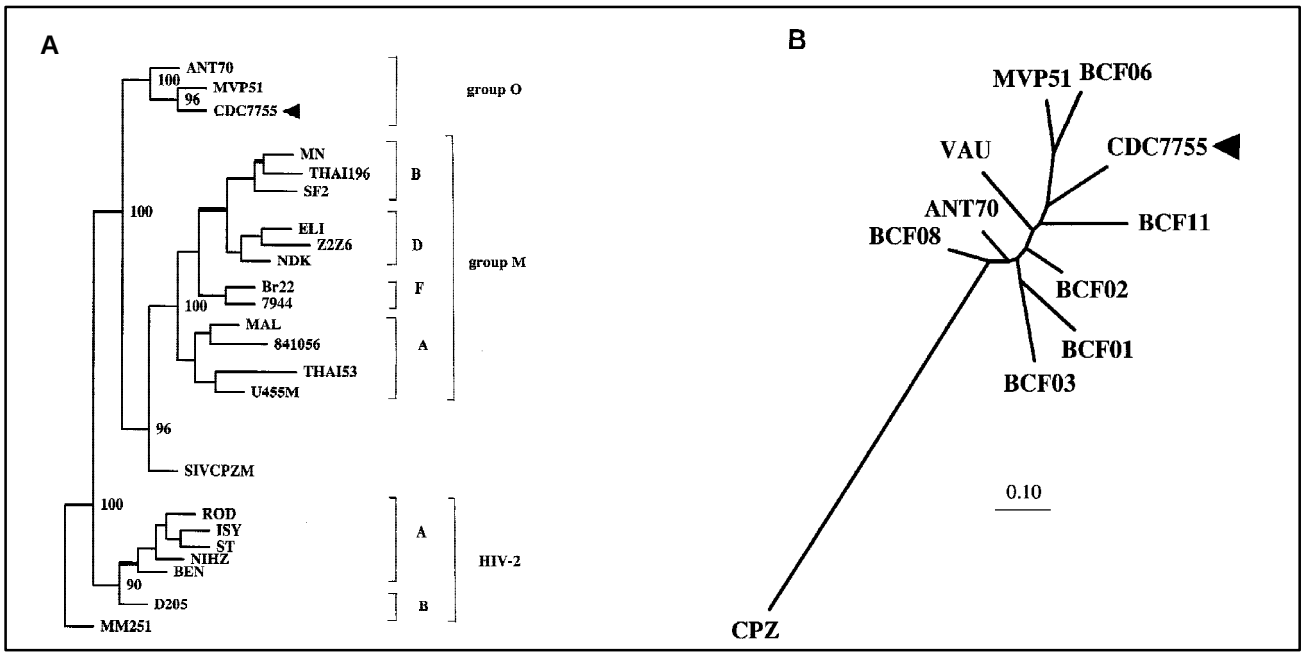

Figure 1. Phylogenetic analysis of the prt and env gene sequences. The patient's isolate is indicated by the arrow. The trees were constructed on the basis of the proviral DNA sequences for the protease gene (A) by the neighbor-joining method in the Phylip 3.5c package (2) and 230 aligned nucleotides from the C2V3 region of the env gene of Group O isolates (B) by using the maximum likelihood method with the fast DNAml program (1). Numbers at the branch nodes within the protease tree indicate bootstrap values. The SIV-MM251 protease gene sequence and the SIV-CPZ env sequence were used as outgroups in trees A and B, respectively. The nucleotide sequence distance among strains can be deduced from the horizontal branch lengths by using the included bar scale. Vertical distances are for clarity only.

among the Group O reference strains. Also of interest is the increased number of amino acid residues in the V3 loop of the CDC7755 strain (37 amino acids)–a feature common to other Group O viruses but two to three residues more than most Group M viruses; however, the significance of these differences is not clear.

To date, nearly all of the Group O infections reported worldwide (fewer than 100), have been detected in persons from West-Central Africa. This is the first Group O case reported in the United States, and the presence of these infections here is believed to be exceedingly low. However, the discovery of any divergent HIV strains should be considered a sentinel event and should prompt the reappraisal of HIV antibody tests used to protect of the blood supply. In the United States guidelines for preventing HIV transmission by blood and blood products include testing for HIV-1 and -2; the refinements necessary to improve the sensitivity of

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CONS-O   K P T V S T Q L I L  N G T L S K G K I R  I M G K N I S E S G  K N I ? V T L N ? T  I N M T C E R P G ?
ANT70    R - - - - - - - - -  - - - - - - - - - -  M - A - D - L - G -  - - - I - - - - S -  L - - - - - - - Q I
MVP5180  - - - - - - - - - -  - - - - - R E - - -  - - - - - - - T - -  A - - - I - - - - T P  - - - - - I - E - I
VAU      - - - - - - - - - -  - - - - - - - - N - T  - - - - - - - - D - -  E - - L I - - - T N  - T I A - - - - - N
CDC7755  - - - - - - H - - F  - - - I - - - - - -  - - V - - - - S N S  D - - L - - - - S -  - - - - - V - - - N

CONS-O   ? ? ? Q E ? R I G P  M A W Y S M G L G R  T ? G ? ? ? ? S R A  A Y C T Y N A T D W  ? K A L ? Q ? A E R
ANT70    D * I - - M - - - - -  - - - - - - - - I -  G - A - N * * S - - -  - - - K - - - - - -  G - I - K - T - - -
MVP5180  A E V - D I Y T - -  - R - R - - T - K -  S N N T S P R - - V  - - - - - - K - V -  E N - - Q - T - I -
VAU      Q T I - K I M A - -  - - - - - - A - S N  - K - D * * * T - -  - - - N - S - - - -  N - - - K N I T - -
CDC7755  N S V - - M - - - -  L - - - - - - - - -  g Y A S * * K - - I  - F - - - - - - E -  K E T - Q G I - - -

CONS-O   Y L E L V N ? N T E  N V T ? T F ? ? S S  G G D A E V T H L H  . . . . . .  . . . . . .
ANT70    - - - - - - * - - G  S I N M - - N H - -  - - - L - - - - - -  . . . . . .  . . . . . .
MVP5180  - - N - - - * Q - -  - - - I I - S R T -  - - - - - - S - - -  . . . . . .  . . . . . .
VAU      - - - - - E Y - Q T  D - - M K - G - H -  - E - - - - - N F F  . . . . . .  . . . . . .
CDC7755  - - - - - - * H - -  - S - I - - K N - T  - - - I - - - R - -  . . . . . .  . . . . . .
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Figure 2. Alignment of deduced amino acid sequences of the env C2V3 region of the CDC7755 strain with those of three representative Group O Cameroonian strains (ANT70C, MVP5180 and FR.VAU). The CONS.O represents the consensus amino acid sequence derived from the four strains presented in this alignment. (?) represents positions where a consensus could not be derived. (-) indicates identical amino acids with the CONS.O and (*) indicates gaps (insertion \ deletions) that were introduced to align the sequences.

these diagnostic tests for the detection of Group O variants are known. Other criteria for donor deferral (e.g., self-deferral for donors at high risk for HIV infection and temporary exclusion of donors from malaria-endemic regions) that may confer a measure of protection against divergent HIV viruses present in equatorial Africa are in place. The identification of Group O infections underscores the potential for the emergence of other highly divergent HIV strains and highlights the importance of maintaining active worldwide surveillance for these variants.

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Two Morbilliviruses Implicated in Bottlenose Dolphin Epizootics

Sequence analysis was performed on viral RNA isolated from bottlenose dolphins (*Tursiops truncatus*) that died during two chronologically and geographically separate epizootics in North America. Both dolphin morbillivirus (DMV) and porpoise morbil-livirus (PMV) were detected in bottlenose dolphins that died during the 1987 U.S. Atlantic coast epizootic. Our results indicate not only that these viruses are not species specific, but also that both viruses were present in North America before outbreaks in the Mediterranean and Irish Seas. Samples taken along the Atlantic coast showed a statistically significant trend with DMV in the north and an increasing incidence of PMV in samples isolated farther south. In the 1993 Gulf of Mexico epizootic, only PMV was detected in bottlenose dolphins that died. Thus, DMV and PMV are implicated as the causes of the earliest known aquatic mammal morbilliviral outbreak, the U.S. Atlantic coast epidemic; PMV is implicated in the Gulf of Mexico epidemic. The presence of two pathogenic morbilliviruses that may circulate together or separately complicates the epidemiology of cetacean morbilliviral diseases.

The only morbilliviruses known before 1989 were human measles virus, canine distemper virus, rinderpest, and peste-des-petits-ruminants virus (1). Recently, newly characterized morbilliviruses have been shown to be epizootic-associated pathogens in pinnipeds and cetaceans. Phocine distemper virus (2) was associated with a massive epizootic of harbor seals (*Phoca vitulina*) in northwestern Europe (3) in 1988. During the harbor seal phocine distemper virus outbreak, PMV was isolated from harbor porpoises (*Phocoena phocoena*) that died along the Irish coast (4). DMV (5), was isolated during an epizootic of striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea (6,7) in 1990-92.

Between June of 1987 and May of 1988, a morbillivirus epizootic caused a tenfold increase in bottlenose dolphin stranding along the U.S. Atlantic coast from New Jersey to Florida (8,9). More than half of the in-shore population of bottlenose dolphins in this area may have died. Morbillivirus-related strandings of bottlenose dolphins along the Gulf of Mexico coasts of Alabama, Mississippi, and Texas were also observed from October 1993 through April 1994 (10,11).

Using reverse transcriptase–polymerase chain reaction (RT-PCR), we examined tissue lysates made from formalin-fixed paraffin-embedded lung tissue from stranded dolphins from the Atlantic epizootic and frozen unfixed lung tissue from the Gulf of Mexico epizootic (11,12). RT-PCR-positive cases were subsequently characterized by sequence analysis of segments of the morbillivirus P gene in regions with the greatest diversity between DMV and PMV.

We sequenced segments of the morbillivirus P gene from 29 dolphin specimens (1 striped and 28 bottlenose dolphins) from the 1987-88 Atlantic epizootic; sequence sufficient for viral identification was obtained in 25 (86%) of 29 cases. Sequence was also obtained from 7 (37%) of 19 bottlenose dolphin specimens stranded in Texas during the Gulf of Mexico epizootic. Because of the advanced state of postmortem decomposition of most of these specimens, histologic and immunophenotypic analysis was not possible (13). Data (stranding date, location, species, and morbillivirus sequence analysis results) for these 32 cases are shown in Table 1. In addition, P gene sequence was obtained from formalin-fixed paraffin-embedded lung tissue from four of four striped dolphins from the 1990 Mediterranean Sea epizootic and from one harbor porpoise recovered off the coast of Northern Ireland in 1988 during the harbor seal epizootic (Table 1).

Morbillivirus P gene sequencing allowed viral identification of 37 stranded cetaceans from four geographically and chronologically separate epizootics. In the U.S. Atlantic Coast 1987-88 epizootic, mixed infection was present involving both recognized cetacean morbilliviruses, DMV and PMV (Table 1). Twelve animals (including the striped dolphin) were infected with DMV, nine with PMV, and four with both. From the later Gulf of Mexico morbillivirus-associated epizootic, only PMV was identified. The morbillivirus infecting striped dolphins in the Mediterranean Sea epizootic was identified as DMV (6). All four striped dolphin specimens examined in this study were typed as DMV-infected. A harbor porpoise stranded on the coast of Northern Ireland during the harbor seal epizootic in northwestern Europe was typed as PMV-infected, consistent with the published analysis of these animals (14).

Partial P gene nucleotide sequence data are presented with published DMV and PMV P gene sequences (Figure 1). Sequence data from the PMV-infected cases (Atlantic epizootic cases, Gulf of Mexico epizootic cases, Irish harbor porpoise)

Table 1. Case summary

Date found	Stranding by state	Dolphin species	Morbillivirus sequence[a]
4 Aug. 1987	New Jersey	*Tursiops truncatus*	DMV
5 Aug. 1987	New Jersey	*T. truncatus*	DMV
9 Aug. 1987	New Jersey	*T. truncatus*	DMV
11 Aug. 1987	New Jersey	*T. truncatus*	DMV
23 Aug. 1987	New Jersey	*T. truncatus*	DMV + PMV
3 Sep. 1987	New Jersey	*Stenella coeruloalba*	DMV
5 Sep. 1987	New Jersey	*T. truncatus*	DMV + PMV
6 Sep. 1987	New Jersey	*T. truncatus*	PMV
14 Aug. 1987	Virginia	*T. truncatus*	PMV
14 Aug. 1987	Virginia	*T. truncatus*	DMV + PMV
15 Aug. 1987	Virginia	*T. truncatus*	PMV
26 Aug. 1987	Virginia	*T. truncatus*	DMV + PMV
29 Aug. 1987	Virginia	*T. truncatus*	PMV
29 Aug. 1987	Virginia	*T. truncatus*	DMV
4 Sep. 1987	Virginia	*T. truncatus*	DMV
5 Oct. 1987	Virginia	*T. truncatus*	DMV
6 Oct. 1987	Virginia	*T. truncatus*	DMV
7 Oct. 1987	Virginia	*T. truncatus*	DMV
21 Dec. 1987	Florida (Atlantic Coast)	*T. truncatus*	PMV
21 Dec. 1987	Florida (Atlantic Coast)	*T. truncatus*	PMV
1 Jan. 1988	Florida (Atlantic Coast)	*T. truncatus*	PMV
18 Jan. 1988	Florida (Atlantic Coast)	*T. truncatus*	PMV
9 Feb. 1988	Florida (Atlantic Coast)	*T. truncatus*	DMV
9 Feb. 1988	Florida (Atlantic Coast)	*T. truncatus*	PMV
10 Feb. 1988	Florida (Atlantic Coast)	*T. truncatus*	DMV
4 Apr. 1993	Texas	*T. truncatus*	PMV
4 Apr. 1993	Texas	*T. truncatus*	PMV
4 Apr. 1993	Texas	*T. truncatus*	PMV
11 Apr. 1993	Texas	*T. truncatus*	PMV
15 Apr. 1993	Texas	*T. truncatus*	PMV
19 Apr. 1993	Texas	*T. truncatus*	PMV
21 Apr. 1993	Texas	*T. truncatus*	PMV
1991	Mediterranean Sea	*S. coeruloalba*	DMV
1991	Mediterranean Sea	*S. coeruloalba*	DMV
1991	Mediterranean Sea	*S. coeruloalba*	DMV
1991	Mediterranean Sea	*S. coeruloalba*	DMV
1988	Northern Ireland	*Phocoena phocoena*	PMV

[a] RNA isolation, RT-PCR, and cycle sequencing were performed (11,20).

showed no within-group variation. Data from all cases showed two single nucleotide changes from the published PMV sequence (12), at positions 206 and 209 (aligned with the measles virus P gene sense mRNA). Likewise, no sequence variation was noted for the DMV-infected animals (Atlantic and Mediterranean epizootics). However, a two-nucleotide inversion, at coding positions 206-207, was consistently noted when compared to the published DMV sequence (12) (Figure). In four cases from the Atlantic epizootic cycle sequencing indicated simultaneous infection with DMV and PMV. To confirm that the two different sequences were present simultaneously, PCR product was cloned, and plasmid DNA from individual colonies was sequenced. In each of the four doubly infected cases, approximately half the clones contained the DMV sequence, and half contained the PMV sequence. No clones containing hybrid sequences were found. Since PMV and DMV cause non-species-specific infections, more appropriate designations for these viruses might be cetacean mor-billivirus 1 for PMV (the first detected cetacean morbillivirus [4]) and cetacean morbillivirus 2 for DMV.

Bottlenose dolphins of the U.S. Atlantic coast migrate seasonally (15). The 1987-88 epizootic moved south from New Jersey to the east coast of Florida with the southerly fall migration. In-shore bottlenose dolphins rarely travel around the southern tip of the Florida peninsula (Randall Wells, pers. comm.), which explains the end of the 1987 epizootic on the east coast of Florida in May of 1988. Sequences were obtained from dolphins stranded during this epizootic in three U.S. states, New Jersey, Virginia, and Florida. From the Gulf of Mexico epizootic, which occurred 5 years later, sequence data from seven dolphins stranded in Texas were obtained. A significant trend (chi square for trends [16], $p = 0.00023$) was noted among the specimens analyzed from the New Jersey, Virginia, and Florida Atlantic coasts in which the percentage of animals infected with DMV decreased, while PMV infection increased (Table 2). In the 1993 die-off, 100% of the animals from which sequence information was obtained were PMV-infected. The epidemiologic significance of this finding is unknown. It is possible that dolphins of the southern Atlantic coast and those in the Gulf of Mexico were more susceptible to infection with (or had an increased death rate from) PMV than with DMV.

To test whether enzootic morbillivirus infection could be detected in bottlenose dolphins, RT-PCR was performed on formalin-fixed paraffin-embedded tissue samples from 11 dolphins stranded along the U.S. Atlantic coast from 1974 to 1985 (before the 1987 epizootic). None of the dolphins had histologic or immunohistochemical evidence of morbillivirus infection (9). Amplifiable RNA was obtained from samples from eight animals, and all were negative for morbillivirus. Tissue specimens from seven bottlenose dolphins stranded on the Florida coast of the Gulf of Mexico in the interval between the Atlantic and Gulf of Mexico epizootics were also ex-

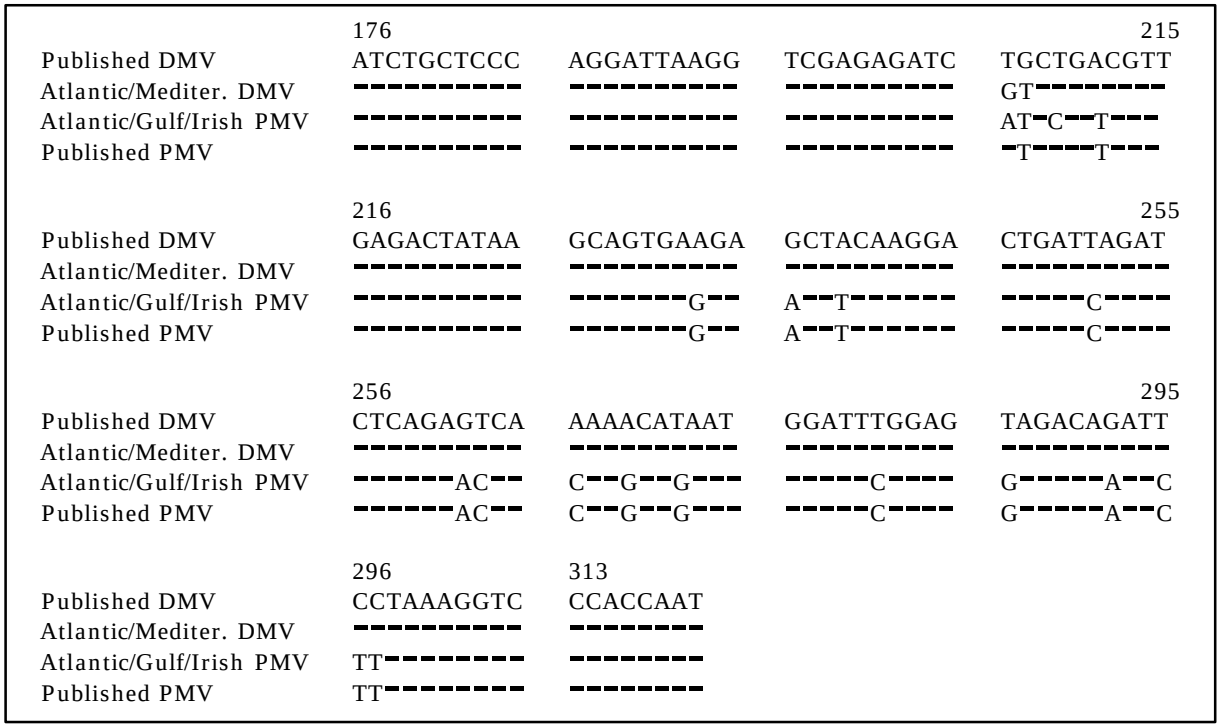

Figure. Partial nucleotide sequence of DMV and PMV morbillivirus P gene compared with published DMV and PMV sequences (12). Primer sequences used were 5'-ATC TGC TCC CAG GAT TAA GGT CGA-3' (forward) and 5'-CGG GAT TGG TGG GAC CTT TA-3' (reverse). RT-PCR was performed (11). PCR products were cycle sequenced (20) or cloned into PDK101 (21) and sequenced by using T7 Sequenase (Amersham Corporation, Arlington Heights, IL) according to the manufacturer's instructions.

amined by RT-PCR for morbillivirus. None of these animals had histologic or immunohistochemical evidence of morbillivirus infection (data not shown). However, one of these samples was weakly positive for the morbillivirus P gene 78 bp product (11). Sequence information, however, could not be obtained from this sample. This result suggests the possibility of an enzootic infection in dolphins along the U.S. coast after the initial 1987 morbillivirus epizootic.

Serum antibodies to canine distemper virus were detected in specimens of 6 of 13 free-ranging bottlenose dolphins during the U.S. Atlantic epizootic in 1987 (17), indicating exposure to morbillivirus. A recent study by Duignan, et al. (18) presented serologic evidence of morbillivirus infection in 11 of 15 cetacean species in the western Atlantic since 1986.

Table 2. Percentage of DM-V and PMV-infected animals by location

Stranding location	No. dolphins	No. DMV infected (%)	No. PMV infected (%)
Atlantic, New Jersey	8	7 (70)[a]	3 (30)[a]
Atlantic, Virginia	10	7 (58)[a]	5 (42)[a]
Atlantic, Florida	7	2 (29)	5 (71)
Gulf of Mexico, Texas	7	0	7 (100)

[a] Including the four cases of simultaneous DMV and PMV infection.

Virus neutralizing titers were higher against DMV and PMV than against peste-des-petits ruminants virus, phocine distemper virus, or canine distemper virus. Enzootic morbillivirus infection of two species of pilot whale (*Globicephala melas* and *G. macrorhynchus*) was recently demonstrated in animals in the western Atlantic (19), with the earliest titer from a pilot whale stranded in 1982. Neutralizing antibody titers in these species were also higher against DMV and PMV, and were observed in 108 of 125 animals tested. However, confirmation of active infection by viral sequence analysis was not performed in either of these studies. It is, therefore, possible that the pilot whale mass strandings reported in 1982 and 1986 were morbillivirus induced, but confirmation of this hypothesis would require histologic and/or viral sequence confirmation.

Geographically and temporally distinct morbillivirus die-offs, as well as the evidence presented in this study that the two previously described cetacean morbilliviruses are not species-specific, raise many questions about the epidemiology of this family of viruses. The viruses implicated in the 1988 European porpoise deaths and 1990 Mediterranean dolphin epizootic were present in bottlenose dolphins

of the 1987 U.S. die-off. Duignan, et al. hypothesized that pilot whales enzootically infected with morbilliviruses could act as long-distance vectors between America and Europe (19). Confirmation would require sequence analysis of pilot whale samples collected in both regions. The distribution of DMV and PMV infection in the U.S. epizootics suggests that the later epizootic may have been initiated by rare contact between PMV-infected Atlantic dolphins and immunologically naive Gulf dolphins. Further investigation of serum antibody titers, PCR evidence of enzootic infection, and sequence identification of morbillivirus species are required for the better understanding of these epizootics.

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An Outbreak of Spotted Fever Rickettsiosis in U.S. Army Troops Deployed to Botswana

Novel infectious diseases are recognized when nonimmune persons move into the ecologic niche of a pathogen and become inadvertent hosts. One population at frequent risk to such emerging pathogens is the military. Historical examples of this phenomenon include outbreaks of scrub typhus during World War II and Korean hemorrhagic fever during the Korean War (1). In this report, we describe a modern example as we document an unusually high attack rate of a rickettsial disease among U.S. troops camping for a brief period in Africa.

In January 1992, U.S. soldiers participated in a 10-day training exercise with the Botswana Defense Force near Shoshong, Botswana. Activities included individual weapons' training and a series of mock battles. The terrain was generally flat with scattered bushes and small trees. The climate was semiarid, and temperatures reached 120°F during the day. During the fieldexercise, one soldier sought medical assistance to remove small "insects" crawling rapidly on his body. No specimens were reliably identified in the field or preserved for subsequent examination. No unusual illnesses were reported to medical personnel during the training exercise. After the field-training exercises, the soldiers spent 2 days in Gaborone, the capital of Botswana. Within 2 days of their return to their home station, approximately 30% of the deployed soldiers sought medical attention with symptoms of fever, headache, and regional lymphadenitis. Several soldiers reported insect bites. An epidemiologic team from Walter Reed Army Institute of Research was sent to Botswana to assist with the outbreak investigation.

From airplane manifests, 169 soldiers were identified as having been deployed to Botswana. One week after the soldiers returned to their home station, a questionnaire requesting information on symptoms, personal protective measures taken against arthropod vectors, reservoir exposures, and other potential risk factors for infection was administered to all available soldiers (n = 140); 132 soldiers underwent directed physical examinations, in which skin lesions, lymphadenopathy, and right upper quadrant tenderness were noted. Clothing and equipment used in the field that had not been cleaned since the exercise were visually examined in detail by a team of entomologists. Blood samples from 126 soldiers were obtained at 1 week and 10 weeks postdeployment. Serum specimens were divided into aliquots and frozen at -20°C for laboratory analyses.

Sera were tested for antibodies to rickettsiae by using an indirect fluorescence antibody technique (IFA) (2). Sera drawn at 10 weeks postdeployment were initially screened for whole immunoglobulin reactivity against *Rickettsia conorii* Moroccan strain, a member of the spotted fever group of rickettsiae that cause boutonneuse fever. Subsequently, sera showing titers ≥1:64 were paired with their respective 1-week samples and titered to the endpoint, defined as the highest dilution showing discernible fluorescing organisms. The rickettsial species used as antigens for antibody titration included cell culture–propagated *R. conorii* (Moroccan, ATCC VR-141), *Rickettsia akari* Hartford, and *Rickettsia typhi* (Wilmington, ATCC VR-144). The latter two microorganisms are the etiologic agents of rickettsial pox and murine typhus, respectively. Slides were read by one technician who was blinded to the clinical history corresponding to the sample.

A case-patient was defined as a soldier who had either a fourfold or greater rise in immunoglobulin G (IgG) titer to *R. conorii* within 9 weeks to a titer ≥1:128 by IFA, or clinically, by the presence of a *tache noire* (eschar) and at least two of the following symptoms: regional lymphadenitis, fever, chills, severe headache, and muscle or joint pains. By this case definition, the overall attack rate was 23% (39 of 169 soldiers). Of 36 case-patients who provided paired serum samples, 24 (67%) seroconverted during the postdeployment period. All 24 case-patients who seroconverted were symptomatic, but six did not have an eschar. Of the 15 case-patients without documented fourfold rises in titer, all had at least one eschar and regional lymphadenitis observed by a physician. Three of the soldiers who did not seroconvert, but were identified as case-patients by the clinical definition, had high standing titers, ≥1:256.

One week after returning from Botswana, the case-patients (n = 36) had a geometric mean reciprocal (GMT) IgG titer to *R. conorii* of 43, which increased to 185 9 weeks later (Student's *t* test paired, $p < 0.0001$). Over the same period, the GMT of non-case-patients (n = 38) who had positive titers on the initial whole immunoglobulin screening remained the same at 38. To determine whether early treatment impaired antibody production, the case-patients were divided into two groups: those who

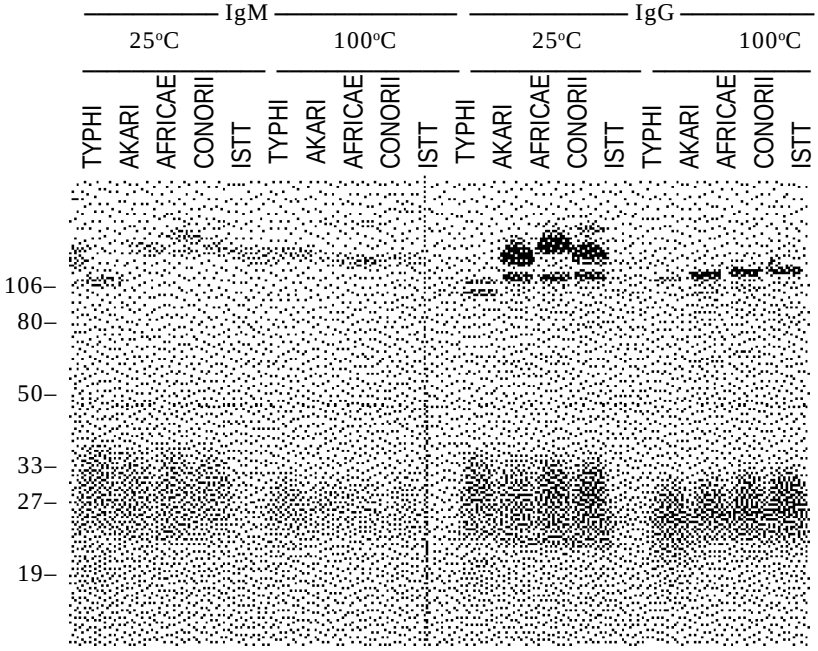

Figure 1. Western blot reactivity of convalescent-phase serum from a patient with spotted fever rickettsiosis with high standing titers. Antigens from the rickettsial isolates were solubilized at room temperature or boiled for 5 minutes before electrophoresis. The darkest large bands indicate *R. africae, R. conorii*, and Israeli tick typhus rickettsiae [ISTT] (no specificity detected). *R. typhi* is a member of the typhus group of rickettsiae, whereas all other isolates are members of the spotted fever group.

received treatment within 2 days of the start of their symptoms (n = 12) and those who received treatment 3 or more days later (n = 21). The group that was treated early had a lower mean GMT at follow-up, but the difference was not statistically significant (early 136, 95% confidence interval [C.I.]: 77, 239: late 197, 95% C.I.: 114, 338).

A Western blot immunoassay was used (3). Rickettsial antigens (*R. typhi* Wilmington, *R. akari*, *Rickettsia africae* F (4,5), *R. conorii* Malish (ATCC VR613), and Israeli tick typhus rickettsiae (T-487)) used in the immunoblots, either boiled or solubilized at room temperature, were electrophoresed on an 8% to 16% gradient PAGE gel. Renografin density gradient purified antigen was applied at 20 µg protein per lane. *R. typhi* was yolk sac–propagated, whereas spotted fever group antigens were grown in irradiated L cells. Patients' serum specimens were reacted with the electrophoresed antigens at 1:250 dilution. Horseradish peroxidase–labeled goat antibodies against human IgG (gamma-chain-specific) and IgM (µ chain-specific) (Calbiochem, San Diego, CA) were used at 1:1000 dilution, and bound antibody was detected with 4-Cl-1-naphthol-H_2O_2 reagent. Prestained SDS-PAGE molecular weight standards (Bio-Rad, Melville, NY) were used to estimate antigen sizes

Western blots were used to characterize the serologic reactivities of 38 case-patients and 37 non–case-patients. Serum specimens were considered positive for reactivity with spotted fever rickettsial antigens if a characteristic broad washboard of spotted fever group lipopolysaccharide reactivity (SFG LPS) was observed in the 20- to 40-kDa region with anti-IgG conjugate (Figure 1). Most of these serum specimens also exhibited pronounced IgM reactivity, which sometimes included *R. typhi* LPS. Serologic specificity toward the individual spotted fever species was determined by the relative IgG reactivity to species-specific antigens (SPAs) solubilized at room temperature found at 125 to 145 kDa. Thirty-two of the 38 case-patient specimens tested by Western blot were positive for spotted fever group IgG LPS.

On Western blot, the sera of 23 case-patients reacted most strongly with *R. africae* SPA (not shown), one with *R. conorii* SPA, and eight showed no specificity (Figure 1). Nine of 37 soldiers who did not meet our case definition were positive for spotted fever group IgG LPS. The specimens of two reacted primarily with *R. africae* SPA, and specimens from the remaining seven had nonspecific patterns. Five of these nine soldiers reported no symptoms. Two soldiers reported a runny nose, another reported a rash on the dorsal portion of the right upper arm, and another had a tender raised papule. By Western blot criteria, these nine soldiers would be considered infected and may represent asymptomatic cases. However, they did not meet our case definition and were not counted as case-patients in our analyses.

Patients' sera showed expected broad cross-reactivity with other spotted fever group rickettsiae by IFA. In sera collected at 10 weeks, 17 (51%) of the case-patients had identical *R. akari* and *R. conorii* titers; a difference of one dilution in titers was observed in12 (36%) of the case-patients. The remaining cases had *R. akari* titers that were fourfold less than their *R. conorii* titer. However, no Western blots were positive for *R. akari*. All but two 10-week serum samples from the case-patients were nonreactive at a titer of <1:32 with *R. typhi*

antigen by IFA, and none were positive by Western blot. Serum from one case-patient had titers of 1:128 o *R. akari*, *R. typhi*, and *R. conorii*. Serum from the second case-patient had titers of 1:512 to *R. conorii* and 1:64 to *R. typhi*.

Median ages of the case-patients and non–case-patients were 23.5 and 22 years, respectively. Although a larger proportion of cases was found among the older age groups, this trend was not significant (χ^2 trend, $p = 0.28$). Most of the soldiers (77%) were non-Hispanic white. There were too few soldiers in other race-ethnic groups to make separate comparisons, but when those from other ethnic backgrounds were combined into one group, the group had an attack rate (22%) similar to that of the non-Hispanic white soldiers (24%).

The illness observed in the soldiers was mild to moderately severe. Although all cases were symptomatic, some came to medical attention only through active case finding. Many case-patients had systemic complaints, commonly described as "flulike." Each of the classic symptoms associated with rickettsial diseases (i.e., fever, chills, headache, myalgia, and arthralgia) was reported by at least 59% of the case-patients but by fewer than 10% of the non–case-patients (Table 1). Gastrointestinal complaints of stomach pain, anorexia, nausea, or vomiting were also reported more frequently by the case-patients than by the non–case-patients. In contrast, diarrhea and upper respiratory symptoms were reported at similar rates among case-patients and non–case-patients. On physical examination, lymphadenitis was observed in 36 (92%) and right upper quadrant tenderness in 17 (44%) of the case-patients. The skin lesions began as a tender erythematous papule, which progressed over 24 to 36 hours to form one to three clear vesicles. The fragile vesicles ruptured, exposing an erythematous base, which darkened into a small eschar. The total duration of the evolution was 72 to 96 hours; 34 (87%) of case-patients had at least one eschar, and 10 of the case-patients had more than one. Of all eschars reported during physical examinations, 50% were located on the trunk; others were located on the head, neck, extremities, penis, and scrotum. No soldiers were hospitalized, and all case-patients responded rapidly to antibiotic treatment.

An incubation period of up to 6 days was estimated from the histories of the first two case-patients (Figure 2). The first case-patient reported onset of symptoms on January 20, 6 days after he arrived in Botswana as a member of an advance team. The second case-patient arrived in Botswana on January 18 and had onset of symptoms 6 days later. Thus, assuming a Botswana exposure, the time from infection to symptoms in these two case-patients could not have exceeded 6 days, an incubation time consistent with the 5- to 7-day period commonly reported for African spotted fever rickettsioses. Finally, since most case-patients reported symptoms during the 3 days before or the 4 days after leaving Botswana, we concluded that the period of high risk exposures occurred during field training in Shoshong and not in Gaborone.

Screening of clothing used during the field training found a crushed trombiculid mite on the boot of one of the case-patients. In addition, a live *Hyalomma marginatum* tick was found on parachute rigging used in Botswana. No rickettsiae were isolated from the tick or any human specimens.

No significant differences were found between case-patients and others in reported exposure to vectors, such as mosquitoes (68% vs. 62%; χ^2, $p = 0.53$) and chiggers (19% vs 10%; χ^2, $p = 0.14$). However, case-patients (68%) were more likely than non–case-patients (40%) to report tick bites (χ^2, $p = 0.007$). Only one soldier reported finding an engorged tick on his body. Fewer than 50% of all deployed soldiers reported using a DEET-containing insect repellent at any time during the exercise, and fewer than 10% of the soldiers reported using permethrin (anacaricide and repellent applied to clothing). No statistical differences were found between case-patients and non–case patients in reported uses of these personal protective measures. Self-reported exposures of case-patients (n = 31) vs non-case-patients (n = 101) to potential reservoirs

Table 1. Symptoms of case-patients and non–case-patients during a spotted fever outbreak

Symptoms	Case–patients; n=32(%)	Non–case-patients; n=107(%)	Risk ratio	95% Confidence interval
Fever	19 (59)	8 (7)	6.06	3.44,10.68
Chills	25 (78)	6 (6)	12.44	5.96,26.00
Headache	23 (72)	8 (7)	8.90	4.61,17.21
Muscle ache	26 (81)	10 (9)	12.40	5.56,27.66
Joint pain	21 (66)	9 (8)	6.94	3.78,12.73
Fatigue	27 (84)	6 (6)	17.35	7.26,41.43
Lymphadenitis	27 (84)	3 (3)	19.62	8.27,46.57
Abdominal pain	9 (28)	4 (4)	3.79	2.26,6.36
Anorexia	11 (34)	3 (3)	4.68	2.90,7.53
Nausea/vomiting	13 (41)	3 (3)	5.26	3.27,8.46
Diarrhea	7 (22)	21 (20)	1.11	0.54,2.30
Cough	4 (12)	9 (8)	1.38	0.58,3.33
Nasal congestion	7 (22)	17 (16)	1.34	0.66,2.74
Sore throat	6 (19)	16 (15)	1.23	0.57,2.63

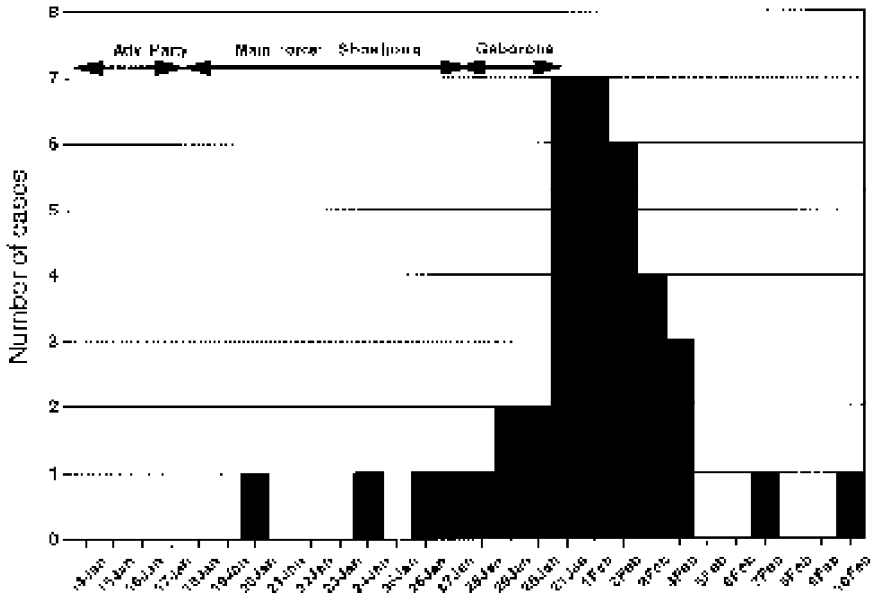

Figure 2. Epidemic curve of a spotted fever outbreak among U.S. troops.

of *R. conorii* (dogs: 52% vs 56%; rodents: 10% vs 35%; antelopes: 3% vs 7%; cattle: 81% vs 87%; horses:16% vs 25%; sheep: 42% vs 38%; and goats: 90% vs 87%) were not statistically different.

More than 85% (n = 117) of the soldiers slept on sleeping pads placed directly on the ground. Few (n = 18) soldiers reported sleeping on cots, litters, hammocks, or pallets. The attack rate was approximately twice as high among men sleeping on the ground (25%) as among those who did not (11%). Because of the small number who slept off the ground, this difference, was not statistically significant (risk ratio = 2.23; 95% C.I.: 0.58, 8.56). The attack rate among soldiers who maneuvered in the field during daily training exercises was lower than the rate among soldiers who remained at base camp (24% vs. 33%, respectively), but the difference was also not statistically significant (odds ratio = 1.40, 95% C.I.: 0.77, 2.56).

This report documents the occurrence of a large focal outbreak of a spotted fever group rickettsiosis among American soldiers participating in a short field-training exercise in Botswana. Little is known about the epidemiology of rickettsial diseases in Botswana. However, during the civil unrest that occurred in neighboring Rhodesia (now Zimbabwe) in the late 1970s, several thousand cases of tick typhus were reported among military personnel. In Zimbabwe, ticks removed from 100 soldiers were all larvae of *Amblyomma hebraeum* (6). In another study, 33% to 75% of *A. hebraeum* ticks, collected from animals or vegetation in four regions of Zimbabwe, were heavily infested with rickettsialike organisms (7). Only the recently characterized new rickettsia *R. africae*, but not *R. conorii*, the etiologic agent of boutonneuse fever, was detected in these ticks (4,7). In contrast, the dog ticks *Rhipicephalus simus* and *Haemaphysalis leachi* were less heavily infected (0% to 21%) and were only infected with *R. conorii*. *R. africae* has also been isolated from a hospitalized patient with fever, severe headache, and regional lymph-adenopathy but no rash other than an erythematous tick bite site(8).

On the other hand, in his original 1934 clinical description of African tick bite fever, Pijper (9) described a patient with a rash and eschar, whose condition he carefully distinguished from bouton-neuse fever. He clearly associated this milder disease with the "hardly visible" swarming larval veld ticks ("little pests") and not dog ticks. He also noted that even if the disease was not severe, it needed to be recognized by physicians and not confused with other potentially more devastating illnesses requiring more aggressive therapy.

Recent surveys of human antibodies in eight African countries have suggested a seroprevalence of antibodies to spotted fever rickettsiae of 0% to 52%, which parallels the distribution of *Amblyomma* ticks (10,11). The etiologic agent appeared to be *R. africae*, based on Western blot analysis (10). IFA analysis has been found inadequate in distinguishing human infections with *R. africae* and *R. conorii* (11).

The collective observations parallel our clinical, epidemiologic, and serologic findings on the Botswana outbreak and are most consistent with disease caused by *R. africae*. *R. africae* is closely related to *R. conorii* but has been proposed as a new species (5). Whether we consider this a newly emerging rickettsiosis or, in deference to the pioneering work of Pijper, a disease reemerging into our consciousness, African tick bite fever caused by *R. africae* may be epidemiologically distinct from urban *R. conorii* infections.

We have established the potential for high attack rates of this spotted fever rickettsiosis, but much more needs to be understood about the clinical severity of this disease and its potential for asymptomatic infections. Physically fit, healthy,

young soldiers may not represent a particularly good model for assessing the severity of disease in populations residing in areas where the disease is endemic.

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A Highly Heterogeneous HIV-1 Epidemic in the Central African Republic

The Central African Republic has been strongly affected by the human immunodeficiency virus (HIV) epidemic. Although itself sparsely populated, the republic borders five other African countries and is crossed by the trans-African highway, which connects West and Central Africa with East Africa (Figure 1). Wide genetic variation reported among HIV-1 subtypes (on the basis of small numbers of samples) suggests multiple introductions of HIV into the republic (1). However, except in small studies and unpublished reports (2), the distribution and serologic reactivities of HIV within the Central African Republic have not been characterized.

The need to conduct HIV surveillance has also been highlighted by reports of highly divergent strains of HIV-1 group O from neighboring Cameroon (3,4) and by recent findings that some of these strains were not reliably detected by current antibody screening tests (5-7). To assess the prevalence and distribution of HIV in the Central African Republic, the local Ministry of Health AIDS Program, assisted by the Centers for Disease Control and Prevention (CDC), initiated a nationwide sentinel surveillance survey in late 1994. Sentinel surveillance has been useful for obtaining information on HIV infection (8). In particular, serial sentinel studies have permitted the spread of HIV to be monitored and trends to be followed over time. In this report, we present findings on the distribution of HIV-1 infections and serologic reactivities of HIV in the Central African Republic.

From October through December 1994, a nationwide sentinel surveillance survey was conducted in 10 cities and towns: Bambari, Bangassou, Bangui, Berberati, Bossangoa, Bozoum, Bria, Gamboula, M'Baiki, and Mobaye (Figure 1). The two primary population groups included were women attending prenatal clinics and male and female attendees at sexually transmitted disease (STD) clinics. Two additional sites in the capital city, Bangui, were included to perform testing for laborers and university students.

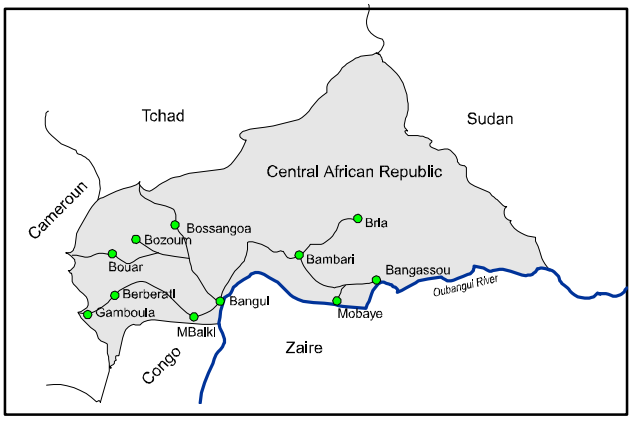

Figure 1. Map of the Central African Republic with location of sentinel sites.

Serum samples, anonymous and without any personal identifiers, were collected and transported to the National Public Health Laboratory in Bangui. Testing for this study was supported by CDC, which used U.S. Public Health Service guidelines for confirmation (9). All sera were tested with the Genetic Systems HIV-1/2-enzyme immunoassay (EIA) kit and all repeat positive sera were confirmed by HIV-1 Western blot (Cambridge Biotech). Specimens positive by initial EIA but negative for HIV-1 by Western blot or indeterminate were further tested for HIV-2 antibodies by an HIV-2 Western blot (Cambridge Biotech). In addition, samples from 11 sites (with sufficient quantity) that were positive by initial EIA were serotyped by using peptides representing the known HIV-1 subtypes A-F and the divergent HIV-1 group O (10,11).

A total of 2,259 persons were tested from 17 sites from 10 cities and towns. Between 2.7% and 30.7%, by site, were positive for HIV-1 by repeat EIA and Western blot confirmation (Table). A higher HIV-1 prevalence (25.3% to 30.7%) was observed among STD clinic attendees, whereas the prevalence among women at prenatal care clinics was generally >5% and as high as 16.7% (the exception was the lower rate in women from the prenatal care clinic in Gamboula. No HIV-2 infection was detected among the 175 persons whose serum was initially positive by EIA but negative for HIV-1 by Western blot or indeterminate by Western blot for HIV-2.

Among 247 samples serotyped by peptide EIA, 173 were HIV-1 positive by Western blot, 60 were indeterminate, and 14 were HIV-1 negative (Figure 2). No divergent HIV-1 group O infection was found among these persons. Serotype C (24%) was most prevalent among the monoreactive specimens. Many of the specimens were reactive to multiple peptides; 16 (9%) did not recognize any of the peptides and may represent more divergent HIV-1 strains.

The high seroprevalences (Table) indicate that HIV-1 infection is a serious public health problem in a number of groups in the Central African Republic. The wide variation of HIV-1 prevalence even

within similar groups in a region illustrates the heterogeneous nature of this epidemic. The HIV-1 prevalence was especially high among STD clinic attendees. Although the number of specimens from STD clinic attendess was relatively small, the high proportion of HIV-infected persons at all four STD clinics is a cause for concern. Because Bangui and Bambari are major crossroads for transport in the Central African Republic as well as on the east-west highway to other countries, the potential for further HIV spread is great.

Since STDs are associated with (and may facilitate) HIV transmission (12), STD diagnosis and control are integral components of HIV prevention. CDC, with financial support from the U.S. Agency for International Development, recently helped establish two pilot STD treatment sites in Bambari and Bria.

Although HIV-2, which is endemic to West Africa, has not been reported from the Central African Republic, reports of HIV-1 Group O from neighboring Cameroon highlighted the importance of monitoring these strains. Although no HIV-2 or divergent HIV-1 group O infections were found in this study, the extremely wide pattern of serologic reactivity among HIV-1-infected persons suggests a very heterogenous distribution of HIV-1 strains in the republic. This is confirmed by the wide variety of genotypes (subtypes) found in the republic in the past (1), and suggests that in certain populations, at least, multiple subtypes of HIV-1 have been introduced over time (7). This finding may be important to the development of effective future HIV vaccines for use in this region.

Table. Prevalence of HIV-1 by sentinel site/population

Sentinel site/Population	Positive by Western blot (%)	Total
Bambari		
Prenatal clinic women	20 (13.3)	150
STD clinic attendees	23 (26.7)	86
Bangassou		
Prenatal clinic women	16 (10.0)	160
Bangui		
Prenatal clinic women A	20 (13.3)	150
Prenatal clinic women B	8 (5.3)	150
STD clinic attendees	20 (25.3)	79
University students	14 (9.3)	150
Workers	23 (15.4)	149
Berberati		
Prenatal clinic women	16 (10.7)	150
Bossangoa		
Prenatal clinic women	9 (6.0)	148
Bozoum		
Prenatal clinic women	20 (13.2)	151
STD attendees	23 (30.7)	75
Bria		
Prenatal clinic women	22 (16.7)	132
STD attendees	24 (30.4)	79
Gamboula		
Prenatal clinic women	4 (2.7)	150
M'Baiki		
Prenatal clinic women	8 (5.3)	150
Mobaye		
Prenatal clinic women	9 (6.0)	150
Total number tested		2,259

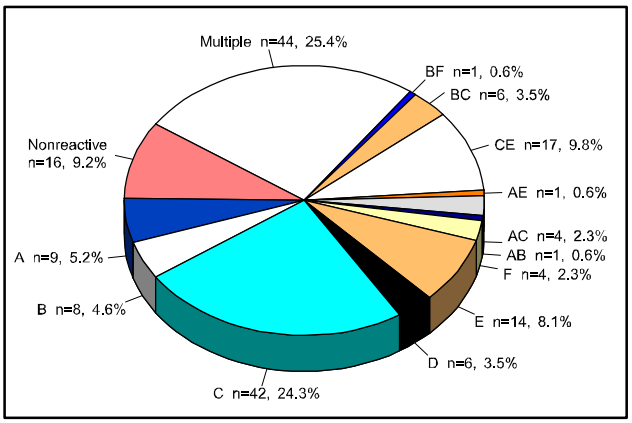

Figure 2. Serotype distribution among 173 HIV-1-infected persons. Letters indicate serologic reactivities to peptides to the given HIV-1 subtypes A-F. Sera reactive to two different subtype peptides are given as a combination of two letters. Multiple refers to sera that reacted to more than 2 different subtype peptides. Nonreactive refers to sera that did not react to any of the peptides used.

Since the quantity of specimens collected in this large-scale survey was not sufficient for genetic analysis, further studies are needed to characterize the genetic diversity of HIV in the Central African Republic. Given the limited search for HIV variants and the diversity of HIV subtypes recognized thus far, both a wider distribution of the HIV strains and the existence of yet more divergent variants seem likely (7). For these reasons, CDC plans to assist the Republic's National AIDS Control Program with further characterization of virus for divergent subtypes; these findings indicate the need for more resources to help the program maintain and expand HIV and STD prevention.

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Legionella-Like Amebal Pathogens––Phylogenetic Status and Possible Role in Respiratory Disease

Legionella-like amebal pathogens (LLAPs) are nonculturable, protozoonotic, gram-negative bacilli. They were named LLAPs because of their ability to infect and multiply intracellularly within amebae (Figure 1) in the same way that legionellae infect and multiply in protozoa and human alveolar macrophages (1-3). However, unlike other known legionellae, LLAPs do not grow routinely on buffered charcoal yeast extract agar (BCYE), or on any other known bacteriologic media.

The first known LLAP was isolated from Polish soil by Drozanski in 1954 (4). Until 1991 when it was described and given the name *Sarcobium lyticum* (5), it was simply referred to as an obligate intracellular bacterial parasite of free-living amebae (6, 7). The next isolation of an LLAP was in England more than 20 years later. Since then, LLAPs have been commonly isolated from various sources and found to infect a variety of amebae (Table 1). Because one LLAP cannot be differentiated from another, each strain is given the name LLAP suffixed by a designated number. Except for *S. lyticum*, LLAP-1, LLAP-5, and LLAP-3, which was a clinical isolate, all LLAPs were originally isolated from sources associated with confirmed cases or outbreaks of Legionnaires' disease (Table 1).

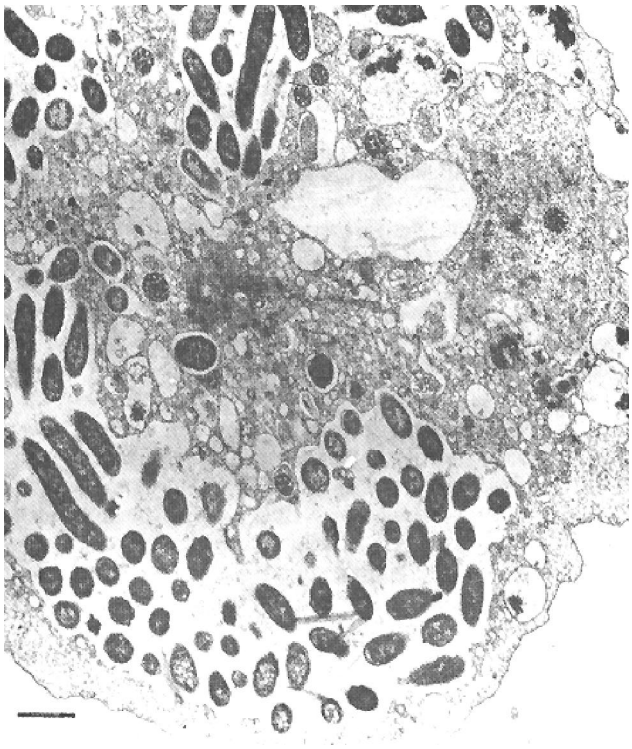

Figure 1. Transmission electron micrograph of *Sarcobium lyticum* within *Acanthamoeba castellanii*. Bar = 1 µm. (Reproduced with permisssion from reference 7).

Amebae are natural hosts for legionellae in the environment (2) and have been used to isolate legionellae from clinical samples (8). The relationship between these organisms is unique in that amebae, which generally use other bacteria as food, are parasitized by legionellae. The mechanism by which legionellae multiply and evade host defenses in amebae simulates the mechanism of infection in humans (9,10). The ability of *Legionella pneumophila* to infect epithelial cells in vitro is also enhanced by prior cultivation of the bacteria in amebae (11). This suggests that there may be a connection between the pathogenicity of legionellae for mammalian cells and animal models and their pathogenicity for amebae.

Whereas members of the genus *Legionella*, particularly *L. pneumophila*, are recognized as important etiologic agents of pneumonia (12), little is known about the clinical relevance of LLAPs; nevertheless, some evidence suggests that they may be an unrecognized and possibly significant cause of respiratory disease. LLAPs' inability to grow or multiply in the absence of amebae makes it difficult to isolate or identify them in clinical samples by conventional techniques. No serologic reagents are currently available for the detection or identification of LLAPs in clinical specimens. It is particularly noteworthy that one LLAP strain was originally isolated by amebal enrichment of a sputum specimen from a patient with pneumonia (13). Clinical samples from the patient were culture and serologically negative for *L. pneumophila* serogroups 1 through 6. However, incubation of the sputum sample with *Acanthamoeba polyphaga* resulted in numerous bacteria-infected amebae. The patient demonstrated a fourfold rise in antibody titer to the bacteria from the infected amebae. Even though the bacteria, now known as LLAP-3, could not be recovered on BCYE, the patient was treated for *Legionella* infection and recovered (13). Attempts to culture LLAP-3 on BCYE or on any other artificial media have been unsuccessful.

Since the discovery of LLAP-3, there has been further serologic evidence for the involvement of LLAPs in pneumonia. Between 1989 and 1993, the Public Health Laboratory (Leeds, UK) routinely

Table 1. Source of *Legionella*-like amebal pathogen (LLAP)

Strain	Host	Temp(°C)	Original source	Year
S. lyticum	AP**	35	Soil isolate[a]	1954
LLAP-1	AP	30^{N35}	Tank of portable water well	1981
LLAP-2	AP**	35	Garage steam cleaning pit	1986
LLAP-3	AP1	35	Sputum from pneumonia patient	1986
LLAP-4	AP	30^{N35}	Hospital whirlpool bath	1986
LLAP-5^{LST}	AP	30	Nursing home plant spray	1988
LLAP-6	AP**	35	Factory liquefier tower	1988
LLAP-7	AP**	35	Hotel whirlpool spa	1991
LLAP-8	HV^{NAP}	35	Hospital shower	1990
LLAP-9	AP**	35	Factory cooling water	1992
LLAP-10	AP	35	Ship air-conditioning system	1994
LLAP-11	AP	30	Factory cooling tower	1993
LLAP-12	AP	30	Factory cooling system	1994

a = first LLAP isolate. AP = infected *Acanthamoeba polyphaga* isolated from original material. AP1 = isolated after coculitivation of sample with *A. polyphaga*. HV = *Hartmannella vermiformis* isolated from original material. ** = also multiplies in *H. vermiformis*. NAP = does not infect *A. polyphaga*. LST = lost strain. N35 = does not multiply at 35°C.

screened sera from patients with suspected Legionnaires' disease for antibodies to LLAP-3 and found more than 10 cases with appropriately timed rising titers and several convalescent-phase sera. A few cases of respiratory disease with rising titers to LLAP-1 and little activity to LLAP-3 were also found (13).

In a study to assess the role of LLAPs in community-acquired pneumonia, 19% of 500 hospitalized patients with pneumonia of unidentified origin demonstrated a ≥ fourfold rise in antibody titer to 1:128 to at least one of nine LLAPs tested (14). These patients had no evidence of infection with known *Legionella* species (15). Cross-reactivity or nonspecific antibody rises may have accounted for some of the cases. Nevertheless, these findings are particularly important since no etiologic agent is identified for an estimated 50% of the 500,000 adult pneumonia cases occurring annually in the United States (16-18).

With the exception of *S. lyticum*, LLAPs have not been named or classified because the inability to cultivate them on laboratory media has limited their genetic and biochemical characterization. In this study, LLAPs were cocultured with *Hartmannella vermiformis* and *Acanthamoeba polyphaga*, depending on the most suitable host for each LLAP. Samples were cultured on blood agar and BCYE without cysteine to rule out bacterial contaminants. Bacteria were separated from amebae by differential centrifugation of cocultures before isolation of genomic DNA for ribosomal DNA sequencing.

The genes that code for rRNA in bacteria are highly conserved and can be used to establish the phylogenetic relationship between organisms (19). Previous 16S rDNA analysis of *S. lyticum* (20) and LLAP-3 (21), showed that they are closely related (91.6% to 95.8% rRNA similarity) to members of the genus *Legionella*. It was, therefore, suggested that LLAPs may have been responsible for the Gen-Probe positive specimens from patients with pneumonic illness, for whom conventional culture and serologic tests for legionellae were negative (13,22,23). The Gen-Probe system is a rapid diagnostic test for legionellae that uses a DNA probe complementary to the rRNAs of *Legionella* species (23,24).

To establish the degree of relatedness of all known LLAPs to each other and to other members of the family *Legionellaceae*, we recently amplified the 16S rRNA gene from five LLAP strains (LLAP-1, LLAP-2, LLAP-7, LLAP-8, and LLAP-9), using primers specific for Eubacteria. The amplified DNA was sequenced and compared with other bacterial sequences (including those of all *Legionella* species and LLAPs) held by GenBank/EMBL. Analysis of the 16S rRNA gene sequences showed that all 12 LLAPs in this study are closely related (91.2% to 97.0% similarity over 1303 bases of 16S rRNA sequence) to members of the genus *Legionella*. This is consistent with the 16S rRNA sequence identity (90.2% to 99.1% over 1303 bases) that exists between the 39 validly described members of the genus.[1]

Phylogenetic analysis showed that all the LLAPs in this study form a coherent cluster with other

[1]Genomic DNA was isolated by standard methods and extracted with phenol/chloroform. A 1400-bp 16S rDNA segment was amplified by using primers specific for Eubacteria and based on a consensus of *Legionella* 16S rRNA sequences. Each strain was sequenced at least 3 times to ascertain accuracy of obtained results. 16S rRNA sequences for all *Legionella* species, those of some previously sequenced LLAPs and *Coxiella burnetii* were obtained from GenBank/EMBL and aligned with sequences generated in this study, using the GCG sequence analysis package (version 8.0). Comparisons were performed at NCBI using the BLAST network service (32). Ambiguous and hypervariable regions were removed before phylogenetic analysis, which was carried out on 53 strains for 1303 nucleotides by the neighbor-joining method from the Phylogeny Inference Package (Phylip) (33), version 3.5. Multiple datasets (X100) were analyzed, and different distance models were compared to ensure reliability. Some of the 16S rRNA sequences included in the data analysis are of unpublished and undescribed stains.

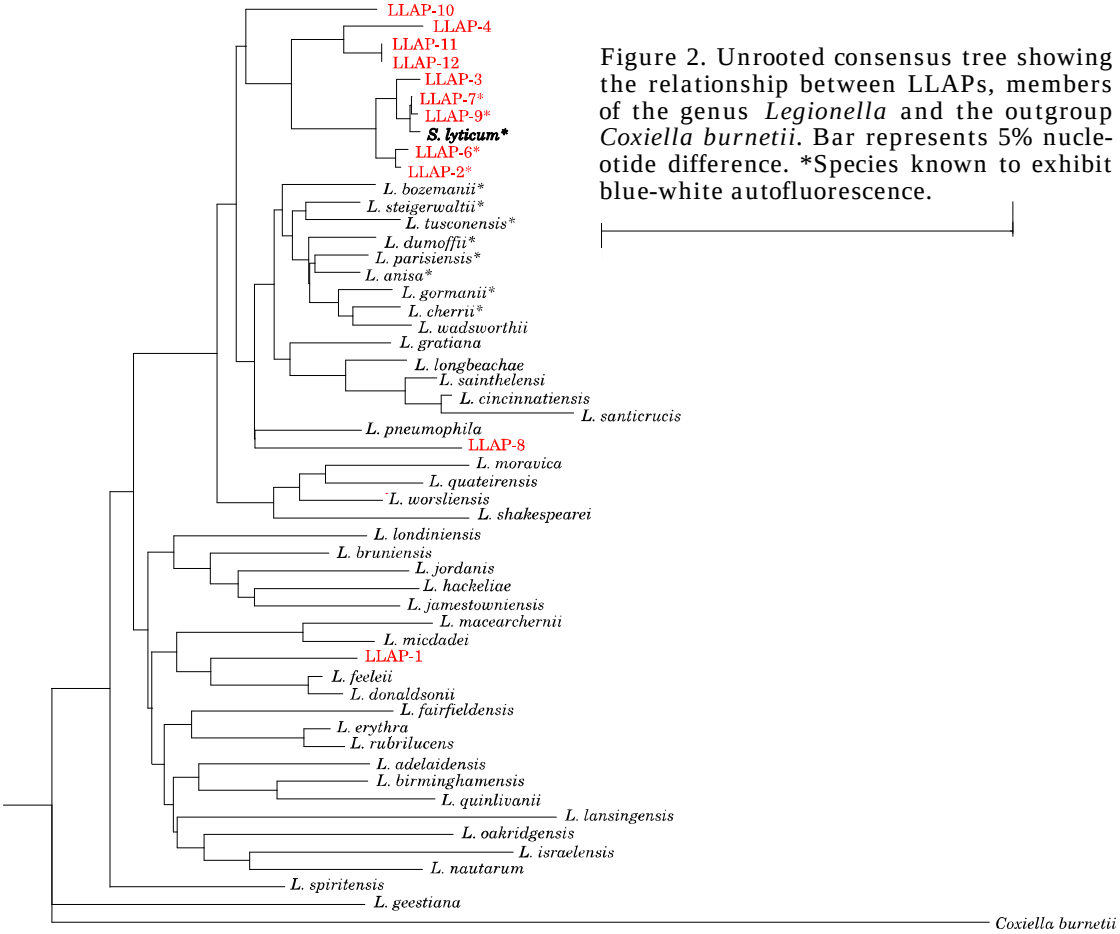

Figure 2. Unrooted consensus tree showing the relationship between LLAPs, members of the genus *Legionella* and the outgroup *Coxiella burnetii*. Bar represents 5% nucleotide difference. *Species known to exhibit blue-white autofluorescence.

members of the family *Legionellaceae* (Figure 2). LLAP-1, LLAP-8, and LLAP-10 were grouped in distinctly different clades, whereas LLAP-2, LLAP-3, LLAP-6, LLAP-7, LLAP-9, and *S. lyticum* formed a single well-defined clade, most closely related (≥96.9% similarity) to another clade consisting of LLAP-4, LLAP-11, and LLAP-12 (Figure 2). LLAP-2, LLAP-3, LLAP-6, LLAP-7, LLAP-9, and *S. lyticum* shared a 16S rRNA sequence similarity of ≥98.9%. LLAP-11 and LLAP-12 had 16S rRNA sequence homology of 100%. Overall 16S rRNA sequence similarity values from some *Legionella* species, all the LLAPs in this study, and *Coxiella burnetii* are shown in Table 2. These results suggest that these 12 LLAP strains may represent up to five new species of *Legionella* and their various subtypes on the basis of a minimum sequence identity of 97.0% between strains of the same species (Table 3). However, they may include new subtypes of previously described species. It is also possible that these strains represent more than five species because, even though 16S rRNA sequence identity can be used routinely to distinguish and establish relationships between genera and well-resolved species, it may not be sufficient to guarantee species identity (25,26). For example, in this study, several *Legionella* species had sequence similarities exceeding 98.0%. *L. cherrii* and *L. wadsworthii* shared a sequence similarity of 98.7%. *L. rubrilucens* and *L. erythra* had a sequence identity of 99.1%. *L. sainthelensi* and *L. cincinnatiensis* were likewise identical by 99.1%. Furthermore, bacterial strains exhibiting more than

Footnote 1 continued

Accession numbers for sequences included in this study are as follows: LLAP-1 NL, LLAP-2 U44909, LLAP-3 X60080, LLAP-4 X97357, LLAP-6 X97357, LLAP-7 U44910, LLAP-8 NL, LLAP-9 U44911, LLAP-10 X97363, LLAP-11 X97362, LLAP-12 X97366. *L. adelaidensis Z49716, L.anisa X733394, L. birminghamensis Z49717, L. brunensis X73403, L. cherrii X73404, L. cincinnatiensis X73407, Coxiella burnetti M21291, L. donaldsonii Z49724, L.dumoffii X73405, L.erythra Z32638, L. fairfieldensis Z49722, L. feelii X73395, L. geestiana Z49723, L. gormanii Z32639, L. gratiana Z49725, L. hackeliae M36028, L. israelensis X73408, L. jamestowniensis X73409, L. jordanis X73396, L. lansingensis Z49727, L. londiniensis Z49728, L. longbeachae M36029, L. maceachernii X60081, L. micdadei M36032, L. moravica Z49729, L. nautarum Z49730, L. oakridgensis X73397, L. parisiensis Z49731, L. pneumophila M59157, L. quateirensis Z49732, L. worsliensis Z49739, L. quinlivanii Z49733, L. rubrilucens X73398, L. santicrucis Z49735, Sarcobium lyticum X66835, L. shakespearei Z49736, L. spiritensis M36030, L. steigerwaltii X73400, L. sainthelensi Z49734, L. tusconensis Z32644, L. wadsworthii X73401.* NL = accession number not yet allocated.

Table. 2. 16S rRNA similarities (%) between members of the genus Legionella and different LLAP strains.

	L1	L2	L3	L4	SL	L6	L7	L8	L9	L10	L11	L12	Lpn	Lfl	Lgr	Lts	Lpr	Lst	Ldm	Lwd	Cox
L1	94.1	93.6	93.9	93.7	94.0	93.7	94.1	93.7	94.4	94.1	94.1	93.8	96.6	93.7	94.0	93.9	94.3	94.1	94.0	85.4
L2		98.9	96.9	99.2	99.8	99.4	95.4	99.3	96.6	97.4	97.4	95.8	94.5	96.2	96.3	96.3	96.7	96.8	96.3	85.6
L3			96.9	99.4	99.0	99.5	94.9	99.5	96.3	97.0	97.0	95.8	93.9	95.7	96.2	96.1	96.5	96.4	96.0	85.6
L4				97.0	97.0	97.2	95.1	97.1	96.0	98.5	98.5	95.5	93.4	95.5	95.9	95.9	96.0	95.9	96.0	85.2
SL					99.1	99.9	94.9	99.8	96.3	97.2	97.2	95.8	94.1	95.5	96.1	95.9	96.4	96.3	96.0	85.6
L6						99.3	95.1	99.2	96.8	97.3	97.3	95.6	94.4	96.1	96.4	96.3	96.5	96.5	96.1	85.5
L7							95.0	99.9	96.5	97.4	97.4	95.9	94.1	95.7	96.3	96.0	96.6	96.5	96.1	85.8
L8								94.9	94.9	95.7	95.7	96.1	95.1	95.9	95.9	95.9	95.8	96.1	95.4	85.5
L9									96.4	97.3	97.3	95.8	94.0	95.6	96.2	95.9	96.5	96.4	96.0	85.8
L10										96.3	96.3	96.7	94.4	96.3	95.9	97.0	96.3	96.4	96.6	85.9
L11											100.0	96.3	94.0	96.3	97.0	96.7	96.9	96.5	96.5	85.0
L12												96.3	94.0	96.3	97.0	96.7	96.9	96.5	96.5	85.0
Lpn													94.3	96.8	96.9	97.3	97.5	97.0	96.8	85.5
Lfl														94.4	94.9	95.0	95.2	95.4	95.1	86.2
Lgr															97.1	97.2	97.4	97.6	96.4	85.5
Lts																97.8	98.1	97.3	96.8	85.8
Lpr																	98.1	98.4	98.0	86.3
Lst																		98.4	97.6	85.7
Ldm																			98.0	86.1
Lwd																				85.8

L. LLAP, Lpn. *L. pneumophila*, Lfl. *L. feeleii*, Lgr. *L. gratiana*, Lts. *L. tusconensis*, Lpr. *L. parisiensis*, Lst. *L steigerwaltii*, Ldm. *L. dumoffii*, Lwd. *L. wadsworthii*, Cox. *Coxiella burnetii*.

exhibiting more than 99.5% rRNA sequence identity may belong to different species by current standards for DNA-DNA hybridization (70% DNA reassociation) (25). DNA hybridization studies are, therefore, needed to determine species identity.

Preliminary fatty acid analysis showed that these LLAPs have branched fatty acids, characteristic of members of the genus *Legionella*. The gas liquid chromatography fatty acid profiles obtained, however, appeared to be distinct from those of other known legionellae (data not shown).

LLAP-2, LLAP-3, LLAP-7, and LLAP-9 reacted positively with antiserum prepared against *S. lyticum*. Fluorescent antibody tests also showed some LLAPs to react positively with the polyvalent *Legionella* antiserum contained in the Remel *Legionella* Poly-ID Test Kit.[2]

Several attempts have been made to culture LLAPs on conventional *Legionella* media, BCYE. *S. lyticum* could occasionally be recovered from a coculture with amebae on alanine supplemented BCYE (27). We attempted to grow 12 LLAP strains on regular BCYE and BCYE supplemented with various amino acids, including alanine, and have recently succeeded in cultivating LLAP-6, LLAP-7, LLAP-9, and LLAP-10 on bacteriologic media for the first time. All colonies had the characteristic cut-glass appearance associated with legionellae. LLAP-1 had previously been cultured on BCYE supplemented with sodium selenate (13,28). In this study, LLAP-1 was successfully cultured on regular BCYE and BCYE supplemented with alanine in the absence of sodium selenate. *S. lyticum*, LLAP-1, LLAP-6, LLAP-7, LLAP-9, and LLAP-10 had a cysteine requirement for growth. Other factors influencing growth are yet to be determined, but sodium selenate and alanine may not necessarily be required for growth. Cultivation of LLAPs on bacteriologic media still cannot be considered a routine process because recovery is not always possible, and extremely low numbers of the plated bacteria produce colonies.

Some species of *Legionell*a are known to produce colonies that autofluoresce on exposure to long-wave ultraviolet (UV) light. This facet of their colonial morphology often assists in identifying and speciating isolates. LLAP-2, LLAP-6, LLAP-7, LLAP-9, and *S. lyticum* exhibited blue-white autofluorescence when exposed to long-wave UV light while LLAP-1 and LLAP-10 did not autofluoresce. This is consistent with the clustering pattern (Figure 2) where the blue-white fluorescent LLAPs formed a well-defined clade.

On the basis of these results, we propose that all 12 *Legionella*-like amebal pathogens be included in the genus *Legionella* as *Legionella* species, with

[2]The Remel Poly-ID kit used in this study is designed to identify 22 species and 31 serogroups of *Legionella* (34,35).

Table 3. Possible number of species represented by *Legionella*-like amebal pathogens (LLAP)

Species 1	Species 2	Species 3	Species 4	Species 5
LLAP-2	LLAP-1	LLAP-8	LLAP-10	LLAP-4
LLAP-3				LLAP-11
LLAP-6				LLAP-12
LLAP-7				
LLAP-9				
S. lyticum				

Note. The table above includes the minimum number of species that may be represented by the 12 LLAPs in this study.

strain specifications. A proposal was recently made for the transfer of the species *S. lyticum* to the genus *Legionella* as *L. lytica* comb. nov. (29). It appeared that *L. lytica* was being proposed for LLAPs. However, our study suggests that LLAPs may represent a minimum of five species. Consequently, the species name *L. lytica*, though appropriate for the strain formerly classified as *S. lyticum*, cannot be applied to all LLAPs.

The genus *Legionella* is defined in *Bergey's Manual of Determinative Bacteriology* as follows:

> Rods measuring 0.3-0.9 x 2.0-20.0 µm or more. Do not form endospores or microcysts and are not encapsulated. Not acid fast. Cells stain gram negative. Motile by one, two, or more straight or curved polar or lateral flagella; non motile strains are occasionally seen. Aerobic. L-Cysteine hydrochloride and iron salts are required for growth. Oxidase test is negative or weakly positive. Nitrates are not reduced. Urease negative. Gelatin is liquified. Branched chain fatty acids predominate cell wall. Chemoorganotrophic, using amino acids as carbon and energy sources. Carbohydrates are neither fermented nor oxidized. Isolated from surface water, mud, and from thermally polluted lakes and streams. There is no known soil or animal source. pathogenic for humans, causing pneumonia (Legionnaires' disease) or a mild, febrile disease (Pontiac fever) (30,31).

The description in *Bergey's Manual* should be amended to include the following statement: "Some legionellae appear to be primarily obligate intracellular parasites of amebae that exhibit little or no growth on current laboratory media."

Further characterization is needed to classify each LLAP strain to species level and assess the need for additional amendments to the present description of the genus *Legionella*. Appropriate diagnostic techniques are needed to determine the clinical relevance of LLAPs, and particularly their possible role in respiratory disease.

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Role of Enterovirus 71 in Acute Flaccid Paralysis After the Eradication of Poliovirus in Brazil

As a result of the successful initiative to eradicate poliomyelitis in the Americas, Brazil is now free of circulating wild poliovirus. The last cases of acute flaccid paralysis (AFP) with confirmed wild poliovirus isolation occurred in March 1989 (1). Since the elimination of wild poliovirus-associated poliomyelitis in Brazil and the American region by intensive mass vaccination campaigns, acute paralytic illnesses from other causes constitute a greater proportion of suspected cases. Enterovirus 71 (EV71) can cause paralytic disease with persistent flaccid paralysis (which may be confused with paralysis caused by wild polioviruses); therefore, the presence of this enterovirus in a community may complicate the evaluation of poliomyelitis control progress. Serologic evidence of EV71 infections (demonstrated by neutralization assays) has been observed in AFP patients in Brazil (9). In addition, EV71 isolates have been obtained from patients with suspected cases of poliomyelitis and their healthy contacts from different areas in Brazil and from AFP patients in Peru and Bolivia (3, Ferreira et al., in preparation).

EV71, the most recently recognized human enterovirus in the family *Picornaviridae*, has been associated with outbreaks of hand-foot-and-mouth disease and central nervous system diseases (e.g., aseptic meningitis, encephalitis, and poliomyelitis-like paralytic diseases) with persistent or transient paralysis (2,4,5). Despite some understanding of the variability in clinical manifestations and epidemiologic pattern, little is known about the contribution of EV71 to overall AFP in different countries.

Immunoglobulin M (IgM) antibody to EV71 was measured by using an μ-capture enzyme immunoassay (EIA) (6). The antigen was prepared from the BrCr strain of EV71, and biotinylated anti-EV71 monoclonal antibody as detector was also prepared (6). The results were expressed as the difference in mean optical density values measured in triplicate wells of positive antigen (P) and negative controls (N). A specimen was considered positive if the observed P-N was ≥ 2 standard deviations (SD) above the mean of the optical densities for the negative control serum (P-N ≥ 0.25).

Serum samples were obtained from 92 infants from different regions of Brazil who had suspected poliomyelitis and symptoms of AFP during 1989 and 1990. The 138 samples included paired serum samples (S1 and S2) from 46 patients (92 sera) and 46 serum samples (S1 only) from 46 patients. Acute-phase serum samples (S1) were obtained 1 to 15 days after the onset of symptoms, and a second group of samples were obtained (S2) 15 to 45 days later; all were from children whose stool specimens were negative for wild poliovirus.

To check the assay reagent cross-reactivity with polioviruses, all 138 serum specimens were tested in parallel by the same described method, but by using poliovirus serotypes 1, 2, and 3 Sabin strains instead of EV71 as the antigen. The assay had also been checked in a limited fashion for heterotypic response due to poliovirus infection by testing sera from a limited number of known vaccine-associated paralytic poliomyelitis cases (4). These vaccine associated case sera showed no evidence of IgM response to EV71 antigen from poliovirus infection.

Specimens from 20 of 92 patients with suspected cases of poliomyelitis had positive IgM responses to EV71 (Table). Three patients (2287, 3805, and 3489) whose specimens were positive had only a single serum specimen (S1) available. Four serum specimens (1819, 2018, 1918, and 1906) only had positive IgM titers for the first serum (S1), and five specimens from Bahia State (429, 784, 1130, 1325, and 780) had an IgM response only for the convalescent-phase sera (S2). The IgM-positive AFP specimens were from residents of eight widely dispersed regions of Brazil with eight specimens collected in 1989 and 12 collected in 1990. Six of 19 AFP patients with IgM positive results had residual paralysis after 60 days, which is considered to be clinically characteristic of poliomyelitis due to poliovirus. The remainder of the AFP patients only had a transient paralysis from which they recovered.

The presence of residual paralysis and EV71 IgM antibodies in 20 (21.7%) of the 92 AFP patients from eight geographic regions indicates that EV71 may be causing AFP-like poliomyelitis throughout Brazil. The incidence of EV71 infection associated with AFP in Brazil is high when compared with its incidence in the United States, where EV71 infections leading to AFP are very uncommon but have rates not much lower than those of vaccine-associated paralytic poliomyelitis (5,8). Poliomyelitis-like paralytic illnesses in Brazil in 1989 and 1990 are not likely attributable to polioviruses; most of these cases occurred after the last confirmed case of wild poliovirus paralysis, and poliovirus was not isolated from any patient's stool specimens. EV71 causes persistent flaccid paralysis; our results link EV71 with

Table. Data from patients positive for enterovirus 71(EV71)

Case	Initial symptom	Brazilian state	EV71 IgM S1	EV71 IgM S2	Year	Sequelae after 60 days
429	AFP	BA	-	+	1989	Yes
784	AFP	BA	-	+	1989	No
1130	AFP	BA	-	+	1989	No
1325	AFP	BA	-	+	1989	Yes
780	AFP	BA	-	+	1989	NF
2287	AFP	BA	+	NA [a]	1990	No
907	AFP	DF	+	+	1989	No
1014	AFP	DF	+	+	1989	No
1394	AFP	RS	+	+	1989	Yes
2593	AFP	RS	+	+	1990	Yes
1826	AFP	SC	+	+	1990	No
1819	AFP	SC	+	-	1990	Yes
1815	AFP	SC	+	+	1990	Yes
1809	AFP	SC	+	+	1990	No
1940	AFP	PE	+	+	1990	No
2018	AFP	RN	+	-	1990	No
1918	AFP	PB	+	-	1990	No
1906	AFP	RN	+	-	1990	No
3805	AFP	RJ	+	NA	1990	No
3849	AFP	RJ	+	NA	1990	No

BA = Bahia State; DF = Distrito Federal; RS = Rio Grande do Sul; SC = Santa Catarina; PE = Pernambuco; RJ = Rio de Janeiro; RN = Rio Grande do Sul; PB = Paraiba.
[a]NA = not available; NF = no follow-up.

some of these AFP cases in Brazil. Subsequent testing on specimens from some of these patients resulted in the isolation of EV71; neutralizing antibodies were found with seroconversion (3, 9,Ferreira et al., in preparation). Before the eradication of wild polioviruses in Brazil, all AFP patients who had residual paralysis as described in this study would have generated concern about undetected wild poliovirus circulation because their cases resembled poliovirus-caused paralysis.

The pattern of results obtained with the acute-phase and convalescent-phase sera in this study is not uncommon for serologic tests of enterovirus infections. Only seven patients with AFP had two positive serum specimens; for nine AFP patients, only one of the two specimens was positive. Some S1 samples were collected during the early stages of the disease when the IgM titers may not have been detectable. Our results are consistent with seroconversion data as has been observed with enterovirus 70 infections and a similar assay (7); however, neutralizing antibodies were detected in this study's convalescent-phase sera (9). Additionaly, late specimen collection or inadequate specimen storage or handling before receipt in the laboratory might have affected IgM titers adversely because these conditions were not carefully controlled. Although cross-reactivity of antibody from other nonpolio enteroviruses cannot be excluded, the possibility can only be addressed directly by additional testing with other serologic assays. However, when the same assay was used to examine specimens from known cases of vaccine-associated paralytic poliomyelitis from the United States, no heterotypic response was observed (4).

Since 1990, for technical and operational reasons, serologic methods have not been used to confirm cases of AFP as caused by wild poliovirus; therefore, serum samples are no longer available for routine poliovirus diagnosis. Our recent efforts have been directed towards isolating and characterizing nonpolio enteroviruses (with emphasis on EV71) from patients with suspected poliomyelitis and towards examining the geographic and temporal relationship between these AFP cases.

Public health personnel and pediatricians should be alerted to the possible role of nonpolio enterovirus infections in the differential diagnosis of AFP or other severe central nervous system diseases, particularly in areas where the circulation of wild poliovirus has been interrupted. The laboratory diagnosis of all AFP cases should routinely include tests capable of detecting EV71 as well as other enteroviruses once the primary objective of poliovirus eradication has been achieved.

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Bancroftian Filariasis Distribution and Diurnal Temperature Differences in the Southern Nile Delta

In recent years the prevalence of Bancroftian filariasis has increased dramatically in the Nile delta (1). The resurgence is believed to be associated with an increasing accumulation, in and near villages, of surface and subsurface water that serves as breeding sites for *Culex pipiens* mosquitoes. This water increase could be due to the growing number of irrigation channels and to more reliable water supply and distribution since the Aswan High Dam was completed in 1971.

A correlation between remotely characterized environmental features and the distribution of some "focal" diseases (2) has been suspected; however, no direct association has been reported. Intermediate factors affecting disease distribution, such as vector abundance (3,4) and risk for disease transmission (5), have been described. Recently, we examined diurnal temperature difference (dT) maps derived from the advanced very high resolution radiometer of the polar-orbiting National Oceanographic and Atmospheric Administration environmental satellite in relationship to the distribution of schistosomiasis in the Nile delta (6). dTs are derived from thermal scanning radiometry, and they indicate surface and subsurface moisture contained in the soil and plant canopy. The distribution of schistosomiasis has been stable for decades, which suggests that infection prevalence relates to intermediate host snail abundance which in turn is influenced by environmental moisture factors. Because dT values are associated with surface moisture and standing water, we hypothesized that those values should correlate well with the distribution of Bancroftian filariasis cases. We report here the initial test of this hypothesis.

First, we determined the prevalence of Bancroftian filariasis between 1985 and 1993, by surveying 297 villages in the southern Nile delta (1). Prevalence was determined on the basis of microfilaremia diagnosis and on the presence of filarial antigenemia and clinical evidence of prior infection, or both. We grouped the prevalence data into four categories (0% to 1%, 2% to 9%, 10% to 19%, and 20^{+}%), and used approximate village coordinates to plot the results on a map. Next, a more accurate version of this map (provided by one of the authors) was digitized to create a file of village latitude and longitude coordinates; this file was overlaid onto the dT map with TeraScan software (SeaSpace Corporation, San Diego, CA) (Figure).

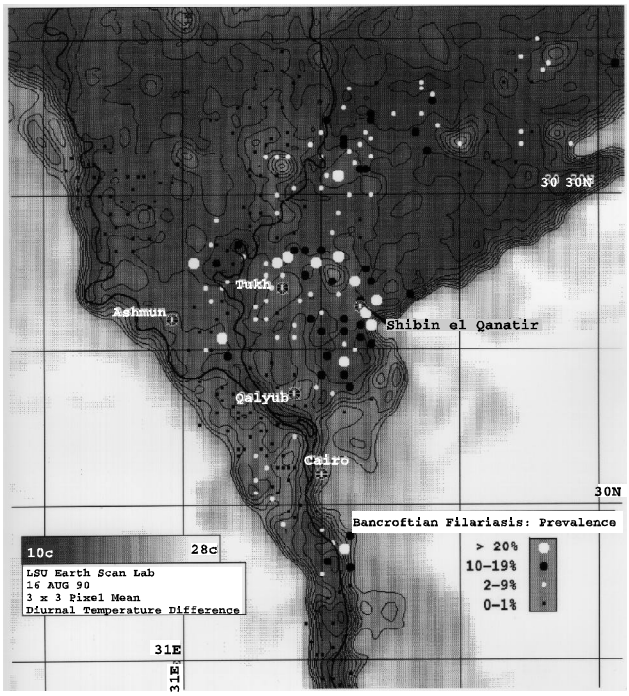

Figure. Mean diurnal temperature differences of southern Nile delta, August 16, 1990, with study village sites superimposed according to Bancroftian filariasis prevalence category.

Satellite image data from the advanced very high resolution radiometer from August 16, 1990, were analyzed by using TeraScan to determine dTs for the southern Nile delta. The time of data collection was approximately 01:00 and 13:00 hours, local time. Point dT values were averaged for 3 x 3 pixel areas (10 km^2), centered on the latitude and longitude of each study site to reduce false precision of individual pixels. Digitized filariasis prevalence data were superimposed and assigned mid-point values for each of the four prevalence categories (0.5%, 5%, 15%, and 25%, respectively). The number of villages assigned to each of these categories, respectively, was 169, 74, 37, and 13. Four villages in the 0% to 1% prevalence category fell beyond the southern border of the computerized dT map.

Single factor analysis of variance between the four categories showed that for the four groups, values were significantly different ($F = 5.42$, $p = .0012$). Stepwise polychotomous logistic regression was used to model prevalence by using the dT value as the

only predictor. The model that predicts prevalence showed significant improvement for each of the three prevalence levels over the (negative) reference category of 0.5% (improvement, $\chi^2 = 17.11$, $p = .002$).

The high degree of correlation between the remotely sensed dT values and the "ground truth" filariasis prevalence data was unexpected, considering the low resolution of the data and the relative imprecision of the village coordinates. We are now gathering data on the precise numerical prevalence level for each village and are using a hand-held global position system receiver in the villages to obtain exact coordinates. Because the effect of random error due to the imprecision of the measurements can only diminish correlations, we anticipate that more precise data will result in even closer associations.

Our efforts are directed towards prospectively determining the predictive power of dT with respect to the village-specific prevalence of Bancroftian filariasis from adjacent parts of the Nile delta for which prevalence data are not yet available. Ultimately, we hope to determine the degree to which dT values, which are available for much of the earth's surface, can be used to signal the presence of Bancroftian filariasis in the Nile delta and perhaps in other disease-endemic regions.

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Sentinel Surveillance for Yellow Fever in Kenya, 1993 to 1995

Yellow fever (YF) is an acute mosquito-transmitted viral infection, endemic and occasionally epidemic in many countries of Africa and South America. Periodic outbreaks of yellow fever in East Africa have been reported since 1940 (1,2) (Figure 1). The largest outbreak, an estimated 300,000 cases, occurred from 1960 to 1962 in Ethiopia (3). In 1966, YF reappeared in Arba-Minch, Ethiopia, east of Lake Abaya, in an area not affected by the outbreak of 1960 to 1962. During the 1966 outbreak, 2,200 cases (450 deaths) were reported (4). Despite the prevalence of anti-YF antibody in northern Kenya (up to 14%) (5,6), no outbreaks of YF were reported in Kenya until 1992 when epidemic YF emerged in Kenya for the first time, with at least 54 cases and 29 deaths (7). Cases of hemorrhagic fever were first reported in September 1992, from the southern parts of Keiyo (Kerio Valley), Baringo, and the Koibatek Districts (former Elgeyo-Marakwet and Baringo districts) in the Rift Valley Province, northwest Kenya (Figure 2). Of the 54 patients with hemorrhagic fever, 35 (67%) were male. Of the 48 patients whose definite age was known, 41 (85%) were 10 to 39 years old. Twenty-one (39%) of the 54 patients had serologic evidence of recent YF infection, and three of the 21 had cases confirmed by isolation of YF virus. The outbreak ended when the entire population of these districts was vaccinated. A surveillance system involving 13 sentinel sites was established 6 months after the epidemic was over. Six health facilities were located within the outbreak area of Keiyo, Koibatek, and Baringo districts. Seven facilities were situated in the surrounding areas of Nakuru, Nyandarua, Kericho, and the Uasin Gishu districts. In 1994, the number of sentinel sites was increased to 18. Sentinel facilities were provided with case investigation forms and equipment for blood collection and storage. Surveillance activities were ongoing, and supervisory visits were conducted once every 6 weeks.

In Kenya, there are three levels of health care delivery: hospitals, health centers, and dispensaries. In addition, missionary hospitals provide independent health services. Surveillance training candidates were selected from district hospitals, health centers, and mission hospitals. Training focused on case recognition, record keeping, use of a YF case investigation form, handling of serum samples, and obtaining exposure information. A field guide for YF surveillance was developed in collaboration with the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC). A 1-day training workshop was followed by a visit to the collaborating surveillance centers. Medical staff at the health facility who could not attend the workshop received on-the-job training. A surveillance team consisted of a medical officer (or scientist from the Virus Research Centre), a laboratory technologist, the provincial public health officer from Rift Valley Province, and a driver. In October 1994, a second 1-day training workshop was offered for five additional sentinel health facilities. For surveillance purposes, a patient was considered to have a case of YF if two of the following symptom complexes were present: jaundice; hemorrhage; signs of encephalitis or renal involvement; a temperature $\geq$ 38°C.

Serum was separated from blood samples on the day of collection and stored frozen, until the serum specimens could be transported by the surveillance team. Vaccine carriers with ice packs were used to transport samples (along the surveillance route) to the Kenya Medical Research Institute (KEMRI). In 1994, a liquid nitrogen container was added for transporting samples. Samples were tested for YF antibodies by the IgM-capture enzyme-linked immunosorbent assay (MAC ELISA) (8) at the Virus Research Centre. Selected acute-phase surveillance samples were also subjected to virus isolation attempts, in which newborn mice and tissue cultures were used. All serologic results were confirmed by either CDC's laboratory in Fort Collins, Colorado, or the Institute Pasteur, Paris. Persons whose illness met the surveillance case definition were considered positive for recent YF infection if

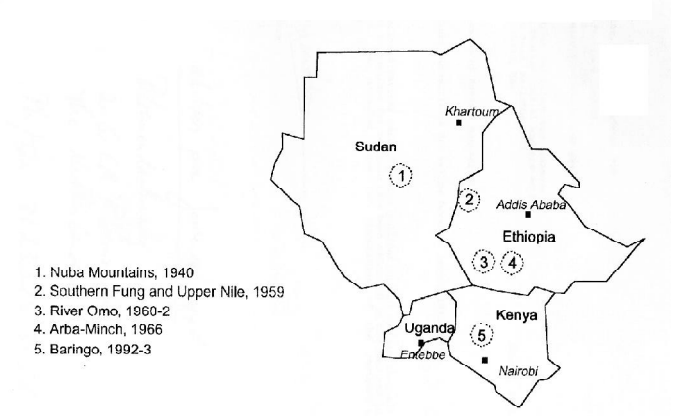

Figure 1. Yellow fever epidemics, East Africa.

YF-specific IgM antibody was present in acute-phase serum samples by MAC ELISA. Samples that were borderline positive by IgM MAC ELISA were tested for neutralizing antibody by the plaque reduction neutralization test with YF virus. Results of this sentinel surveillance from October 7, 1993, to December 31, 1995, are reported here.

One hundred-fifty persons, of whom 131 (87%) had jaundice, met the surveillance case definition. These were identified by 17 health facilities, during a 27-month period (average, 5.5 per month; range, 0-12); 85 (57%) were male. Of the 138 persons whose age was known, 117 (85%) were under 40 years of age (average age, 21.4 years; range, 1 to 70 years).

Ten serologically confirmed cases were identified by four health facilities. All attempts to isolate the virus were unsuccessful. The onset of the first confirmed case was March 1, 1994. The onset of the latest case was May 15, 1995. Seven confirmed cases were from an area not known to be affected during the 1992 to 1993 epidemic. The homes of six patients border the districts where no YF vaccinations are given (Figure 2). One patient (1994, Songhor, Nandi District) lived far outside this area and had no history of travel to the known YF-endemic area. Five patients with confirmed cases were male: eight (80%) were under 40 years of age (average age, 26.5 years; range, 8 to 48 years). All patients with confirmed cases had jaundice, and five (50%) hemorrhaged from the nose or gums. Three patients died (two were female). One was not vaccinated for YF, and the vaccine status of two was unknown. The case-fatality rate for patients with confirmed YF was 30%. All surviving patients had been vaccinated (Table).

Timely reporting of disease emergencies is not feasible through the existing health information system in Kenya. When YF emerged there in September 1992, disease investigation was delayed, and the diagnosis was not confirmed until January 1993. In the case of a limited health information system, sentinel surveillance for YF, with active follow-up by collaborating centers, seems appropriate for monitoring disease after an epidemic.

Table. Characteristics of patients with confirmed YF cases, October 7, 1993, to December 31,1995

Characteristics	Number	Percentage (%)
Sex, male	5	50
female	5	50
Age, < 40 yeas	8	80
Jaundice	10	100
Hemorrhage	5	50
Deaths	3	30
History of YF vaccination	7[a]	70

[a]Only non–fatal cases.

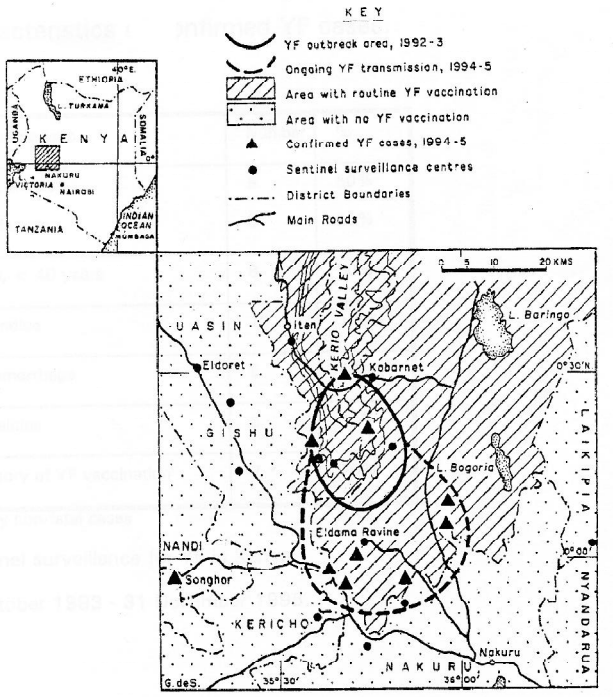

Figure 2. Yellow fever surveillance in the Rift Valley, Kenya.

Most patients surveyed had fever and jaundice. All patients with confirmed YF cases had jaundice, and 50% hemorrhaged from the nose and gums. Mild, nonspecific, and nonfatal clinical forms of yellow fever may have gone unnoticed as they did during the 1992 to 1993 epidemic when YF virus was isolated from a 12-year-old girl, who had fever, headache and joint pains but no jaundice or signs of hemorrhagic illness. (Outbreak of YF in Kenya, 1992-1993 Epidemiological Investigations, Sanders et al., in preparation).

The continued low-level of transmission of YF virus would likely have gone unnoticed if the enhanced surveillance and diagnostic capacity instituted by the government of Kenya after the 1992-93 epidemic was not in place. When cases of YF were confirmed from an area not known to have been affected during the outbreak, the Kenya Ministry of Health quickly recognized the problem and resumed vaccinations (9,10).

Operational costs of the field visits to 18 sentinel sites are relatively high. Since nine (90%) of the confirmed cases of YF were recognized by three hospitals, which had cared for YF patients during the previous epidemic, limiting the number of sentinel

sites may be considered. Distributing materials to obtain blood from persons with suspected cases does not seem feasible countrywide.

The WHO initiative to create a global surveillance program for recognition of and response to emerging diseases emphasizes that a critical element of surveillance includes a network of reference centers that assist national services in disease diagnosis and investigation (11). In Kenya, the first steps have been taken to establish such a reference center at KEMRI in Nairobi. In 1994, two technologists were trained for an extensive period at CDC's laboratory in Fort Collins, Colorado, in the diagnosis of YF and other arboviral diseases. An epidemiologic support unit was established at the KEMRI laboratory to provide the link between possible disease occurrences in the field and the diagnostic facilities at the distant laboratory.

Will this effort ensure that future epidemics will be recognized? It is often argued that the critical element of surveillance is disease recognition by front-line health-care providers, subsequent investigation, sample collection, and the forwarding of samples to available diagnostic facilities. In Kenya there is no focus on what diseases should be reported immediately (monthly disease reporting at district level requires health care workers to compile information on 37 diseases). Communication between the field and laboratory is often difficult. Once a specimen is forwarded, results often do not reach the clinician who submitted the sample in a timely fashion. Separate disease monitoring systems have been initiated by different health programs, but they rely on the same district officers to fill out the forms. Admittedly, sentinel surveillance for YF has not been integrated into one of the existing disease control programs, and its sustainability as anything more than a research program is questionable.

Hemorrhagic fever surveillance can be improved by 1) arranging sentinel sites according to the presumed southward extension of the YF-endemic area, 2) managing supervision of the sentinel sites at provincial level, 3) integrating disease reporting for YF in the surveillance system for EPI target diseases, 4) clearly defining which diseases need to be actively monitored by health care providers, 5) instituting improved diagnostic capabilities, and 6) forming a disease outbreak team.

Transmission of YF poses a potential threat for the largely unvaccinated population of Kenya and other East African countries, especially since the YF-endemic area appears to be expanding (12). YF vaccination appears to be the only practical public health intervention. Vaccination of children 6 months of age and older in the YF-endemic districts (Keiyo, Marakwet, Baringo, and Koibatek) should be continued. Districts adjacent to the disease-endemic area (Uasin Gishu, Kericho, Nandi, and Nakuru) should be included in the vaccination program.

Acknowledgments

We thank our colleagues at the Centers for Disease Control and Prevention (CDC) and at the Institute Pasteur for confirming our preliminary serologic results; the medical staff at the sentinel surveillance facilities, the Kenya Ministry of Health, and the Kenya Medical Research Institute (KEMRI) for their continued support for this program. Special thanks go to Dr. Duane Gubler and his staff at the CDC laboratory in Fort Collins, Colorado, for training two of KEMRI's senior technologists during a 4-month period in 1994.

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Serologic Evidence for the presence in Pteropus Bats of a Paramyxovirus Related to Equine Morbillivirus

Two outbreaks of a previously unknown disease in horses and humans occurred in Queensland in 1994. The outbreaks occurred within 1 month of each other in Brisbane and Mackay, which are approximately 1000 km apart. In the Brisbane incident, 21 horses were infected of which 14 died or were euthanized after severe clinical signs of an acute respiratory disease. Two human cases were in patients with less well defined clinical signs; one patient died (1,2). In the Mackay incident two horses became seriously ill and died, and one person also died (3). Although it is now known that the two outbreaks occurred in August and September 1994, knowledge of the Mackay outbreak did not occur until late 1995 when the infected person died of a relapsing encephalitis. The name equine morbillivirus (EMV) has been proposed for a paramyxovirus isolated from four of the Brisbane horses and the first patient who died (2).

In both locations, the index case appears to have been in a mare in late pregnancy, on pasture. The mode of transmission to other horses and to humans is unknown. In spite of intensive investigations, no connection has been established between the two incidents. At both locations, initial serologic studies of in-contact horses and humans failed to show evidence of neutralizing antibody, and it was concluded that infection by contact is uncommon. A subsequent state-wide serologic survey of 2,411 horses has also shown no evidence of infection (4). At present, only seven horses have had antibody, and all were involved in the Brisbane outbreak.

To evaluate the theory that EMV originated from a wildlife source, a trapping program was initiated, focusing on the location of the index cases, first in Brisbane and later in Mackay when the details of that incident became known. A total of 5,264 sera from 46 species were tested, none of which showed any evidence of antibody.

Further information from the Mackay cases enabled us to think more logically about a possible reservoir host. Although virus was not isolated from the two horses, it was possible to amplify part of the EMV matrix protein gene by PCR. A comparison of the sequence of this PCR product with PCR product from the Brisbane cases showed that they were identical, indicating a common source of origin (5). In our consideration of possible reservoir hosts, the following criteria were applied to prioritize species for investigation: 1) The species should be present in both the Brisbane and Mackay areas; 2) the species should be capable of migrating between these areas, and 3) contact with horses should be possible. The two groups of animals which readily fit this description were birds (especially migratory waders) and flying foxes. Because EMV is a mammalian virus and transmission of paramyxoviruses from birds to mammals is uncommon, flying foxes were given a higher priority than birds.

In Australia four species of bats belong to the Suborder Megachiroptera (fruit bats or flying foxes). The spectacled fruit bat (*Pteropus conspicillatus*) occurs in the northern and eastern parts of Queensland while the black fruit bat (*P. alecto*) has a much wider distribution across the northern part of Australia. The little red (*P. scapulatus*) is found across northern and eastern Australia while the grey-headed *(P. poliocephalus*) occurs in eastern and southern areas of the country.

Serologic sampling of these species soon showed the presence of antibody to EMV. At present, 224 samples have been tested of which 20 have neutralizing antibody to EMV (prevalence rate of approximately 9%). Positives have been detected in all species but insufficient samples are available to provide any meaningful estimate of comparative prevalence. Animals with positive results have been recorded along the whole of the eastern coast of Queensland, from Cairns to Brisbane. Again, insufficient data are available to compare the seroprevalence in different locations. Titers vary from 1/5, the lowest dilution tested, to 1/640. For comparison, the neutralization titer of the control positive equine sera is 1/160.

In relation to the origin of EMV, the significance of finding antibody in fruit bats remains to be determined. Our interpretation of the neutralization test results is that a virus closely related to EMV is circulating in fruit bats. We propose referring to this virus as bat paramyxovirus to emphasize that its relationship to EMV is uncertain.

A serologic survey of persons who have had prolonged and close contact with fruit bats is under way. To our knowledge there have been no unexplained occurrences of severe infectious disease in fruit bats, nor have there been any recorded cases of severe disease in persons who have had extensive exposure to these animals.

We will continue sampling birds and other ani-

mals because fruit bats may not be the only reservoir of this virus. Also transmission from bats to another species may be required before spillover occurs in horses. A high priority will be to isolate the bat paramyxovirus and compare it to EMV. The natural history of bat paramyxovirus infection should be investigated so that natural routes of infection can be established. This information should then lead to testable hypotheses about how infection of other species occurs.

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Acute Cervical Lymphadenopathy

To the Editor: Acute cervical lymphadenopathy has not been reported as a symptom of *Mycobacterium genavense* infection. In June 1994, a 32-year-old injecting drug user, who had been monitored since 1987 for human immunodeficiency virus (HIV) infection at the outpatient clinic of the Infectious Disease Institute, Perugia, Italy, was admitted to a hospital with fever (39°C) and progressive swelling over the submandibular region and neck. In addition to being febrile, upon physical examination the patient had tender left submandibular and cervical lymphadenopathy approximately 3 cm in diameter, with redness and edema of the overlying skin. The CD4+ lymphocyte count was 0.01 x 10^9/L. A specimen obtained by needle aspiration of the submandibular lymph node contained numerous acid-fast bacilli, and the patient was treated with isoniazid, rifampin, ethambutol, and amikacin for presumed *Mycobacterium tuberculosis* with a good response; however, 10 days later, the patient's submandibular pain recurred along with abdominal pain and bowel irregularities. Gastroscopy showed superficial duodenal erosions, and acid-fast bacilli were visualized by microscopy. Shortly thereafter, pain and swelling of the patient's right ankle developed, and small lesions were noted on the dorsum of the right foot. Clarithromycin was substituted for the amikacin for suspected without a clear response, and a course of steroids was initiated with clinical improvement. Symptoms recurred when the steroids were tapered. Ciprofloxacin was substituted for isoniazid, and amikacin was readministered. Material from a repeat needle aspiration of the submandibular node 1 month later also showed acid-fast bacilli by microscopy.

Cultures of the initial submandibular aspirate demonstrated poor growth in Bactec 13A broth and did not grow on solid media. The specimen was sent to a reference laboratory where acid-fast bacilli were successfully isolated 10 weeks later in Middlebrook agar containing mycobactin J. These acid-fast bacilli were subsequently identified as *M. genavense* by high-pressure liquid chromatography and nucleic acid sequencing of the 16S rRNA. By this point, the patient had improved on a regimen of isoniazid, pyrazinamide, clofazimine, and amikacin for presumed *M. genavense* infection, and this regimen was continued. The patient died 19 months later; cultures for mycobacteria were persistently negative even when antimycobacterial drugs were discontinued 16 months after the initial episode.

M. genavense is a novel mycobacterial species that causes serious disseminated infections with massive involvement of the small intestine, spleen, liver, and abdominal lymph nodes in profoundly immunocompromised persons. Cultures with Bactec 13A vials containing radiometric liquid medium are generally positive but subcultures on solid media are unsuccessful (1). Lowering the pH of medium to six enhances its growth (1), while adding mycobactin J to Middlebrook 7H11 (2) solid media can help in the isolation. The suppression of growth of *M. genavense* by NAP can cause confusion with the *M. tuberculosis* complex; however, *M. genavense* can be easily distinguished by its slow growth and its dysgonic nature. At present, the way to identify *M. genavense* is by 16S rRNA sequencing (3). High-pressure liquid chromatography can be used (4).

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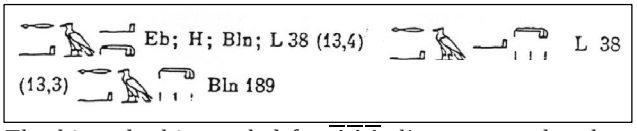
AIDS: Déjà Vu in Ancient Egypt?

To the Editor: The recent letter by Olson et al. (1) on the plague of Athens and the "re-emergence (?)" of Ebola virus prompts brief reiteration of an earlier communication (2) placing human immunodeficiency virus (HIV) back in history, possibly in the time of the pharaohs.

Translations of the Papyrus Ebers from ancient Egyptian literature repeatedly make reference to difficulties in the diagnosis of conditions under the names *$\overline{A}\overline{A}\overline{A}$ disease, uxedu-disease,* and *uha-disease* (3). The interpretation of these diseases has been inconclusive among Egyptologists. However, the many remedies proposed for the ravages of *$\overline{A}\overline{A}\overline{A}$*, whether by itself or complicated by *uxedu-* or *uha-disease*, brand it as a scourge of first magnitude. *$\overline{A}\overline{A}\overline{A}$* is mentioned 50 times in four papyri (28 times in Papyrus Ebers, 12 times in Papyrus Berlin, 9 times in Papyrus Hearst, and once in the London Papyrus [4]). The hieroglyphic symbol for *$\overline{A}\overline{A}\overline{A}$* (5) is shown below.



The hieroglyphic symbol for *$\overline{A}\overline{A}\overline{A}$ disease,* translated as "semen" or "poison" (From: Von Deines and Westeneorf [5])

Could this be documentation of HIV, or more accurately its prototype, occurring in Egypt during the time of the pharaohs?

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References

1. Olson PE, Hames CS, Benenson AS, Genovese EN. The Thucydides syndrome: Ebola déjà vu? (or Ebola reemergent?). Emerging Infectious Diseases 1996;2:155-6.
2. Ablin RJ, Immerman RS, Gonder MJ. AIDS: a disease of ancient Egypt? NY State J Med 1985;85:200-1.
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4. Ghalioungui P. Magic and medical science in ancient Egypt. London, UK: Hodder and Stoughton, 1963.
5. Von Deines H, Westeneorf W. Wöterbuch der medizimischen texte. Berlin, Germany: Akademie-Verlag, 1961;7,Part 1:129.

International Meeting on Research Advances and Rabies Control in the Americas

The "VIIth, Annual International Meeting on Research Advances and Rabies Control in the Americas" will be convened at the Centers for Disease Control and Prevention in Atlanta during 9–13 December 1996. The Conference will focus on both basic scientific contributions and applied research in the understanding and control of this fatal viral disease. Conference sessions are designed fro a diverse public health and biomedical audience, particularly professionals in Canada, the United States, and Latin America; the sessions will explore research advances, diagnostic issues, viral pathobiology, oral immunization, descriptive and molecular epizootiology, host ecology, and human preexposure and postexposure vaccination. Workshops detailing techniques used in the diagnosis and surveillance of rabies by conventional and molecular methods will also be held.

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Symposium Notice

The Infectious Disease Association of California (IDAC) will hold its fall symposium on Saturday, October 19, 1996, at the Hyatt Regency in Irvine, California.

Registration, including lunch, is free to IDAC members; $50 for non-members.

For information, call

(310) 398-5931 or e-mail: IDAC@aol.com.