Vol. 2, No. 4, October-December 1996

Emerging Infectious Diseases

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

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Social Inequalities and Infectious Diseases — P. Farmer

Molecular Mechanisms of Bacterial Virulence — J. Mecsas

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Guarding Against the Most Dangerous Emerging Pathogens: Insights from Evolutionary Biology

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Control of emerging infectious diseases will be difficult because of the large number of disease-causing organisms that are emerging or could emerge and the great diversity of geographic areas in which emergence can occur. The modern view of the evolution of pathogen virulence—specifically its focus on the tradeoff between costs and benefits to the pathogen from increased host exploitation—allows control programs to identify and focus on the most dangerous pathogens (those that can be established with high virulence in human populations).

Studies of emerging diseases have focused chiefly on the spectrum of different emerging pathogens, epidemiologic reasons for emergence, and interventions to control emergence. The feasibility of disease control is hampered by the potentially vast number of emerging and reemerging pathogens, the diversity of geographic sources, the potential for rapid global dissemination from these sources, and numerous ecologic and social factors influencing emergence (1-4). Disease control could be made more manageable if the most dangerous pathogens could be singled out for the most intense study, surveillance, and control efforts. Experts who have addressed this problem from an epidemiologic but not an evolutionary perspective disagree about the feasibility of predicting and preventing the emergence of the most damaging new pathogens (5-8). In this perspective, I argue that improved understanding of the evolution of virulence (defined broadly as the harmfulness of an infection) can make this goal more feasible in two ways: 1) by facilitating identification and blocking of pathogens that represent the greatest threat should they become established in human populations (e.g., *Yersinia pestis* during the Middle Ages and human immunodeficiency virus [HIV] during recent decades) and 2) by providing methods for inhibiting the emergence of particularly virulent variants of pathogens that are already established in human populations (e.g., the pathogen that caused the 1918 influenza pandemic and virulent, antibiotic-resistant strains of *Staphylococcus aureus)*.

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Modern understanding of the evolution of virulence focuses on a tradeoff to which pathogens are subjected: the competitive benefits that pathogens accrue through increased exploitation of hosts and the costs that result from any effects of disease that reduce infectious contact between infected and susceptible hosts. The traditional view presumed that natural selection would favor evolution toward benign coexistence between host and parasite (9-12). The modern view, however, stresses that such benign coexistence will be unstable if pathogens that exploit hosts to a greater degree have more overall success across transmission cycles than those that achieve benign coexistence (13-17).

The primary assumption of this evolutionary argument is that increased virulence is correlated with increased pathogen propagation (manifested as increases in pathogen reproduction within hosts and/or pathogen shedding from infected hosts). This correlation need not be strong across host/pathogen associations for the arguments to be valid; differences in pathogenic mechanisms, for example, could make the correlation virtually undetectable when extremely different kinds of

pathogens are compared. Rather, the tradeoff argument states that for a given pathogen (with its particular tropisms and pathogenic mechanisms), mutations that increase the level of host exploitation tend to increase harmfulness. The association between virulence, exploitation, and pathogen propagation is expected among "wild type" mutants, but not among novel laboratory-generated virulent ones. Because there are many routes to increased virulence and laboratory-generated variants are often not selected on the basis of competitive superiority in vivo, the increased virulence of variants generated in the laboratory may not be linked to propagative superiority. In contrast, natural selection should eliminate any variants for which increases in virulence are not linked to increases in pathogen fitness.

The connection between virulence, host exploitation, and pathogen propagation may be indirect or direct. If the pathogenic mechanism involves toxin production, a positive association is expected between toxin production and pathogen propagation. In *Vibrio cholerae*, for example, high toxin production is associated with increased densities of vibrios in the fecal material, apparently as a result of the toxin's flushing of competing organisms from the intestinal tract (17). In other organisms, the association between virulence, host exploitation, and pathogen propagation is more direct. The human plasmodia that reproduce more extensively often cause more severe illness and are more life-threatening (16). Similarly, more virulent strains of vector-borne dengue virus reproduce more extensively in cell culture (18). Growth rates of *Salmonella typhimurium* were reduced by eliminating one of its virulence plasmids and inhibiting the plasmid's expression; introduction of an 8-kb region encoding the spv genes restored increased growth rate (19). Comparison of *Shigella* species suggests a similar association between virulence and pathogen reproduction (20).

Sexually transmitted pathogens show analogous associations. For the best studied pathogen, HIV, more rapidly replicative HIVs are associated with greater cellular destruction in vitro, more rapid destruction of the immune system, and more rapid onset of AIDS (21-35). Similarly, the more oncogenic serotypes of human papilloma-viruses (HPV) generate greater numbers of progeny by interfering with the cell's mechanisms for restricting cell division (36). For both viruses, increased viral loads are associated with increased probability of transmission to contacted persons (37-39), and HIV-1, which propagates to higher densities than HIV-2, is more transmissible per contact (40).

The association between virulence and viral propagation in pathogens circulating naturally in human populations therefore supports the modern emphasis on a tradeoff between the fitness benefits and the costs accrued by pathogens as a function of changes in host exploitation.

Transmission Associated with High Virulence

Transmission from Immobile Hosts

Like the traditional view of host/parasite coevolution, the modern view identifies host illness as a potential liability for the pathogen. When pathogens rely on the mobility of their current host to reach susceptible hosts, the illness caused by intense exploitation typically reduces the potential for transmission. The modern perspective on host/parasite coevolution differs from the traditional one, however, in its emphasis on weighing these setbacks against the benefits of exploitation: high virulence can contribute to evolutionary stability if the costs incurred by parasites from exploitation-induced damage are particularly small and/or the benefits obtained from exploitation are particularly big. Thus, if host immobilization has little negative effect on transmission, pathogen variants that exploit the host so intensely that it is immobilized will reap the benefits of exploitation. Put more generally, when the costs incurred from transmission associated with immobilization are small, the costs of exploitation should outweigh the benefits at a higher level of exploitation—and hence virulence—than would occur if immobilization severely impaired transmission (16).

Recognizing this version of the general tradeoff led to several predictions: Because vector-borne parasites can be transmitted effectively from immobilized hosts, they should evolve to a higher level of virulence than

directly transmitted parasites (16). Similarly, aspects of human behavior and culture can form "cultural vectors," which transmit pathogens from immobile to susceptible hosts (41). For example, diarrheal pathogens that are largely waterborne should evolve to relatively high levels of virulence because effective transmission can occur even when infected hosts are mobilized: persons carrying contaminated clothing and bedding, the water used for washing bed sheets, and the movement of contaminated water into drinking water together act like a swarm of mosquitoes, transmitting pathogens from the immobilized host. Attendant-borne pathogens should also become virulent. Attendant-borne transmission often occurs in hospitals, when nurses and physicians transmit pathogens from one immobilized patient to another. A reciprocal process occurs when parasites rely on the mobility of susceptible persons rather than the mobility of the infected hosts to reach the susceptible persons. Parasites that are durable in the external environment should thus evolve toward a higher level of virulence than nondurable pathogens because durable pathogens may remain viable in the environment until the movement of susceptible individuals brings them into contact with the pathogens.

Each of these hypotheses has been evaluated and in each case the expected association occurred: virulence is positively associated with vector-borne transmission, waterborne transmission, attendant-borne transmission, and durability in the external environment (Table 1). This evolutionary framework, therefore, explains the diversity of human parasites in a way that contrasts starkly with the traditional view. Instead of being seen as a sign of maladaption, the severity of diseases such as malaria, tuberculosis, smallpox, cholera, and typhoid fever is seen as a consuequence of evolutionary adaptation because the causative parasites do not rely on host mobility for transmission. The tradeoffs between the benefits and costs of exploitation, therefore, favor evolution of relatively high levels of exploitation for such pathogens and hence high degrees of harm to the host.

Sexual Transmission

The evolutionary tradeoffs associated with virulence in sexually transmitted diseases involve the requirements for sexual transmission imposed on the pathogens by the sexual behavior of the host. Short durations of infections would be ineffective for most sexually transmitted pathogens. If people changed sex partners once per year, for example, a pathogen that was rendered noninfectious by immunologic defenses or the host's death within a few weeks would have little chance of being transmitted. To survive, the pathogen must be transmissible for a period that extends into the time of the next sexual partnership. To prosper, the pathogen must be transmissible for periods that span more than one change in sex partners; therefore, sexually transmitted pathogens may often need cell and tissue tropisms that keep them from being eliminated by the immune system for relatively long periods.

The evolutionary effects of changes in sexual behavior on virulence may be strongly influenced by tropisms that were present before the behavior change. Increased potential for sexual transmission should favor pathogen variants that reproduce more extensively sooner after the onset of infection. If the preexisting tropisms target nonessential cell types, this selection for earlier reproduction will have relatively little effect on virulence. If, for example, people changed sex partners every few days, the sexually transmitted pathogen should evolve virulence levels much like those of respiratory tract pathogens, which rely on host mobility for transmission. Examples of such pathogens are sexually transmitted unicellular pathogens such as *Neisseria gonorrheae* and *Chlamydia trachomatis*, which tend to infect mucosal tissues and, therefore, have relatively minor negative effects on the survival of adult hosts. If, however, the tropisms involve critical cells, the damage associated with increased levels of host exploitation should be more severe to the host. HIV provides an example: HIV has a tropism for helper T cells, which are critical regulators of immunologic responses. Although a high level of replication in these cells can be tolerated over short periods, it eventually leads (by mechanisms that are still being clarified) to the decimation of this category of cells and the collapse of the immune system.

If these arguments about evolutionary forces and tissue tropisms are applicable to

HIV, HIVs should be more virulent in areas where the potential for sexual transmission is greater. In accordance with this prediction, HIV-2 tends to be less virulent than HIV-1; moreover, evidence indicates that during the early years of HIV infection in Africa, HIV-2 tended to be transmitted in populations having a lower potential for sexual transmission (17,20). The overall validity of this approach to HIV virulence, however, will be better tested as different variants of HIV emerge in different geographic regions. Information about the potential for sexual transmission can help predict the evolution of HIV virulence in different geographic areas. On the basis of the evolutionary tradeoffs mentioned above, for example, the type E HIV-1s that are circulating in Thailand (where the potential for sexual transmission has been great) are predicted to be particularly virulent (17). Although this prediction needs to be evaluated rigorously, recently gathered data support the prediction: the decline in CD4+ cell counts of persons infected with HIV and the progression of illness in these patients appear to be particularly rapid in Thailand (43-44).

The most important application of this evolutionary approach to HIV, however, pertains to interventions that can be used to control the future evolution of HIV. If the inherent virulences of HIVs depend evolutionarily on the potential for sexual transmission, interventions that reduce this potential should have a long-term evolutionary effect, as well as widely recognized short-term epidemiologic effects—in addition to reducing the spread of HIV infection, such interventions should reduce the harmfulness per infection. Follow-up of persons infected with HIV-1 for more than a decade without deterioration of the immune system indicates that the mildness of the infections is sometimes attributable to inherently mild viruses (45-47). The raw material for this evolutionary change, therefore, appears to be already present in the HIV gene pool.

In Japan, which has a relatively low potential for sexual transmission (48), type E HIV-1s have recently been introduced from Southeast Asia. If a low potential for sexual transmission favors evolution toward mildness, the Japanese type E viruses should become milder over the next few decades.

Assessing the Threat Posed by Pathogens

Assessment Goals

Focusing investigative and intervention efforts on the most significant disease threats makes sense only if the threats can be reliably assessed. The long-term threat depends on the evolutionary stability of high pathogen virulence, and the most dangerous pathogens are those that threaten widespread persistence with severely damaging manifestations. One of the most important tasks in controlling emerging diseases is to identify and block such pathogens during the early stages of emergence, or better yet, before they emerge. If the most dangerous pathogens—the future analogs of the causes of AIDS, malaria, smallpox, tuberculosis, and cholera—could be effectively blocked, the effort against emerging diseases would be successful. If not, the effort may be looked on as a failure in spite of successes against pathogens that are less able to effectively penetrate human populations or relatively benign when they do establish

Table 1. Categories of pathogens that pose threats of being stably harmful in human populations because of reduced dependence on host mobility

Characteristics allowing transmission from immobile hosts	Association with lethality	Reference
arthropod-borne transmission	lethality higher among arthopod-borne pathogens than among directly transmitted pathogens	(16)
water-borne transmission	lethality of diarrheal bacteria correlated with tendencies for waterborne transmission	(42)
attendant-borne transmission	lethality of *E. coli* correlated with duration of attendant-borne cycling	(20, 41)
durability in the external environment	lethality of respiratory-tract pathogens correlated with durability	*

*B. A. Walther and P. W. Ewald, unpublished manuscript

Table 2. First-level checklist for identifying the most dangerous emerging pathogens. If the answer to any of the questions is yes, the potential for continuous transmission between humans should be assessed. If this potential is high, the pathogen should be considered particularly dangerous.

- Does it have a tendency for waterborne transmission?
- Is it vector-borne with the ability to use humans as part of the life cycle?
- If it is directly transmitted, is it durable in the external environment?
- Is it attendant-borne?
- Is it needle-borne?*
- If it is sexually transmitted, is it mutation-prone with a tropism for critical cell types or does it have invasive or oncogenic tendencies?

*The hypothesized importance of needleborne transmission has not yet been tested; it has been included in this listing on the basis of the harmfulness of needleborne pathogens and the hypothetical assocations between needleborne transmission and virulence (17).

themselves. The emergence, spread, and persistence of pathogens with the characteristics of rhinoviruses, for example, would not be looked on as a great failure. The establishment of such pathogens would hardly be noticed against the current backdrop of mild to moderately severe respiratory tract pathogens.

To identify pathogens that must be studied and controlled most intensively, each pathogen should be assessed for two characteristics that are associated with high virulence: 1) an ability to spread well from human to human (directly or indirectly through vectors) rather than infecting humans as dead-end hosts, and 2) transmission features that select for high levels of virulence.

The existing associations between virulence and transmission characteristics (Table 1) can be used to make such identifications. Table 2 offers a checklist that could be applied to each emerging pathogen to determine whether it makes the first cut in the process of identifying the most dangerous candidates. Subsequent analyses of the pathogens would then assess the nature of any barriers that limit the establishment of pathogens in human populations (e.g., the absence of suitable arthropod vectors for large proportions of the year).

Durability

Although durability in various external environments was quantified in detail by microbiologists during the first half of this century (49), modern studies have paid this attribute little attention. Evolutionary considerations, however, indicate that it should be one of the first variables quantified when a new pathogen is being studied. If a new, directly transmitted pathogen can remain viable in the external environment for many days to many weeks, it falls in the category of especially dangerous pathogens. If, for example, Ebola virus were viable upon natural desiccation for weeks instead of hours, its level of host exploitation and potential for transmission from exploited hosts would not be so mismatched, and it, like smallpox virus, would pose a much more serious threat. Durability in the external environment depends largely on environmental conditions (49), and thus assessments of viability should cover all feasible environmental conditions.

Vector-borne Transmission

The most serious threat involved in vector-borne transmission comes from pathogens that can be maintained by human/mosquito cycles but are absent from suitable areas because of historical accidents or past eradication campaigns. Dengue and malaria are members of this category; they have the potential to spiral out of control immediately upon release into areas with suitable vectors. Nonevolutionary analyses of emerging infections recognize the threat posed by these pathogens because their damaging effects on human populations are known.

Vector-borne pathogens that have not used humans as the primary vertebrate host but may be capable of doing so represent less easily recognized threats. Evolutionary considerations heighten concern because such vector-borne pathogens are expected to become increasingly harmful as they become adapted to human/vector cycles of transmission (16).

Rift Valley fever virus provides an example. For most of this century, this virus was believed to infect humans only as dead-end hosts. Although it was vector-borne in ungulates, humans were seen as acquiring the

infection either when involved in the slaughtering process or when bitten by mosquitoes that had acquired infection from other vertebrates. Recent outbreaks have spread to an extent consistent with substantial human/mosquito cycling, but the existence of such cycling has not been conclusively documented. If human/mosquito cycling is occurring, the door is open for further adaptation to humans and for evolution of increased virulence in humans, increased efficiency of human/vector transmission, and increased spread through human populations. Rift Valley fever virus viremias seem sufficient for human/mosquito cycling, and the lethality of the largest outbreaks was particularly high, as one would expect if some evolution toward increased virulence accompanied a temporary establishment of human/mosquito cycles (50-51). To assess the long-term threat posed by Rift Valley fever virus and to block this virus should it prove to be particularly threatening, we need to emphasize the following research priorities: 1) study the transmission of Rift Valley fever virus in human/mosquito cycles, 2) assess the potential for such transmission over extended periods, and 3) evaluate the effects of such transmission on virus virulence.

All emerging vector-borne pathogens need not be viewed as equally threatening. For example, *Borrelia burgdorferi*, the agent of Lyme disease (an emerging vector-borne pathogen in human populations in North America), does not need to be monitored to avoid its establishment as a human pathogen because once emerged, it does not threaten to spiral out of control; it is tick-borne, and ongoing human/tick cycles are not feasible because of the limited exposure of infected humans to susceptible tick populations of the appropriate instar. Tick- and mite-borne rickettsiae do not present a great threat for similar reasons.

Sexual Transmission

The tradeoff concerning sexually transmitted pathogens may prove particularly useful in identifying pathogens that are capable of sexual transmission and have cell tropisms that would cause severe damage if host exploitation increased but have not had high potential for sexual transmission. Human T-cell lymphotropic virus (HTLV) is in this category, even though by nonevolutionary criteria it could be dismissed because it has been geographically widespread in humans for a long time (1). HTLV type 1 (HTLV-I) is less damaging than HIV; it kills or severely handicaps 5% to 10% of the people it infects, generally decades after infection. Although HTLV-I and HIV infections share many characteristics, HTLV does not have HIV's high mutation rate and hence does not have the potential for staying ahead of immune responses and eventually decimating the immune system. Instead, HTLV relies on modes of transmission that do not expose it to the immune system: proviral replication through stimulation of host cell proliferation and transmission through cell-to-cell contact. A concern with HTLV is that a high potential for sexual transmission may favor increased rates of viral replication leading to increased exposure to the immune system and increased mutation rates (48).

A preliminary step toward evaluating the threat posed by the emergence of particularly virulent HTLVs is assessing whether HTLVs exposed to different levels of potential for sexual transmission vary in virulence. HTLV-I infections tend to lead to leukemias and lymphomas at younger ages in Jamaica, where the potential for sexual transmission is high, than in Japan, where potential for sexual transmission is low (48). This difference also occurs among North Americans of Japanese and Caribbean descent (52), who presumably are infected predominantly (if not exclusively) by Japanese and Caribbean HTLVs, respectively. The inherent virulence and mutation-proneness of the Japanese and Caribbean HTLVs need to be assessed. Similarly, HTLV virulence needs to be better studied in regions of Africa where it has been long endemic to determine whether variations in HTLV virulence are correlated with the potential for sexual transmission.

Although mutation-prone sexually transmitted viruses that infect critical cell types are particularly threatening, sexually transmitted viruses in general deserve special attention. Even if a sexually transmitted virus invades only epithelial cells and replicates with low mutation rates, a high potential for sexual

transmission may lead to evolution of increased lethality. Death caused by HTLV-induced lymphomas and leukemias is one manifestation of the danger posed by an RNA virus that replicates substantially in its DNA form and hence is in a middle area within the spectrum of mutation-proneness. HPVs illustrate dangers posed by sexually transmitted viruses that, because they are DNA viruses, are even further away from HIV on the mutation-proneness continuum. The mechanism by which HPV nudges infectious cells toward cancer is associated with increased viral replication; moreover, high potential for sexual transmission (as indicated by the number of lifetime sex partners) is a strong risk factor for infection with the more oncogenic HPV serotypes but not for the mild HPV serotypes (53). This association supports the idea that reductions in the potential for sexual transmission should cause evolution of reduced HPV virulence. Specifically, as the potential for sexual transmission decreases, the risk for acquiring the oncogenic serotypes (vs benign serotypes) should disproportionately decrease. Similarly, if interventions prevent the potential for sexual transmission from increasing, the emergence of oncogenic HPV serotypes should be disproportionately suppressed.

Waterborne Transmission

Although such pathogens as *Vibrio cholerae* O139 and *Shigella dysenteriae* type 1 threaten emergence in countries with inadequate water supplies, the threat is much lower in countries with safe water supplies. Although such pathogens continue to be brought into the countries with safe water supplies by travelers and commerce, the pathogens show little potential for emergence. For example, a major epidemic of *S. dysenteriae* type 1 spread from Guatemala through Central America during the early 1970s. It entered the United States in several places but dissipated without any great effort at containment. Its transmission was studied in a Los Angeles neighborhood, where each infection gave rise on average to about 0.4 new infections (54). Without amplification by waterborne transmission, this outbreak, like other introductions in the United States, was self-limited (54). The situation at the other end of Central America was similar. The *S. dysenteriae* epidemic dissipated as it moved into Costa Rica, where water supplies were relatively pure (L. J. Mata, pers. comm.).

Attendant-borne Transmission

Emerging hospital-acquired pathogens may pose one of the greatest and most controllable threats to people in countries like the United States, where more than 5% of hospital admissions and about 14% of intensive care patients acquire infections during their stay (55-57). According to some estimates, nosocomial infections rank among the ten leading causes of death in the United States (56), with dangerous bloodstream infections approximately doubling during the 1980s (58).

Although high virulence has been documented in pathogens involved in nosocomial outbreaks (59-63), the damage caused by nosocomial pathogens has generally been attributed to the state of hospitalized patients, who may be compromised by underlying disease, immunosuppressive drugs, and invasive procedures. These factors, however, do not explain why nosocomial pathogens, such as *Staphylococcus aureus* often cause symptomatic infections in hospital staff (60) but rarely in persons in the outside community. They also do not explain the association between the extent of nosocomial transmission and the virulence of infection, or the differences in symptomatic infections among otherwise healthy babies (17,20,41). In a New York City hospital, for example, where attendant-borne transmission rates were very low, only approximately one of 30 babies with *S. aureus* were symptomatic (64). Among nosocomial outbreaks of endemic disease, the analogous proportion may be 5- to 10-fold higher (65).

Without an evolutionary framework for understanding pathogen virulence, researchers would have no reason for expecting to find particularly virulent endemic pathogens in hospitals. The only serious attempts to explain the apparently high-level of pathogen virulence in hospitals involved the linking of virulence to another characteristic associated with hospitals: antibiotic resistance. The emergence of antibiotic-resistant organisms in hospitals in concert with the use of the antibiotics (66) led researchers to conclude that high levels of antibiotic use caused the emergence of resistant organisms and to speculate that antibiotic-resistant organisms might be inher-

ently more virulent than their antibiotic-sensitive counterparts (67). Yet when infections caused by resistant nosocomial organisms are compared with sensitive (generally nosocomial) infections, the former are only sometimes found to be associated with more severe infections. Even when they are associated with more severe disease (62,63), any differences in inherent virulence tend to be confounded with other factors, such as increased severity due to lowered effectiveness of antibiotics. The increased severity of disease, however, is sometimes associated with resistance to antibiotics other than the one being used (61), suggesting that the increased damage is not simply a result of ineffective antibiotics. The presence of virulence-enhancing bacterial characteristics in damaging, resistant nosocomial strains (63,68) also suggests a link between nosocomial transmission, antibiotic resistance, and virulence: antibiotic-resistant strains may have been particularly virulent because they were nosocomial, but this virulence was not apparent in many of the comparisons because the sensitive strains were also nosocomial.

Although the controversy regarding virulence and antibiotic resistance in hospital-acquired infections can be explained by the hypothesized connection between attendant-borne transmission and the evolution of both virulence and antibiotic resistance, none of the investigations of the topic made measurements that would allow assessment of the connection between attendant-borne transmission and the emergence of variants with increased virulence. The critical measure is the harmfulness per person housing the organisms in question, and the critical comparison is between nosocomial and community-acquired strains. Among persons that harbor nosocomial strains of *S. aureus*, for example, the proportion that show symptomatic infection could be compared with the analogous proportion of matched persons who are harboring community strains. After virulence-enhancing mechanisms are well understood, pathogens can be assayed for their virulence directly. Thus *Clostridium difficile* pathogens isolated from prolonged nosocomial outbreaks are predicted to be more toxigenic than *C. difficile* isolated from the outside community. Similarly, nosocomial *Escherichia coli* are predicted to have virulence-enhancing characteristics (e.g., invasiveness, adherence) (69) more often than community strains.

Further knowledge about virulence enhancing mechanisms and development of techniques for rapid detection (e.g., [72-75]) should offer opportunities for carefully controlled experiments to test whether reduction in attendant-borne transmission causes a greater decline in the inherent virulence of nosocomial pathogens in experimental hospitals than in control hospitals in which interventions are not imposed. Long-term follow-up should clarify the degree to which attendant-borne transmission may foster the emergence of virulent variants among both established human pathogens (e.g., *S. aureus, E. coli*) and new or newly recognized pathogens (e.g., *Serratia* spp., and *Pseudomonas aeruginosa*).

Harmful, often antibiotic-resistant, hospital-acquired pathogens can readily emerge beyond a hospital's boundary, when patients are moved, or attendants move between hospitals; the documentation is particularly strong for dangerous variants of *E. coli* and *S. aureus* (62,74-78). The degree to which emerging nosocomial pathogens spill over to generate outbreaks in the outside community is not well understood, but evidence suggests that this spillover represents a substantial threat when the organisms can infect healthy people. When large-scale communitywide epidemics of pathogenic *E. coli* have occurred, for example, transmission in hospitals often was strongly implicated. During 1953 and 1954, an *E. coli* epidemic advanced up the East Coast of the United States from the Carolinas through New England; "As it spread, explosive outbreaks were limited to institutions, hospital wards, and newborn nurseries" (59). A focal study of the U.S. Army Hospital at Fort Belvoir, Virginia, indicated that the epidemic strain was brought into the hospital by infected people in the community, with the proportion of inpatient to outpatient cases reversing dramatically during the hospital's 5-month outbreak (59). Similarly, during the winter of 1961, in an outbreak in Chicago and adjacent communities in Indiana, about 5% of the infants were affected, and nearly half of the affected infants had direct or indirect contact with one of the 29 involved hospitals just before

their illnesses (75).

Studies of *S. aureus* have also shown that nosocomial and community outbreaks are sometimes synchronous with transmission occurring in both directions between the hospital and the outside community (79-80). The long-term consequences of emergence of nosocomial strains for the outside community, however, still need to be assessed. The possibility that nosocomial pathogens may tend to be not only more resistant to antibiotics, but also more inherently virulent lends some urgency to this need.

Almost no work has been done to determine the potential of pathogens thought to be almost exclusively associated with nosocomial infection (e.g., *Enterococcus, C. difficile*) to take hold in the outside community. The high durability in the external environment of many nosocomial pathogens heightens the need for additional information. Durable pathogens that can infect uncompromised hosts (e.g., antibiotic-resistant *S. aureus* and to a lesser extent *C. difficile*) possess the basic characteristics that damaging organisms need to spread in the outside community. Durable organisms unable to infect healthy people pose a relatively low threat, but this inability is often presumed. Any transmission of durable nosocomial organisms like *P. aeruginosa* from patients after discharge heightens the threat to the outside community by providing an avenue for further adaptation to humans. Molecular analyses that allow reconstruction of epidemiologic patterns (e.g., molecular phylogenetics) could be used to improve assessments of the degree to which nosocomial pathogens can emerge in the outside community; such studies need to provide quantitative assessments not only of the threats posed by nosocomial pathogens in their current state, but also of their potential to breach by evolution the barriers that have inhibited their broader spread in the past.

Conceptual Innovation, Explanatory Power, and Precision

Dangerous Emergences of the Past

Each of the organisms that caused devastating epidemics over the past 5 centuries, would have been identified as an extremely dangerous pathogen by the criteria proposed here. *Y. pestis*, for example, is durable in the external environment (49) and is vector-borne. Its threat is lower now than centuries ago when fleas and rats were abundant domiciliary inhabitants, but it still represents a threat where these hosts are present.

The periodic emergence of yellow fever in European and American cities during the 18th and 19th centuries took a heavy toll; the 1878 epidemic, for example, killed about a quarter of the population of Memphis, Tennessee (81). If yellow fever virus were first encountered today, it would be recognized as an important threat because it is vector-borne and can be transmitted indefinitely through human/mosquito cycles.

With regard to the emergence of virulent variants from established pathogens, the influenza viruses circulating at the Western Front during World War I would be considered dangerous because barriers to transmission from immobile hosts were removed by cultural practices and because influenza virus is mutation prone (17,20). It is, therefore, not surprising that the Western Front has been identified as the source of the highly lethal variants of the 1918 influenza pandemic and that a pandemic of this severity has never recurred (17). More importantly, evolutionary considerations suggest that such a lethal pandemic will not recur unless influenza viruses are again exposed to opportunities that allow transmission from immobile hosts, as they are on poultry farms where highly lethal influenza outbreaks periodically emerge (17).

Uncertainty about the Dangerous Epidemics of the Future

These arguments about the evolution of virulence provide only coarse approximations of the selective processes in pathogen populations. To determine whether the implications of these arguments need to be substantially modified, we need empirical studies that evaluate these arguments against alternative explanations. Considering the current state of uncertainty, some might argue that it is dangerous to incorporate the current coarse understanding of the evolution of virulence into policy making. But failing to incorporate this understanding is dangerous.

If we do not adjust investments to take into account the evolutionary arguments, and the arguments prove correct, the reduction in death and illness per unit investment will be lower than it could have been. If we do adjust investments on the basis of these evolutionary arguments, and the arguments prove wrong, the nonevolutionary benefits of the investments would still be obtained.

Although the precise mechanisms that increase virulence in pathogens in the high-risk categories still need to be clarified, the associations (Table 1) are strong. One could argue, for example, that durable or waterborne pathogens are more harmful because hosts tend to pick up a greater diversity of genotypes from the environment when pathogens are more durable or are mixed in water; if the within-host genetic variability of such pathogens is greater, they would have more potential for within-host competition, which could favor the evolution of increased virulence. By this argument, factors such as durability, vector-borne transmission, and waterborne transmission would increase virulence indirectly by increasing within-host genetic variation. With regard to the prevention of the emergence of highly virulent disease, uncertainties about mechanisms are not critical. Whether the effects of these factors are direct or indirect, elimination of the factors should discourage the emergence of severe disease and favor the decrease of highly virulent pathogens.

Decisions to invest in interventions without certainty about mechanisms is not new to the health sciences. The hygienic interventions to control hospital acquired diseases and the purification of water supplies to control cholera were appropriately advocated on the basis of epidemiologic data (from Ignaz Semmelweis and John Snow) a half century before the causative agents of these or any other infectious diseases were first identified. Jenner's smallpox vaccine program was accepted globally more than a century before viruses were discovered or the mechanisms by which vaccines provide protection were understood. Even now the mechanisms by which the immune system provides protection encompass major areas of uncertainty. This uncertainty is evidenced, for example, by the controversies about the importance of the different legs of the immune system (such as cytotoxic T cells, neutralizing antibody, and subsets of helper T cells) in HIV pathogenesis.

If the evolutionary arguments are correct, the emergence of the most harmful diseases can be countered not only for pathogens that are recognized as threats but also for those posing threats that are not yet recognized. Providing pure water supplies, reducing attendant-borne transmission, and reducing vector-borne transmission preferentially from ill people (e.g., by providing mosquito-proof houses [17]) should guard against the emergence of virulent pathogens, whether the pathogens are unidentified or are highly virulent variants of identified human pathogens. An understanding of the evolutionary determinants of virulence may thus make surveillance and prompt intervention much more manageable.

The emphasis thus is on suppression of the emergence of particularly virulent variants rather than suppression of the emergence of new disease organisms. The expectation is that the frequency of disease will drop even though the frequency of individuals harboring organisms may decline little if at all. The data on decentralization of nursery/maternity wards, for example, indicate that the rates of nosocomial infection decline among mothers and babies, even though the rates at which babies harbor pathogens (colonization plus infection) do not decline (82). Indeed the disagreement about the value of rooming-in as a mode of infection control (82) can be attributed to a failure to distinguish the prevalence of disease organisms from the prevalence of disease. Controversies about the value of waterborne transmission can be traced to a similar failure (17).

The lead article of the first issue of this journal was entitled, "Emerging infections: getting ahead of the curve" (4). I propose that integrating evolutionary principles with epidemiology would enhance our ability to stay ahead of the curve. Evolutionary insights should increase our ability to distinguish emerging pathogens according to the long-term threat that they pose and thereby adjust investments in accordance with the threat. Knowledge of the evolution of virulence should also guide us to identify for each pathogen the critical data that will allow us to make this assessment. Finally, evolutionary considerations should allow identification of

infrastructural investments that will guard against the most dangerous pathogens, even if they are not blocked by surveillance and containment efforts and even if they have not yet been identified or are never identified as emerging pathogens.

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Social Inequalities and Emerging Infectious Diseases

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Although many who study emerging infections subscribe to social-production-of-disease theories, few have examined the contribution of social inequalities to disease emergence. Yet such inequalities have powerfully sculpted not only the distribution of infectious diseases, but also the course of disease in those affected. Outbreaks of Ebola, AIDS, and tuberculosis suggest that models of disease emergence need to be dynamic, systemic, and critical. Such models—which strive to incorporate change and complexity, and are global yet alive to local variation—are critical of facile claims of causality, particularly those that scant the pathogenic roles of social inequalities. Critical perspectives on emerging infections ask how large-scale social forces influence unequally positioned individuals in increasingly interconnected populations; a critical epistemology of emerging infectious diseases asks what features of disease emergence are obscured by dominant analytic frameworks. Research questions stemming from such a reexamination of disease emergence would demand close collaboration between basic scientists, clinicians, and the social scientists and epidemiologists who adopt such perspectives.

The past decade has been one of the most eventful in the long history of infectious diseases. There are multiple indexes of these events and of the rate at which our knowledge base has grown. The sheer number of relevant publications indicates explosive growth; moreover, new means of monitoring antimicrobial resistance patterns are being used along with the rapid sharing of information (as well as speculation and misinformation) through means that did not exist even 10 years ago. Then there are the microbes themselves. One of the explosions in question—perhaps the most remarked upon—is that of "emerging infectious diseases." Among the diseases considered "emerging," some are regarded as genuinely new; AIDS and Brazilian purpuric fever are examples. Others have newly identified etiologic agents or have again burst dramatically onto the scene. For example, the syndromes caused by Hantaan virus have been known in Asia for centuries but now seem to be spreading beyond Asia because of ecologic and economic transformations that increase contact between humans and rodents. Neuroborreliosis was studied long before the monikers Lyme disease and *Borrelia burgdorferi* were coined, and before suburban reforestation and golf courses complicated the equation by creating an environment agreeable to both ticks and affluent humans. Hemorrhagic fevers, including Ebola, were described long ago, and their etiologic agents were in many cases identified in previous decades. Still other diseases grouped under the "emerging" rubric are ancient and well-known foes that have somehow changed, in pathogenicity or distribution. Multidrug-resistant tuberculosis (TB) and invasive or necrotizing Group A streptococcal infection are cases in point.

Like all new categories, "emerging infectious diseases" has benefits and limitations. The former are well known: a sense of urgency, notoriously difficult to arouse in large bureaucracies, has been marshaled, funds have been channeled, conferences convened, articles written, and a journal dedicated to the study of these diseases has

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been founded. The research and action agendas elaborated in response to the perceived emergence of new infections have been, by and large, sound. But the concept, like some of the diseases associated with it, is complex. Its complexity has, in some instances, hampered the learning process. A richly textured understanding of emerging infections will be grounded in critical and reflexive study of how learning occurs. Units of analysis and key terms will be scrutinized and defined more than once. This process will include regular rethinking not only of methods and study design, but also of the validity of causal inference and reflection on the limits of human knowledge. This study of the process, loosely known as epistemology, often happens in retrospect, but many of the chief contributors to the growing research in emerging infectious diseases have examined the epistemologic issues surrounding their work and are familiar with the multifactorial nature of disease emergence: "Responsible factors include ecological changes, such as those due to agricultural or economic development or to anomalies in the climate; human demographic changes and behavior; travel and commerce; technology and industry; microbial adaptation and change; and breakdown of public health measures" (1). A recent Institute of Medicine report on emerging infections does not even categorize microbial threats by type of agent, but rather according to factors held to be related to their emergence (2).

In studying emerging infectious diseases, many thus make a distinction between a host of phenomena directly related to human actions—from improved laboratory techniques and scientific discovery to economic "development," global warming, and failures of public health—and another set of phenomena, much less common and related to changes in the microbes themselves. Close examination of microbial mutations often shows that, again, human actions have played a large role in enhancing pathogenicity or increasing resistance to antimicrobial agents. In one long list of emerging viral infections, for example, only the emergence of Rift Valley fever is attributed to a possible change in virulence or pathogenicity, and this only after other, social factors for which there is better evidence (1). No need, then, to call for a heightened awareness of the sociogenesis, or "anthropogenesis," of emerging infections. Some bench scientists in the field are more likely to refer to social factors and less likely to make immodest claims of causality about them than are behavioral scientists who study disease. Yet a critical epistemology of emerging infectious diseases is still in its early stages of development; a key task of such a critical approach would be to take existing conceptual frameworks, including that of disease emergence, and ask, What is obscured in this way of conceptualizing disease? What is brought into relief? A first step in understanding the "epistemological dimension" of disease emergence, notes Eckardt, involves developing "a certain sensitivity to the terms we are used to" (3).

A heightened sensitivity to other common rubrics and terms shows that certain aspects of disease emergence are brought into relief while others are obscured. When we think of "tropical diseases," malaria comes quickly to mind. But not too long ago, malaria was an important problem in areas far from the tropics. Although there is imperfect overlap between malaria as currently defined and the malaria of the mid-19th century, some U.S. medical historians agree with contemporary assessments: malaria "was the most important disease in the country at the time." In the Ohio River Valley, according to Daniel Drake's 1850 study, thousands died in seasonal epidemics. During the second decade of the 20th century, when the population of 12 southern states was approximately 25 million, an estimated million cases of malaria occurred each year. Malaria's decline in this country was "due only in small part to measures aimed directly against it, but more to agricultural development and other factors some of which are still not clear" (4). These factors include poverty and social inequalities, which led, increasingly, to differential morbidity with the development of improved housing, land drainage, mosquito repellents, nets, and electric fans—all well beyond the reach of those most at risk for malaria. In fact, many "tropical" diseases predominantly affect the poor; the groups at risk for these diseases are

often bounded more by socioeconomic status than by latitude.

Similarly, the concept of "health transitions" is influential in what some have termed "the new public health" and in the international financial institutions that so often direct development efforts (5). The model of health transitions suggests that nation-states, as they develop, go through predictable epidemiologic transformations. Death due to infectious causes is supplanted by death due to malignancies and to complications of coronary artery disease, which occur at a more advanced age, reflecting progress. Although it describes broad patterns now found throughout the world, the concept of national health transitions also masks other realities, including intranational illness and death differentials that are more tightly linked to local inequalities than to nationality. For example, how do the variables of class and race fit into such paradigms? In Harlem, where the age-specific death rate in several groups is higher than in Bangladesh, leading causes of death are infectious diseases and violence (6).

Units of analysis are similarly up for grabs. When David Satcher, director of the Centers for Disease Control and Prevention (CDC), writing of emerging infectious diseases, reminds us that "the health of the individual is best ensured by maintaining or improving the health of the entire community" (7), we should applaud his clearsightedness but go on to ask, What constitutes "the entire community"? In the 1994 outbreak of cryptosporidiosis in Milwaukee, for example, the answer might be "part of a city" (8). In other instances, community means a village or the passengers on an airplane. But the most common unit of analysis in public health, the nation-state, is not all that relevant to organisms such as dengue virus, *Vibrio cholerae* O139, human immunodeficiency virus (HIV), penicillinase-producing *Neisseria gonorrhoeae*, and hepatitis B virus. Such organisms have often ignored political boundaries, even though their presence may cause a certain degree of turbulence at national borders. The dynamics of emerging infections will not be captured in national analyses any more than the diseases are contained by national boundaries, which are themselves emerging entities—most of the world's nations are, after all, 20th-century creations.

Here I have discussed the limitations of three important ways of viewing the health of populations—tropical medicine, "the" epidemiologic transition, and national health profiles—because models and even assumptions about infectious diseases need to be dynamic, systemic, and critical. That is, models with explanatory power must be able to track rapidly changing clinical, even molecular, phenomena and link them to the large-scale (sometimes transnational) social forces that manifestly shape the contours of disease emergence. I refer, here, to questions less on the order of how pig-duck agriculture might be related to the antigenic shifts central to influenza pandemics, and more on the order of the following: Are World Bank policies related to the spread of HIV, as has recently been claimed (9)? What is the relationship between international shipping practices and the spread of cholera from Asia to South America and elsewhere in the Western Hemisphere (10,11)? How is genocide in Rwanda related to cholera in Zaire (12)?

The study of anything said to be emerging tends to be dynamic. But the very notion of emergence in heterogeneous populations poses questions of analysis that are rarely tackled, even in modern epidemiology, which, as McMichael has recently noted, "assigns a primary importance to studying interindividual variations in risk. By concentrating on these specific and presumed free-range individual behaviors, we thereby pay less attention to the underlying social-historical influences on behavioral choices, patterns, and population health" (13). A critical (and self-critical) approach would ask how existing frameworks might limit our ability to discern trends that can be linked to the emergence of diseases. Not all social-production-of-disease theories are equally alive to the importance of how relative social and economic positioning—inequality—affects risk for infection. In its report on emerging

infections, the Institute of Medicine lists neither poverty nor inequality as "causes of emergence" (2).

A critical approach pushes the limits of existing academic politesse to ask harder and rarely raised questions: What are the mechanisms by which changes in agriculture have led to outbreaks of Argentine and Bolivian hemorrhagic fever, and how might these mechanisms be related to international trade agreements, such as the General Agreement on Tariffs and Trade and the North American Free Trade Agreement? How might institutional racism be related to urban crime and the outbreaks of multidrug-resistant TB in New York prisons? Does the privatization of health services buttress social inequalities, increasing risk for certain infections—and death—among the poor of sub-Saharan Africa and Latin America? How do the colonial histories of Belgium and Germany and the neocolonial histories of France and the United States tie in to genocide and a subsequent epidemic of cholera among Rwandan refugees? Similar questions may be productively posed in regard to many diseases now held to be emerging.

Emerging How and to What Extent? The Case of Ebola

Hemorrhagic fevers have been known in Africa since well before the continent was dubbed "the white man's grave," an expression that, when deployed in reference to a region with high rates of premature death, speaks volumes about the differential valuation of human lives. Ebola itself was isolated fully two decades ago (14). Its appearance in human hosts has at times been insidious but more often takes the form of explosive eruptions. In accounting for recent outbreaks, it is unnecessary to postulate a change in filovirus virulence through mutation. The Institute of Medicine lists a single "factor facilitating emergence" for filoviruses: "virus-infected monkeys shipped from developing countries via air" (2).

Other factors are easily identified. Like that of many infectious diseases, the distribution of Ebola outbreaks is tied to regional trade networks and other evolving social systems. And, like those of most infectious diseases, Ebola explosions affect, researchers aside, certain groups (people living in poverty, health care workers who serve the poor) but not others in close physical proximity. Take, for example, the 1976 outbreak in Zaire, which affected 318 persons. Although respiratory spread was speculated, it has not been conclusively demonstrated as a cause of human cases. Most expert observers thought that the cases could be traced to failure to follow contact precautions, as well as to improper sterilization of syringes and other paraphernalia, measures that in fact, once taken, terminated the outbreak (15). On closer scrutiny, such an explanation suggests that Ebola does not emerge randomly: in Mobutu's Zaire, one's likelihood of coming into contact with unsterile syringes is inversely proportional to one's social status. Local élites and sectors of the expatriate community with access to high-quality biomedical services (viz., the European and American communities and not the Rwandan refugees) are unlikely to contract such a disease.

The changes involved in the disease's visibility are equally embedded in social context. The emergence of Ebola has also been a question of our consciousness. Modern communications, including print and broadcast media, have been crucial in the construction of Ebola—a minor player, statistically speaking, in Zaire's long list of fatal infections—as an emerging infectious disease (16). Through Cable News Network (CNN) and other television stations, Kikwit became, however briefly, a household word in parts of Europe and North America. Journalists and novelists wrote best-selling books about small but horrific plagues, which in turn became profitable cinema. Thus, symbolically and proverbially, Ebola spread like wildfire—as a danger potentially without limit. It emerged.

Emerging From Where? The Case of TB

TB is said to be another emerging disease, in which case, emerging is synonymous with reemerging. Its recrudescence is often attributed to the advent of HIV—the Institute of Medicine lists "an increase in immunosuppressed populations" as the sole

factor facilitating the resurgence of TB (2)—and the emergence of drug resistance. A recent book on TB, subtitled "How the battle against tuberculosis was won—and lost," argues that "Throughout the developed world, with the successful application of triple therapy and the enthusiastic promotion of prevention, the death rate from tuberculosis came tumbling down" (17). But was this claim ever documented? Granted, the discovery of effective anti-TB therapies has saved the lives of hundreds of thousands of TB patients, many in industrialized countries. But TB—once the leading cause of death among young adults in the industrialized world—was already declining there well before the 1943 discovery of streptomycin. In the rest of the world, and in pockets of the United States, TB remains undaunted by ostensibly effective drugs, which are used too late, inappropriately, or not at all: "It is sufficiently shameful," notes one of the world's leading authorities on TB, "that 30 years after recognition of the capacity of triple-therapy . . . to elicit 95%+ cure rates, tuberculosis prevalence rates for *many* nations remain unchanged" (18). Some estimate that more than 1.7 billion persons are infected with quiescent, but viable, *Mycobacterium tuberculosis* and, dramatic shifts in local epidemiology aside, a global analysis does not suggest major decreases in the importance of TB as a cause of death. TB has retreated in certain populations, maintained a steady state in others, and surged forth in still others, remaining, at this writing, the world's leading infectious cause of adult deaths (19).

At mid-century, TB was still acknowledged as the "great white plague." What explains the invisibility of this killer by the 1970s and 1980s? Again, one must turn to the study of disease awareness, that is, of consciousness and publicity, and their relation to power and wealth. "The neglect of tuberculosis as a major public health priority over the past two decades is simply extraordinary," wrote Murray in 1991. "Perhaps the most important contributor to this state of ignorance was the greatly reduced clinical and epidemiologic importance of tuberculosis in the wealthy nations" (20). Thus TB has not really emerged so much as emerged from the ranks of the poor (21,22). An implication, clearly, is that one place for diseases to hide is among poor people, especially when the poor are socially and medically segregated from those whose deaths might be considered more important.

When complex forces move more poor people into the United States, an increase in TB incidence is inevitable. In a recent study of the disease among foreign-born persons in the United States, immigration is essentially credited with the increased incidence of TB-related disease (23). The authors note that in some of the immigrants' countries of origin the annual rate of infection is up to 200 times that registered in the United States; moreover, many persons with TB in the United States live in homeless shelters, correctional facilities, and camps for migrant workers. But there is no discussion of poverty or inequality, even though these are, along with war, leading reasons for both the high rates of TB and for immigration to the United States. "The major determinants of risk in the foreign-born population," conclude the authors, "were the region of the world from which the person emigrated and the number of years in the United States."

Going Where? The Case of HIV

To understand the complexity of the issues—medical, social, and communicational—that surround the emergence of a disease into public view, consider AIDS. In the early 1980s, the public was informed by health officials that AIDS had probably emerged from Haiti. In December 1982, for example, a physician affiliated with the National Cancer Institute was widely quoted in the popular press stating that "We suspect that this may be an epidemic Haitian virus that was brought back to the homosexual population in the United States" (24). This proved incorrect, but not before damage to Haitian tourism had been done. Result: more poverty, a yet steeper slope of inequality and vulnerability to disease, including AIDS. The label "AIDS vector" was also damaging to the million or so Haitians living elsewhere in the Americas and certainly hampered public health efforts among them (25).

HIV disease has since become the most extensively studied infection in human

history. But some questions are much better studied than are others. And error is worth studying, too. Careful investigation of the mechanisms by which immodest claims are propagated (as regards Haiti and AIDS, these mechanisms included "exoticization" of Haiti, racism, the existence of influential folk models about Haitians and Africans, and the conflation of poverty and cultural difference) is an important yet neglected part of a critical epistemology of emerging infectious diseases. Also underinvestigated are considerations of the pandemic's dynamic. HIV may not have come from Haiti, but it was going to Haiti. Critical reexamination of the Caribbean AIDS pandemic showed that the distribution of HIV does not follow national borders, but rather the contours of a transnational socioeconomic order. Furthermore, much of the spread of HIV in the 1970s and 1980s moved along international "fault lines," tracking along steep gradients of inequality, which are also paths of migrant labor and sexual commerce (26).

In an important overview of the pandemic's first decade, Mann and coworkers observe that its course "within and through global society is not being affected—in any serious manner—by the actions taken at the national or international level" (27). HIV has emerged but is going where? Why? And how fast? The Institute of Medicine lists several factors facilitating the emergence of HIV: "urbanization; changes in lifestyles/mores; increased intravenous drug abuse; international travel; medical technology" (2). Much more could be said. HIV has spread across the globe, often wildly, but rarely randomly. Like TB, HIV infection is entrenching itself in the ranks of the poor or otherwise disempowered. Take, as an example, the rapid increase in AIDS incidence among women. In a 1992 report, the United Nations observed that "for most women, the major risk factor for HIV infection is being married. Each day a further three thousand women become infected, and five hundred infected women die" (28). It is not marriage per se, however, that places young women at risk. Throughout the world, most women with HIV infection, married or not, are living in poverty. The means by which confluent social forces, such as gender inequality and poverty, come to be embodied as risk for infection with this emerging pathogen have been neglected in biomedical, epidemiologic, and even social science studies on AIDS. As recently as October 1994—15 years into an ever-emerging pandemic—a *Lancet* editorial could comment, "We are not aware of other investigators who have considered the influence of socioeconomic status on mortality in HIV-infected individuals" (29). Thus, in AIDS, the general rule that the effects of certain types of social forces on health are unlikely to be studied applies in spite of widespread impressions to the contrary.

AIDS has always been a strikingly patterned pandemic. Regardless of the message of public health slogans—"AIDS is for Everyone"—some are at high risk for HIV infection, while others, clearly, are at lower risk. Furthermore, although AIDS eventually causes death in almost all HIV-infected patients, the course of HIV disease varies. Disparities in the course of the disease have sparked the search for hundreds of cofactors, from *Mycoplasma* and ulcerating genital lesions to voodoo rites and psychological predisposition. However, not a single association has been compellingly shown to explain disparities in distribution or outcome of HIV disease. The only well-demonstrated cofactors are social inequalities, which have structured not only the contours of the AIDS pandemic, but also the course of the disease once a patient is infected (30-33). The advent of more effective antiviral agents promises to heighten those disparities even further: a three-drug regimen that includes a protease inhibitor will cost $12,000 to $16,000 a year (34).

Questions for a Critical Epistemology of Emerging Infectious Diseases

Ebola, TB, and HIV infection are in no way unique in demanding contextualization through social science approaches. These approaches include the grounding of case histories and local epidemics in the larger biosocial systems in which they take shape and demand exploration of social inequalities. Why, for example, were there 10,000 cases of diphtheria in Russia from 1990 to 1993? It is easy enough to argue that the excess cases were due to a failure to

vaccinate (35). But only in linking this distal (and, in sum, technical) cause to the much more complex socioeconomic transformations altering the region's illness and death patterns will compelling explanations emerge (36,37).

Standard epidemiology, narrowly focused on individual risk and short on critical theory, will not reveal these deep socioeconomic transformations, nor will it connect them to disease emergence. "Modern epidemiology," observes one of its leading contributors, is "oriented to explaining and quantifying the bobbing of corks on the surface waters, while largely disregarding the stronger undercurrents that determine where, on average, the cluster of corks ends up along the shoreline of risk" (13). Neither will standard journalistic approaches add much: "Amidst a flood of information," notes the chief journalistic chronicler of disease emergence, "analysis and context are evaporating . . . Outbreaks of flesh eating bacteria may command headlines, but local failures to fully vaccinate preschool children garner little attention unless there is an epidemic" (38).

Research questions identified by various blue-ribbon panels are important for the understanding and eventual control of emerging infectious diseases (39,40). Yet both the diseases and popular and scientific commentary on them pose a series of corollary questions, which, in turn, demand research that is the exclusive province of neither social scientists nor bench scientists, clinicians, or epidemiologists. Indeed, genuinely transdisciplinary collaboration will be necessary to tackle the problems posed by emerging infectious diseases. As prolegomena, four areas of corollary research are easily identified. In each is heard the recurrent leitmotiv of inequality:

Social Inequalities

Study of the reticulated links between social inequalities and emerging disease would not construe the poor simply as "sentinel chickens," but instead would ask, What are the precise mechanisms by which these diseases come to have their effects in some bodies but not in others? What propagative effects might social inequalities per se contribute (41)? Such queries were once major research questions for epidemiology and social medicine but have fallen out of favor, leaving a vacuum in which immodest claims of causality are easily staked. "To date," note Krieger and co-workers in a recent, magisterial review, "only a small fraction of epidemiological research in the United States has investigated the effects of racism on health" (42). They join others in noting a similar dearth of attention to the effects of sexism and class differences; studies that examine the conjoint influence of these social forces are virtually nonexistent (43,44).

And yet social inequalities have sculpted not only the distribution of emerging diseases, but also the course of disease in those affected by them, a fact that is often downplayed: "Although there are many similarities between our vulnerability to infectious diseases and that of our ancestors, there is one distinct difference: we have the benefit of extensive scientific knowledge" (7). True enough, but Who are "we"? Those most at risk for emerging infectious diseases generally do not, in fact, have the benefit of cutting-edge scientific knowledge. We live in a world where infections pass easily across borders—social and geographic—while resources, including cumulative scientific knowledge, are blocked at customs.

Transnational Forces

"Travel is a potent force in disease emergence and spread," as Wilson has reminded us, and the "current volume, speed, and reach of travel are unprecedented" (45). Although the smallpox and measles epidemics following the European colonization of the Americas were early, deadly reminders of the need for systemic understandings of microbial traffic, there has been, in recent decades, a certain reification of the notion of the "catchment area." A useful means of delimiting a sphere of action—a district, a county, a country—is erroneously elevated to the status of explanatory principle whenever the geographic unit of analysis is other than that defined by the disease itself. Almost all diseases held to be emerging—from the increasing number of drug-resistant dis-

eases to the great pandemics of HIV infection and cholera—stand as modern rebukes to the parochialism of this and other public health constructs (46). And yet a critical sociology of liminality—both the advancing, transnational edges of pandemics and also the impress of human-made administrative and political boundaries on disease emergence—has yet to be attempted.

The study of borders qua borders means, increasingly, the study of social inequalities. Many political borders serve as semipermeable membranes, often quite open to diseases and yet closed to the free movement of cures. Thus may inequalities of access be created or buttressed at borders, even when pathogens cannot be so contained. Research questions might include, for example, What effects might the interface between two very different types of health care systems have on the rate of advance of an emerging disease? What turbulence is introduced when the border in question is between a rich and a poor nation? Writing of health issues at the U.S.-Mexican border, Warner notes that "It is unlikely that any other binational border has such variety in health status, entitlements, and utilization" (47). Among the infectious diseases registered at this border are multidrug-resistant TB, rabies, dengue, and sexually transmitted diseases including HIV infection (said to be due, in part, to "cross-border use of 'red-light' districts").

Methods and theories relevant to the study of borders and emerging infections would come from disciplines ranging from the social sciences to molecular biology: mapping the emergence of diseases is now more feasible with the use of restriction fragment length polymorphism and other new technologies (48). Again, such investigations will pose difficult questions in a world where plasmids can move, but compassion is often grounded.

The Dynamics of Change

Can we elaborate lists of the differentially weighted factors that promote or retard the emergence or reemergence of infectious diseases? It has been argued that such analyses will perforce be historically deep and geographically broad, and they will at the same time be processual, incorporating concepts of change. Above all, they will seek to incorporate complexity rather than to merely dissect it. As Levins has recently noted, "effective analysis of emerging diseases must recognize the study of complexity as perhaps the central general scientific problem of our time" (49). Can integrated mathematical modeling be linked to new ways of configuring systems, avoiding outmoded units of analyses, such as the nation-state, in favor of the more fluid biosocial networks through which most pathogens clearly move? Can our embrace of complexity also include social complexity and the unequal positioning of groups within larger populations? Such perspectives could be directed towards mapping the progress of diseases from cholera to AIDS, and would permit us to take up more unorthodox research subjects—for example, the effects of World Bank projects and policies on diseases from onchocerciasis to plague.

Critical Epistemology

Many have already asked, What qualifies as an emerging infectious disease? More critical questions might include, Why do some persons constitute "risk groups," while others are "individuals at risk"? These are not merely nosologic questions; they are canonical ones. Why are some approaches and subjects considered appropriate for publication in influential journals, while others are dismissed out of hand? A critical epistemology would explore the boundaries of polite and impolite discussion in science. A trove of complex, affect-laden issues—attribution of blame to perceived vectors of infection, identification of scapegoats and victims, the role of stigma—are rarely discussed in academic medicine, although they are manifestly part and parcel of many epidemics.

Finally, why are some epidemics visible to those who fund research and services, while others are invisible? In its recent statements on TB and emerging infections, for example, the World Health Organization uses the threat of contagion to motivate wealthy nations to invest in disease surveillance and control out of self-interest—an age-old public health approach acknowl-

edged in the Institute of Medicine's report on emerging infections: "Diseases that appear not to threaten the United States directly rarely elicit the political support necessary to maintain control efforts" (2). If related to a study under consideration, questions of power and control over funds, must be discussed. That they are not is more a marker of analytic failures than of editorial standards.

Ten years ago, the sociologist of science Bruno Latour reviewed hundreds of articles appearing in several Pasteur-era French scientific reviews to constitute what he called an "anthropology of the sciences" (he objected to the term epistemology). Latour cast his net widely. "There is no essential difference between the human and social sciences and the exact or natural sciences," he wrote, "because there is no more science than there is society. I have spoken of the Pasteurians as they spoke of their microbes" (50) (Here, perhaps, is another reason to engage in a "proactive" effort to explore themes usually relegated to the margins of scientific inquiry: those of us who describe the comings and goings of microbes—feints, parries, emergences, retreats—may one day be subjected to the scrutiny of future students of the subject).

Microbes remain the world's leading causes of death (51). In "The conquest of infectious diseases: who are we kidding?" the authors argue that "clinicians, microbiologists, and public health professionals must work together to prevent infectious diseases and to detect emerging diseases quickly" (52). But past experience with epidemics suggests that other voices and perspectives could productively complicate the discussion. In every major retrospective study of infectious disease outbreaks, the historical regard has shown us that what was not examined during an epidemic is often as important as what was (53,54) and that social inequalities were important in the contours of past disease emergence. The facts have taught us that our approach must be dynamic, systemic, and critical. In addition to historians, then, anthropologists and sociologists accountable to history and political economy have much to add, as do the critical epidemiologists mentioned above (55-58).

My intention, here, is ecumenical and complementary. A critical framework would not aspire to supplant the methods of the many disciplines, from virology to molecular epidemiology, which now concern themselves with emerging diseases. "The key task for medicine," argued the pioneers Eisenberg and Kleinman some 15 years ago, "is not to diminish the role of the biomedical sciences in the theory and practice of medicine but to supplement them with an equal application of the social sciences in order to provide both a more comprehensive understanding of disease and better care of the patient. The problem is not 'too much science,' but too narrow a view of the sciences relevant to medicine" (59).

A critical anthropology of emerging infections is young, but it is not embryonic. At any rate, much remains to be done and the tasks themselves are less clear perhaps than their inherent difficulties. The philosopher Michel Serres once observed that the border between the natural and the human sciences was not to be traced by clean, sharp lines. Instead, this border recalled the Northwest Passage: long and perilously complicated, its currents and inlets often leading nowhere, dotted with innumerable islands and occasional floes (60). Serres' metaphor reminds us that a sea change is occurring in the study of infectious disease even as it grows, responding, often, to new challenges—and sometimes to old challenges newly perceived.

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Molecular Mechanisms of Bacterial Virulence: Type III Secretion and Pathogenicity Islands

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Recently, two novel but widespread themes have emerged in the field of bacterial virulence: type III secretion systems and pathogenicity islands. Type III secretion systems, which are found in various gram-negative organisms, are specialized for the export of virulence factors delivered directly to host cells. These factors subvert normal host cell functions in ways that seem beneficial to invading bacteria. The genes encoding several type III secretion systems reside on pathogenicity islands, which are inserted DNA segments within the chromosome that confer upon the host bacterium a variety of virulence traits, such as the ability to acquire iron and to adhere to or enter host cells. Many of these segments of DNA appear to have been acquired in a single step from a foreign source. The ability to obtain complex virulence traits in one genetic event, rather than by undergoing natural selection for many generations, provides a mechanism for sudden radical changes in bacterial-host interactions. Type III secretion systems and pathogenicity islands must have played critical roles in the evolution of known pathogens and are likely to lead to the emergence of novel infectious diseases in the future.

Discovery of Two Traits Used by a Broad Range of Bacterial Pathogens

In the past decade, there has been an explosion of new information about bacterial pathogens as researchers have begun to examine the molecular and genetic bases of microbial pathogenicity. Because microbes invade many niches in humans and cause a wide variety of syndromes, it initially appeared that each disease might be created by a distinct molecular mechanism. However, the spectrum of methods is not as broad as first imagined; rather, bacteria exploit a number of common molecular tools to achieve a range of goals (1). Among these tools are pathogenicity islands, which enable bacteria to gain complex virulence traits in one step, and type III secretion systems, which provide a means for bacteria to target virulence factors directly at host cells. These factors then tamper with host cell functions to the pathogens' benefit.

Early in the search for virulence genes, researchers discovered that many of these genes resided on plasmids or phages; however, it was also clear that these genes did not produce all of the physiologic changes induced in host cells by various pathogens (2). Thus, researchers searched the chromosome. Surprisingly, as when found on plasmids, virulence genes often clustered in functionally related groups. Furthermore, these groups often appeared to have been acquired from another organism, as features of their DNA sequence differed from the bulk of the genome. These observations gave rise to the concept of pathogenicity islands—discrete segments of DNA that encode virulence traits and often appear to have a foreign origin (3,4).

Researchers found that a particular set of virulence genes appeared several times on both plasmids and pathogenicity islands (5-8). These genes were discovered in both plant and animal pathogens and were homologous

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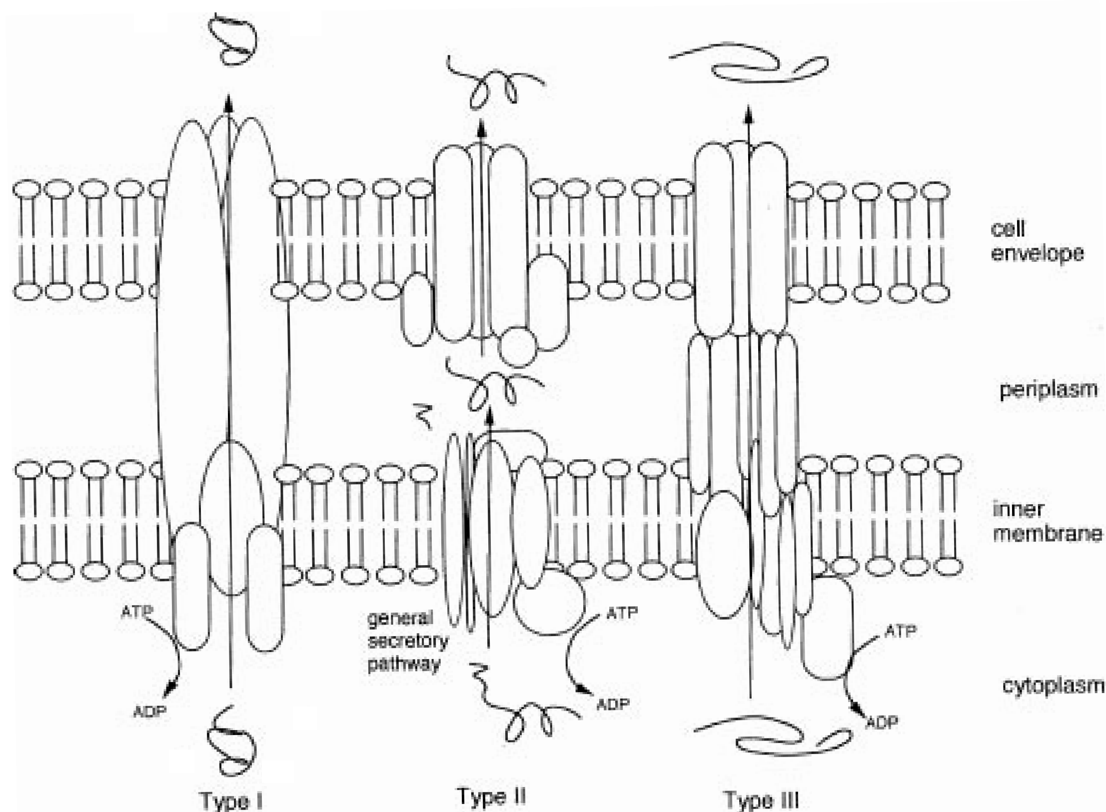

Figure 1: Schematic diagram of type I, type II, and type III secretion systems. All systems use the energy of ATP hydrolysis to drive secretion. Type I and type III secrete proteins across both the inner membrane and the cell envelope (outer membrane) in one step; secreted proteins do not make an intermediate stop in the periplasm, as they do in type II secretion. Type I and type III systems are also similar in that they do not remove any part of the secreted protein. In contrast, the N-terminus of proteins secreted by the general secretory pathway is removed upon transfer to the periplasm; the N-terminal signal sequence can be seen in the periplasm, and the extracellular protein is clearly different from the intracellular protein by virtue of its absence. Type I systems are composed of far fewer components than type III systems; this is indicated by the number of distinct proteins (indicated by shape and size) in the figure. Type II and type III systems share a similar cell envelope component, as indicated by sequence homology; this is reflected in the shape of a cell envelope component in the figure.

to genes encoded on a virulence plasmid of pathogenic *Yersinia spp.* (Table 1) (9-12). The *Yersinia* proteins are the components of a novel secretion system (13), called type III (14). This machinery propels effector molecules toward host cells where they alter host physiology (15,16). The homology suggested that many divergent bacterial pathogens had acquired a similar system from a common source. Pathogens use the type III system to secrete different effector molecules that influence host cells in a variety of ways (16-19).

Secretion Systems in Bacteria

Secreted or surface-exposed bacterial proteins have long been known to play central roles in bacterial-host interactions. In gram-negative bacteria, these proteins must pass through two membranes: the inner membrane, which surrounds the cytoplasm, and the outer envelope, which encloses the periplasm and acts as a barrier to the environment (Figure 1). The general secretory pathway transports proteins to the periplasm. Before the *Yersinia* secretion system was identified, two other specialized secretion systems, type I and type II, were

known to transport molecules to the cell surface (14,20,21). Proteins secreted by the Type I system cross directly from the cytoplasm to the cell surface, bypassing the general secretory pathway completely (Figure 1). Type II-secreted proteins use the general secretory pathway to reach the periplasm and then traverse the outer membrane through distinct channel proteins. Both type I and type II systems secrete proteins involved in various functions, including pathogenesis. For example, α-hemolysin of *E. coli* uses a type I system and bundle-forming pili of enteropathogenic *E. coli* (*EPEC*) and enterotoxigenic *E. coli* (*ETEC*) use type II systems for export.

In the past 5 years, the highly conserved, multicomponent type III secretion system has been found in many gram-negative bacteria that cause disease in animals and plants (8). This secretion system is responsible for transporting effector molecules directly from the cytoplasm to the cell surface, where they interact with mammalian cells and modify host cell proteins (13). This one-step secretion process is reminiscent of the mechanism used by type I systems (Figure 1). The genes that encode many components of type III systems are homologous to those that encode flagellar export machinery in both gram-negative and gram-positive bacteria (Table 1) (22-24). Indeed, these two systems share many structural and functional features. The differences reside at the outer membrane. Flagellar components pass through an outer ring structure that is part of the flagellum itself (23), whereas pathogenic effector molecules traverse the outer membrane through a channel protein that is homologous to those used in type II secretion systems (20,25). While our discussion of type III secretion systems will focus primarily on those used by human pathogens, many characteristics are common among systems found in plant pathogens and bacteria that produce flagella.

Type III Systems Secrete Effector Proteins Upon Contacting Host Cells

In contrast to the secretion process in type I and type II systems, type III secretion is triggered when a pathogen comes in close contact with host cells (18,19,26,27), and hence, has been called contact-dependent secretion (28). Temperature, growth phase, and salt conditions are environmental cues known to induce synthesis of the secretion apparatus and effector molecules in various pathogens (29-31). When the pathogen comes into close contact with tissue culture cells, effector molecules move to the external surface of the bacterium, sometimes forming appendages suggestive of flagellae (18). In some cases, the bacterium binds to the host cells and these molecules are delivered into the host cell (32). The effector molecules cause changes in host cell function, which facilitate the pathogen's ability to survive and replicate (15-17,33).

How Pathogens Use Type III Secretion Systems in the Host

The best studied bacterial pathogens that use type III secretion are *Yersinia pestis*, which causes plague, and a number of enteropathogens. Although these various enteropathogens (*Yersinia spp., Salmonella spp., Shigella spp.*, and EPEC) cause diarrhea and, in some cases, systemic disease, they produce distinct syndromes because their secreted proteins target different host cells and molecules (Table 1) (34).

Yersinia spp. use their effector molecules to destroy key functions of immune cells and render them innocuous (35). When these bacteria bind to tissue culture cells, approximately 10 different effector molecules are secreted (13) and at least three are injected into cells (27,36-38). Two of these injected molecules, YopE and YopH, modify macrophage proteins and destroy the cells' abilities to engulf and kill bacteria (16,39). During the course of disease, immune cells are presumably neutralized by these effector molecules, which enables *Yersinia spp.* to flourish in the reticuloendothelial environment.

While effector molecules in *Yersinia* destroy normal cellular functions, those from *Shigella spp.* and from one of the *Salmonella spp.* type III secretion systems, encoded by genes located in SPI I (Table 2), stimulate

Table 1. Function and location of components in Type III systems[a]

Yersinia (p)[b]	*S. typhimurium* SPI I(c)	SPI II(c)	*S. flexneri* (p)	EPEC (c)	Flagella[c] (c)	Plant[d] (c)	Possible Function[e]	Possible Cellular Location[f]	Reference
YscN	SpaL	orf1	MxiB	SepB	FliI	HrpE	ATPase	cytoplasm, associated with inner membrane (I.M.)	8, 43, 63
LcrD	InvA	orf9	MxiA	SepA	FlhA	HrpO	structural/ regulatory	I.M.	28, 43, 63
YscR	SpaP	orf5	Spa24		FliP	HrpT	structural	I.M.	8, 43
YscS	SpaQ	orf6	Spa9		FliQ	HrpU	"	"	28, 43
YscT	SpaR	orf7	Spa40		FliR	HrpC	"	"	8, 43
YscU	SpaS	orf8	Spa40		FhlB	HrpN	"	"	8, 43, 63
YscC	InvG	orf11	MxiD	SepC		HrpA	structural/ channel forming	outer membrane (O.M.)	28, 43, 63
YscJ	PrgK	orf10	MxiJ	SepD	FliF	HrpI	lipoprotein/ structural	O.M.	8, 28, 43, 63
VirG							"	"	52
YscD							essential, but unknown	I.M.	48
YscE							"	unknown	51
YscF	PrgI		MxiH				needed for YopB and D secretion	"	28, 51
YscG							essential, but unknown	equally distributed between membrane and soluble fractions	48
YscI							"	unknown	51
YscK							"	"	51
YscL						HrpF	"	"	8
YscO	SpaM	orf2	Spa13				unknown	"	12, 28, 43
YscP	SpaN	orf3	Spa32				"	"	12, 28, 43
YscQ	SpaO	orf4	Spa33		FliN	HrpQ	SpaO needed for secretion of Sips (see below)	Spa O: secreted	8, 28, 43
YopB	SipB		IpaB				homology to pore forming toxins/ delivery of effector molecules to host cells	secreted/targeted to host cell	26, 28, 55

[a]Listed are names of proteins in type III secretion systems
[b]p-proteins are encoded by genes on a plasmid; c-proteins are encoded by genes on the chromosome.
[c]Protein names are those from the *S. Typhimurium* flagella; for list of flagellar protein names from other bacteria, see ref. 23.
[d]Protein names are those from the plant pathogen *Pseudomonas solanacearum*; for a list of components of type III secretion systems in other plant pathogens, see ref. 8.
[e]These are the possible functions for these factors in *Yersinia*, unless noted.
[f]These are the possible locations for these factors in *Yersinia*, unless noted.

Table 1. Function and location of components in Type III systems[a] (continued)

Yersinia (p)[b]	*S. Typhimurium* SPI 1(c)	SPI II(c)	*S. Flexneri* (p)	EPEC (c)	Flagella[c] (c)	Plant[d] (c)	Possible Function[e]	Possible Cellular Location[f]	Reference
YopD							delivery of effector molecules to host cells	secreted	55
	SipD		IpaD				"	secreted	26
SycD	SicA		IpgC				chaperone for YopB and D/ IpaB and IpaC in *Shigella*	cytoplasm	56
SycE							chaperone for YopE	"	56
SycH							chaperone for YopH	"	56
YopN	InvE		MxiC				regulatory: cell-contact sensing pathway	O.M./secreted	28
LcrG							"	cytosol/I.M.	59, S. Straley pers. comm.
LcrQ							regulatory	cytoplasm/ secreted	54
VirF	InvF		MxiE			HrpB	regulatory: temperature	cytoplasm	28
YopE							effector molecule: depolymerizes actin anti-phagocytic	host cell	27
YopH							effector molecule: tyrosine phosphatase anti-phagocytic	"	36
	SipC		IpaC				effector molecule: induces entry into epithelial cells	secreted	17, 28
				EspB (EaeB)			effector molecule: induces AE lesions		33, 63

[a]Listed are names of proteins in type III secretion systems

[b]p-proteins are encoded by genes on a plasmid; c-proteins are encoded by genes on the chromosome.

[c]Protein names are those from the *S. Typhimurium* flagella; for list of flagellar protein names from other bacteria, see ref. 23.

[d]Protein names are those from the plant pathogen *Pseudomonas solanacearum;* for a list of components of type III secretion systems in other plant pathogens, see ref. 8.

[e]These are the possible functions for these factors in *Yersinia*, unless noted.

[f]These are the possible locations for these factors in *Yersinia*, unless noted.

Table 2. Characteristics of several pathogenicity islands

Organism	Name	Location	Borders	Stable ?	Foreign origin G+C: % island/ % chromosome	Functions	Size	Ref.
Uropathogenic *E. coli* 536	Pathogenicity island I, Pai I	*selC*[a], 82'	•16 bp direct repeats, derived from *selC* • shared motif with Pai II repeats	no	•direct repeats •absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin I	70 kb	3, 82, 84
	Pai II	*leuX*[a], 97'	•18 bp direct repeats, derived from *leuX* • shared motif with Pai I repeats	no	•direct repeats •absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin II •prf (fimbriae: adherence to host cells) •transcriptional activators of chromosomal genes	190 kb	3,4, 67, 82, 84
Uropathogenic *E. coli* J96	Pai I	near *pheV*[a], 64'			•absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin I •pap (fimbriae: adherence to host cells) •IS element sequences •R plasmid sequences •P4 phage sequences	>170 kb	96
	Pai II	*pheR*[a], 94'	135 bp imperfect direct repeats	no	•direct repeats •absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin II •prs (fimbriae: adherence to host cells) •cytotoxic necrotizing factor type 1 •IS element sequences •P4 phage sequences •OmpR homolog	106 kb	4, 96, 97
Entero-pathogenic *E. coli* (EPEC)	Locus of enterocyte effacement, LEE	*selC*[a], 82'	no repeats or IS elements found	yes[b]	•G+C: 39%/51% •not present in closely related, non-AE-producing bacteria	•mediates formation of AE lesions •type III secretion system	35 kb	63, 83
Salmonella typhimurium	*Salmonella* pathogenicity island 1, SPI 1	between *flhI* and *mutS*, 63'	no repeats or IS elements found in *S. typhimurium*; IS3 on one border in certain *Salmonella* serotypes	yes[b,c]	•G+C: 42%/52% •absent from *E. coli*	•invasion into cultured epithelial cells •type III secretion system	40 kb	28, 68

[a]tRNA gene
[b]apparently
[c]unstable in certain serotypes
[d]additional information received in personal communication with M. Stein
[e]Censini S, et al., 1996, submitted for publication

Table 2. Characteristics of several pathogenicity islands (continued)

Organism	Name	Location	Borders	Stable ?	Foreign origin G+C: %island/ %chromosome	Functions	Size	Ref.
	SPI 2	between *ydhE* and *pykF*, 31'		yes[b]	•G+C: 45%/52% •absent from *E. coli*; conserved among *Salmonella*	•type III secretion system	40 kb	43
	Salmonella induced filament gene A, *sifA*	*potB*/ *potC*	14 bp direct repeats	yes	•G+C: 41%/52% •direct repeats •absent in *E. coli*; conserved among *Salmonella*	•required for formation of structures associated with *Salmonella*-associated vacuoles within epithelial cells	1.6 kb	69[d]
Yersinia pestis	Ability to adsorb exogenous pigments, Pgm	*phoE*	2.2 kb direct repeats (=IS100)	no	•G+C: hemin storage region 47%/46-50%; yersiniabactin receptor/ iron-regulated protein region 56-60%/46-50% •direct repeats	•hemin and congo red binding •pesticin sensitivity •iron acquisition •growth at 37 C in defined medium	102 kb	70, 71
Helicobacter pylori	Cytotoxin-associated gene region, Cag	*glr*	•31 bp direct repeats, derived from glutamate racemase gene •IS605 on one end	see text	•G+C: 35%/38-45% •IS elements •not present in type II strains	•induction of IL-8 secretion •homologues to membrane-associated proteins: environmental sensors, translocases, permeases, pilus and flagella assembly proteins •IS elements	40 kb	e
Vibrio cholerae O139	*otnA otnB*	*rfb*	flanked by two different IS elements		•IS elements •not present in *Vibrio cholerae* O1 El Tor	•capsule and O antigen synthesis (by homology)	35 kb	93, 98
Listeria monocytogenes		between *prs* and *ldh*	No IS elements found	yes[b]	•not present in several nonpathogenic species	•escape from vacuole •intra-/inter-cellular spread	9.6 kb	99

[a]tRNA gene
[b]apparently
[c]unstable in certain serotypes
[d]additional information received in personal communication with M. Stein
[e]Censini S, et al., 1996, submitted for publication

cells to perform functions in addition to those in their usual repertoires. Studies in epithelial tissue culture systems show that these bacteria induce their own entry into normally nonphagocytic cells by using effector molecules secreted by their type III systems (28,40). During the course of disease, *Shigella spp.* enter and replicate in the mucosal epithelial cells of the large intestine, while *Salmonella spp.* gain entry into the peritoneal cavity by passing through the epitheloid-like M cells in the small intestine (41). In the murine model for typhoid fever, *S. typhimurium* that are defective in this secretion system are attenuated for infection when administered orally, but not intraperitoneally (42). Presumably this attenuation is a consequence of reduced entry into the M cells in the small intestine, a barrier that is bypassed by intraperitoneal delivery.

Although neither its effector molecules nor target host cells have been identified, a second type III secretion system, encoded by genes located in SPI II (Table 2), has been described in *S. typhimurium*, on the basis of sequence homology (43). The genes in SPI II, in contrast to those in SPI I, are required for systemic disease regardless of the route of infection (43,44). Presumably the factors encoded in SPI II act after the bacteria have crossed the epithelial barrier of the small intestine.

Although the functions and sites of action of the secretion systems differ among these enteric pathogens, effector molecules from one system can be secreted by other systems, provided the appropriate chaperones are present. Such heterologously expressed effector molecules can induce the same cellular response as when expressed from their native systems. For instance, an effector molecule from *Yersinia* that causes actin depolymerization has the same effect on tissue culture cells when secreted from *Salmonella* (45). Likewise, proteins of *Shigella* and *Salmonella* involved in bacterial uptake into cultured epithelial cells are functionally interchangeable (46).

The observation that *Salmonella spp.* have two contact-dependent systems that function at distinct stages to cause disease raises several interesting questions. Why do *Salmonella spp.* need different type III secretion apparatuses when it is clear that effector molecules can be secreted from heterologous systems? Could one suffice if the two sets of effector molecules were expressed at appropriate times during the course of infection? Alternatively, do these two sets of effector molecules need to be delivered to different target cells in a specific manner, which is only possible with distinctly customized machinery? Answers to these questions will illuminate issues about both the course of salmonellosis and the basic mechanics of the secretion apparatus.

The Nuts and Bolts of Type III Secretion

The type III secretion apparatus in *Yersinia spp.* has been the most intensively investigated. However, this work has been done in three *Yersinia spp*; thus several proteins have been shown to be essential for effector molecule secretion in one species but have not yet been examined in others. Our analysis of these studies assumes that proteins essential in one will play a similar role in all (Table 1).

One essential feature of any secretion system is that energy must be provided to move molecules through the membrane (14). Only one protein in the system, YscN, has been shown to hydrolyze ATP and thus is a likely candidate for generating energy to drive secretion (47). YscN is predicted to be a cytoplasmic protein, closely associated with the inner membrane.

Several proteins essential to secretion including LcrD, YscD, R, S, T, and U, are known or predicted to reside in the inner membrane (10,12,48-50). At the outer membrane, only one protein, YscC (48), and two lipoproteins, YscJ and VirG (51,52), appear essential for proper secretion. The roles and subcellular locations are not known for several more essential proteins, YscE, F, G, I, K, and L (9,48,51). How all of these proteins interact with one another to form the secretion apparatus is not yet understood. It is clear, however, that correct assembly of the apparatus is required not only for secretion, but also for normal synthesis of effector molecules (47,48). If one component of the export machinery is missing, production of the effector molecules is altered. This

feedback regulation also occurs in systems that produce flagella (53). How it works in *Yersinia* is under investigation; thus far, only one protein, LcrQ, has been implicated (54).

Two proteins, YopB and YopD, are loosely associated with the outer membrane (55) and are crucial for efficient delivery of effector molecules into target cells. These two proteins use the type III secretion system to reach the bacterial cell surface. Without YopB, which has homology to pore-forming toxins (55), and YopD, effector molecules are secreted but not efficiently internalized by host cells; thus, their activities on host cells are severely abrogated (27,36). Presumably, YopB and YopD form a pore in the mammalian cell through which effector molecules pass.

Several proteins, called chaperones, play critical roles in secretion by binding to effector molecules in the bacterial cytoplasm. Chaperones have several proposed functions (56-58). Chaperone binding may stabilize and prevent proteins from folding into conformations that are impossible to secrete. Alternatively, as has been shown for *Shigella*, they may prevent effector molecules from improperly associating with one another before secretion (58). Lastly, chaperones may deliver molecules to the secretion apparatus.

In addition to the feedback regulation mentioned above, the synthesis of and secretion from the *Yersinia* type III system is regulated by two networks that respond to environmental cues (29,35). A temperature-sensing network induces synthesis of the apparatus at 37°C and includes VirF and YmoA (35). A host cell-sensing network increases both synthesis of and secretion from the type III system when *Yersinia* binds to target cells. This regulatory system is called the low-calcium response network—low calcium presumably mimics some signal generated by cell contact—and includes YopN, LcrG, and LcrQ (32,54,59,60). YopN localizes to the outer membrane, where it senses cell contact and transduces this signal to the cytoplasm by an unknown mechanism (60). The role of LcrG has not yet been elucidated. LcrQ functions as a repressor of the Yops. When *Yersinia* comes into close contact with host cells, LcrQ is secreted from the cytoplasm through the type III secretion system. This lowers the intracellular concentration of LcrQ and results in an increase in synthesis and secretion of the Yops (32). Flagella synthesis is controlled in a similar manner (61).

Many of the structural components of the *Yersinia* system have homologues in *Shigella*, *Salmonella*, and *EPEC* (Table 1). A comparison of proteins found in each system shows that certain core structural components are present in all type III apparatuses, whereas others may exist in only one or a subset. These differences may be due to particular functions of each system.

Several studies have examined whether structural components from different bacteria are interchangable. In general, core constituents from *Shigella* and SPI I of *Salmonella*, which both facilitate bacterial uptake by epithelial cells, are interchangable with one another, but not with those in the *Yersinia* system (7,62). These results may be due to the observation that factors from *Shigella* and SPI I of *Salmonella* are predicted by sequence homology to be more structurally similar to each other than to those in *Yersinia* (7). Alternatively, some of the regulatory cues for secretion and assembly may be different for *Yersinia* than for *Salmonella* and *Shigella*.

Most proteins in the type III secretion systems, including effector proteins, regulatory proteins, structural proteins, and chaperones (Table 1), are encoded by genes that belong to several large operons, which are clustered together (7,9,12,63). These operons are on plasmids in some species, and on the chromosome in others (Table 1). In some cases, such as in *Shigella* and SPI I of *Salmonella*, the order of the genes within operons and the arrangement of the operons with respect to each other are conserved (7). These observations suggest that type III systems were inherited en masse, and likewise, could be transmitted to other bacteria en masse. One can speculate that the acquisition of a type III secretion system could allow a bacterium to adapt to different environments or hosts. For instance, a new pathogen could perhaps arise if a skin-commensal bacterium were to acquire the means to penetrate and survive in the skin-

associated lymphoid tissue by obtaining a type III secretion system from an enteropathogen.

Where Do Type III Secretion Systems Come From?

It seems plausible that the original type III secretion system for virulence factors evolved from those for flagellar assembly (22,53). The bacterial flagellum exists in a wide range of eubacteria and some archaebacteria, which indicates that it probably emerged well before gram-negative bacteria, the hosts of the type III virulence factor secretion systems identified thus far.

Attempts to establish any of the known type III secretion systems as the progenitor have been fruitless. On the basis of degrees of homology among different type III systems and well-established evolutionary relationships between the bacteria, each organism can be ruled out as the source (64). For example, *Shigella* emerged from *E. coli* after *Salmonella* and *E. coli* diverged from a common ancestor; thus, *Shigella* cannot have provided the type III systems conserved in the *Salmonella spp*. Conversely, as *Shigella* type III apparatus sequences have a G+C content well below that of *Salmonella* and of the bulk of the *Shigella* chromosome, *Salmonella* could not have been the source of the *Shigella* genes (Table 2). Thus, the ability to secrete effector molecules by this mechanism seems to have been introduced independently into each of these bacteria.

Examination of homologous genes in the epithelial cell invasion loci of *Salmonella* and *Shigella* shows that some are highly conserved, while others display much lower levels of homology (65). Li and colleagues have found a relationship between evolutionary rate of change and subcellular location: genes encoding several secreted proteins are hypervariable in relation to genes encoding several proteins located in the bacterial inner membrane (65). In principle, hypervariability could reflect antigenic variation or adaptations to diverse host environments; however, neither of these explanations appears to pertain to the particular proteins examined (28,65).

Type III systems sometimes provide much of what distinguishes particular organisms from closely related, often nonpathogenic, species. As described above, many operons encoding type III secretion machinery are clustered. DNA sequence analysis has shown that these loci are often distinguishable from the bulk of the genomic DNA. The loci that are chromosomally located represent “pathogenicity islands” (66).

History and Definition of “Pathogenicity Islands”

The phrase “pathogenicity island” was first used to describe two large, unstable pieces of chromosomal DNA, unique to uropathogenic *E. coli*, that encode a number of genes required for virulence (3,4). Since its conception, the term has evolved to include regions of chromosomal DNA essential for pathogenicity that do not appear to “belong” (Table 2). Not all pathogenicity islands are genetically unstable, but each one shows an indication of foreign origin. These pieces of DNA are often missing in closely related, nonvirulent bacteria. Many pathogenicity islands differ from the bulk of the genome in G+C content and codon usage, and their borders are often marked by repeated sequences or insertion elements, which suggests that some kind of recombination event delivered them to the chromosome. Several encode multiple proteins that collaborate to confer a single, complex virulence property to the bacterial host.

The definition of pathogenicity islands includes chromosomal location. As such, the plasmid-borne type III gene clusters of *Yersinia* and *Shigella* do not qualify (Table 1). This seems somewhat arbitrary. Indeed, phages and a number of plasmids can easily insert into and excise from the chromosome. Similarly, many transposable elements replicate and function equally as well in the chromosome as on an extrachromosomal element. It seems to us that a block of apparently foreign genes found uniquely in pathogenic members of a genus and required for virulence is a more useful and relevent defining feature of a pathogenicity island than location. Thus, it makes sense to include the loci encoding type III secretion systems, regardless of whether they reside on a plasmid or chromosome. In the

discussion below, however, we adhere to the established definition that includes chromosomal location.

Pathogenicity Islands Contain Virulence Genes and Regulatory Elements

Pathogenicity islands also contain virulence genes other than those encoding type III secretion systems; a common theme appears to be inclusion of genes for secreted or cell surface-localized proteins such as hemolysins, fimbriae, and hemin-binding factors (Table 2). In fact, the similarities between pathogenicity islands extend further: examination of the large ones shows that many also contain genes that encode a secretion system and environmental sensors. They also can include proteins that regulate expression of genes that lie outside the pathogenicity island. For example, pathogenicity island II (Pai II) of uropathogenic *E. coli* contains genes that encode transcriptional activators of S-fimbrial genes that reside at a chromosomal locus remote from either of the known pathogenicity islands in this species (67).

Pathogenicity Islands Can Exist in Various Structural Types and Numbers Within a Bacterium

A single bacterial strain can harbor more than one pathogenicity island. *Salmonella* contains at least five: the gene clusters encoding the two type III secretion systems described above, *sifA* (see below), and two groups of genes that are activated by the two-component regulator, PhoP/PhoQ. These loci vary in size and complexity and reside at distinct chromosomal locations (43,68,69, and S. Miller, pers. comm.).

Pathogenicity islands themselves can be composed of distinct segments. For example, an unstable 102-kb region of DNA that encodes several traits important for virulence of *Y. pestis* appears to consist of several regions (70-72). One contains the hemin storage genes and has a G+C content similar to that of the bulk of the chromosome (R. Perry, pers. comm.); the other contains genes encoding the Yersiniabactin receptor and iron-regulated proteins and has a significantly higher G+C content (R. Perry, pers. comm) (73). Although the 102 kb region often deletes entirely, the two regions can also act independently. In some strains the chromosomal region containing the hemin storage genes spontaneously deletes from the chromosome at a significant frequency, while the Yersiniabactin receptor/iron-regulated protein region appears stable (72). Furthermore, only the Yersiniabactin receptor/iron-regulated segment is present in *Y. enterocolitica* (70).

Even more complex pathogenicity islands are harbored by strains of *Helicobacter pylori*, the causative agent of gastritis and peptic ulcer disease in humans. Strains of *H. pylori* have been divided into two classes: type I strains express the cytotoxin-associated gene A (CagA) antigen and induce secretion of the neutrophil attractant IL-8 by epithelial cells in vitro, while type II strains lack both of these properties. Patients with duodenitis, duodenal ulcers, and gastric tumors are most often infected by type I strains. Likewise, type I strains are more likely than type II strains to cause gastric injuries in murine model systems. Analysis of the chromosomal region that contains the *cagA* gene has shown that it is a pathogenicity island of approximately 40 kb of DNA, missing in type II strains, and that mutations in this region abolish IL-8 induction in gastric epithelial cell lines (Censini, S et al. A pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated factors. 1996; submitted for publication).

Different type I strains display considerable heterogeneity in the cag region (Censini, S et al. A pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated factors. 1996; submitted for publication). In some isolates, the cag region is interrupted by one or more insertion sequences. In a small number of strains, there is an additional 20-kb sequence that is also present in type II strains. Partial deletions of the cag region have been detected as well. Thus, the cag pathogenicity island appears to be undergoing dynamic changes in natural *Helicobacter* populations. Further study of the cag region may elucidate details of pathogenicity island

acquisition and help correlate regions of the pathogenicity island with disease symptoms in the murine model system.

Since its establishment, the definition of pathogenicity islands has evolved to include genetic regions that are neither large nor complex; single genes of apparently foreign origin can also be inserted into chromosomal DNA. *S. typhimurium* has recently been shown to contain such a gene, called *sifA*, which is required for formation of distinctive structures associated with *Salmonella*-containing vacuoles within epithelial cells and contributes to pathogenicity in the murine typhoid fever model system (Table 2) (69).

Possible Origins of Pathogenicity Island DNA and Mechanisms of Transfer and Insertion

While the sources of pathogenicity islands are unknown, their presence in a wide variety of organisms (Table 2) indicates that bacteria can acquire DNA despite multiple barriers to chromosomal gene transfer between species. The existence of "foreign" genomic DNA is particularly intriguing as sequence divergence is a major limitation to such transfers because it severely limits the potential for homologous recombination (74).

Although the identity of the vectors that transport pathogenicity islands from donor to recipient organisms is unknown, any number of mobile genetic elements are candidates. Clear evidence showing an extrachromosomal stage of a pathogenicity island is lacking; however, it is intriguing that the G+C contents of the *Helicobacter* pathogenicity island and plasmid are similar to each other and distinct from the chromosome (Censini, S et al. A pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated factors. 1996; submitted for publication). Phages, plasmids, transposons, integrons, and even free DNA carry genes from one organism to another (74). Indeed, many phages and plasmids contain virulence genes, and often these loci seem alien to the bacterial species in which they reside (75). Furthermore, the animal host environment may be particularly conducive to DNA transfer events. For example, the phage that encodes cholera toxin infects *V. cholerae* more efficiently within the gastrointestinal tract of a mammalian host than under laboratory conditions (76).

Pathogenicity islands insert into the chromosome by an unknown mechanism; however, the existence of insertion elements and repeated DNA motifs at the boundaries of several pathogenicity islands suggest that recombination events are involved. Recombination has recently been shown to be the major factor governing the divergence of a group of *E. coli* strains and is a significant driving force for evolution (74,77). Although the genetic material comprising pathogenicity islands may be introduced into a new host organism in a single step, the events that generate known pathogenicity islands are unlikely to be simple insertions, because DNA rearrangements and alterations are common in the flanking chromosomal regions.

The identification of several tRNA genes as insertion sites for pathogenicity islands is also notable, although the significance of this remains obscure (Table 2). tRNA genes serve as integration sites for a variety of prokaryotic genetic elements, including several phages and transmissible plasmids (78-80). Perhaps the conserved portion of tRNA genes is a useful landmark for mobile genetic elements that inhabit a variety of prokaryotic hosts; in addition, the regions of dyad symmetry characteristic of all tRNA genes could serve as binding sites for enzymes involved in recombination.

Relative Advantages of Instability and Stability

Some pathogenicity islands can excise from the chromosome and are apparently lost from the host bacterium (Table 2) (3,4,70). Such instability may provide an adaptive advantage. Virulence properties may be dispensable at certain stages of infection, and the coordinated loss of these characteristics could be beneficial to the bacterium. Indeed, expression of particular genes at inappropriate times can be detrimental to bacterial pathogens (81). Natural selection of strains with deleted

virulence regions can occur in specific environments: diabetic patients are more susceptible to uropathogenic *E. coli* strains not exhibiting virulence phenotypes (4).

On the other hand, particular virulence traits could provide a continual adaptive advantage, resulting in stable pathogenicity islands. The "foreign nature" of pathogenicity islands may reflect this benefit; foreign DNA may be actively maintained in the population because of its limited ability to recombine with related organisms (75). It is not clear whether stable islands exist because of a divergence of sequences at the borders (for example, repeated elements that are no longer recognizable as such), an integration mechanism completely different from that of the unstable islands, or because of a lack of excision machinery.

Deletion of pathogenicity islands can affect gene expression by altering the chromosomal site of insertion and by removing the genes contained in the island. The locus of enterocyte effacement (LEE) comprising the type III secretion apparatus in *EPEC* and pathogenicity island I (Pai I) of uropathogenic *E. coli* both insert at the selenocysteine tRNA (*selC*) gene (82,83). The presence of Pai I does not interfere with *selC* expression. However, excision from the chromosome appears to occur by a recombination event between the repeated sequences in *selC* and the distal end of Pai I. This recombination event results in deletion of part of the tRNA gene and inhibits anaerobic growth due to the cell's inability to produce formate dehydrogenase, which contains selenocysteine (84). Similarly, in Pai II deletion strains, the *leuX* tRNA gene at the insertion site is disrupted, which interferes with its ability to act as a global regulator of several virulence factors that lie outside the pathogenicity island (84).

Bacteria may be able to have the best of both the stable and the unstable worlds. The phenotypic loss of the enteroinvasive *E. coli* and *Shigella flexneri* virulence plasmids is sometimes due to plasmid insertion into a specific site on the chromosome (85,86). After integration, excision also can be detected; strains containing precisely excised plasmids regain virulence, while those with imprecisely excised plasmids remain noninvasive. Several of the *Y. pestis* plasmids exhibit similar behavior (87-89). Integration, which simultaneously maintains these plasmids in the bacterial genome while downregulating their genes, may represent a sophisticated adaptation to the requirements of different environments or may represent? stages in the bacterial life cycle. Furthermore, integration and excision remind us that strict definitions of "chromosomal" versus "plasmid-borne" do not always reflect biological reality.

Foreign DNA Is a Significant Determinant in Recently Emerged Pathogens

Horizontal gene transfer has been invoked to explain the origin of enterohemorrhagic *E. coli* (*EHEC*), which causes hemorrhagic colitis and hemolytic uremic syndrome (90). Like *EPEC*, *EHEC* induces striking morphologic changes–called attaching and effacing (AE) lesions–in host cells of the small intestine; however, unlike *EPEC*, *EHEC* contains Shiga-like toxins. After analyzing the genetic relationships between many *E. coli* strains, Whittam and colleagues proposed that *EHEC* arose from an *EPEC*-like progenitor strain, which then acquired the prophage-encoded Shiga-like toxins, thus becoming a new pathogen that expresses both sets of traits (90).

The new epidemic *Vibrio cholerae* O139 strain may have emerged after acquisition of a pathogenicity island (91). Although it appears that *V. cholerae* O139 arose from a strain of the same serotype (O1) that is causing the ongoing cholera pandemic (O1 El Tor) (92), *V. cholerae* O139 contains an additional piece of DNA that replaces part of the O antigen gene cluster of O1 strains (93). The inserted DNA contains open reading frames homologous to proteins involved in capsule and O antigen synthesis, two factors that distinguish O139 and O1 El Tor, and are thought to mediate activities important for pathogenesis and evasion of immunity.

Benefiting From Information About Type III Secretion and Pathogenicity Islands

Pathogenic bacteria continue to exhibit impressive genetic flexibility and exchange and use these abilities to adapt to varied

types of lifestyles within host organisms. It should be possible to use the information from studies of pathogenicity islands and type III secretion systems in the ongoing characterization of bacterial infections. When a novel pathogen is isolated, it may be worthwhile to identify chromosomal regions specific to it by comparing the gross genomic structure with that of related organisms, which may provide a shortcut to the identification of virulence genes. Likewise, simple molecular techniques can determine whether bacteria contain type III secretion systems, because genes encoding particular components are highly conserved; perhaps this procedure should be part of our standard investigative arsenal as well.

Our knowledge of type III secretion systems may yield therapeutic benefits. The contact-dependent systems appear to reside in pathogenic and not in commensal bacteria. If this observation reflects a general truth, antibiotics that target type III systems may specifically attack intruding bacteria and spare the normal flora; therefore, these antibiotics might produce minimal side effects. In addition, type III secretion systems will provide new targets for therapeutic drugs that might not kill the bacterium but would inhibit the disease process. We also may be able to exploit this secretion system, by using appropriately attenuated bacteria, to prime immunity. Chimeric proteins—fusions between effector and other proteins—can be secreted in large quantities by the type III secretion machinery and be internalized by host cells; furthermore, these proteins can elicit an antibody response in mice (38,94).

The virulence traits of pathogenic microorganisms at the genetic and molecular level remind us that bacterial pathogenicity does not arise by slow adaptive evolution but by "quantum leaps" (95); therefore, microbes can acquire complete systems that radically expand their capabilities to exploit and flourish in different host environments. History teaches us that infectious diseases may change in severity and form, but they will not simply disappear. Microrganisms are, after all, survivors, and there is no escaping our destiny—to be consumed by them in one way or another. However, the more we learn about the microbial tactics of survival, the longer we forestall this destiny.

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New Vaccines for the Prevention of Pneumococcal Infections

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Streptococcus pneumoniae is a major cause of acute otitis media, pneumonia, bacteremia, and meningitis. Because in recent years antibiotic-resistant pneumococcal strains have been emerging throughout the world, vaccination against pneumococcal infections has become more urgent. The capsular polysaccharide vaccine that has been available is neither immunogenic nor protective in young children and other immunocompromised patients. Several pneumococcal proteins have been proposed as candidate vaccines, but no human studies associated with them have been reported. Clinical trials of first-generation pneumococcal conjugate vaccines have shown that covalent coupling of pneumococcal capsular polysaccharides to protein carriers improves the immunogenicity of the polysaccharides. The protective efficacy of the conjugate vaccines against carriage, acute otitis media, and invasive infections is being studied.

Streptococcus pneumoniae (pneumococcus [Pnc]) is a common bacterial agent found in mild mucosal as well as severe systemic infections. Local infections, such as acute otitis media, are rather common; every child has at least one during the first 2 years of life (1), and Pnc is the causative agent in approximately half of the bacterial culture-positive cases (2). Pneumonia is another disease often caused by Pnc, both in industrialized and developing countries. Pneumonia, which causes more than one million deaths per year, is the most common cause of childhood death in the developing world (3); pneumococcal pneumonia is a serious problem among the elderly in industrialized countries. Pnc also causes frequent invasive infections, especially among children. In Finland, the incidence of bacteremic pneumococcal infections at 0 to 4 years of age has been 24.2 per 100,000 per year (4). The corresponding rate was 42 per 100,000 per year in Israel (5) and 66 per 100,000 per year in the United States (6). In addition to the young and the elderly, some of the other groups at increased risk for Pnc infection are patients with chronic cardiac or pulmonary diseases, immunocompromised patients, and especially persons with functional or anatomic asplenia (7).

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The treatment of recently emerged Pnc strains that are resistant to penicillin and other antibiotics (8) is becoming a challenge. Because of the high rates of illness and death associated with pneumococcal infections and the rapidly increasing resistance of organisms that cause these infections to antimicrobial drugs, development and use of effective pneumococcal vaccines is of high priority. The progress has been rapid; in addition to polysaccharide(PS)-protein conjugate vaccines, vaccines containing pneumococcal proteins are also being developed.

Pneumococcal Capsular Polysaccharide Vaccine

Pnc can be divided into at least 90 serotypes according to the structure of the PS in the capsule surrounding the bacterium. The capsule seems to be the most important virulence factor; all strains isolated from infections are encapsulated. The capsule helps the bacterium escape the host defense mechanisms. However, only a small fraction of all capsular types are common causes of pneumococcal infections. The list of the most common groups/types (4, 6, 7, 9, 14, 18, 19, and 23) that cause

childhood infections is similar in most parts of the world. Types 1 and 5 are, however, more common in the developing world than in industrialized countries (9).

Antibodies to capsular PSs protect from infection by opsonizing Pnc for phagocytosis by neutrophils. A capsular PS vaccine containing 23 of the most common serotypes/ groups has proven protective in immunocompetent adults and in some groups at risk (7,10,11); it has also been shown to have an impact on death rates due to pneumonia in Papua New Guinea (12). Among the immunocompromised and in preventing acute otitis media, (13) its efficacy has been only marginal.

The reason for the vaccine's poor immunogenicity and its lack of efficacy in children is thought to be the nature of the PS antigen. PS antigens are type 2 T-cell independent (TI) antigens, which stimulate mature B cells without the help of T cells. In humans, the B cells of newborns do not respond to most of the PS antigens. Responsiveness develops only slowly during the first years of life. Furthermore, the TI antigens do not induce immunologic memory and the maturation of the immune response; anti-PS antibodies have low avidity and the switch from one isotype to another does not happen even after repeated immunizations. The TI antigens induce mainly IgM responses, especially in mice. However, in humans the response also contains the IgG and IgA components (14). Furthermore, the IgG response to PS antigens contains a greater proportion of IgG2 (15,16) than found in a response to protein antigens. The lack of memory has some important implications for the vaccination. Because of the rapid decline of antibodies, revaccination is often necessary (7).

Pneumococcal Protein Vaccine Candidates

Several ways have been and are being tried to solve the problem of poor immunogenicity of pneumococcal PS vaccines in infancy. In addition to the capsule, other pneumococcal virulence factors have been considered as promising vaccine candidates or as carrier proteins in pneumococcal conjugate vaccines (see above). The prime vaccine candidates are enzymes and toxins that are excreted or released after the bacterium has autolyzed or surface proteins whose exact functions are not known. Pneumococcal proteins studied as potential vaccines include neuramididase, autolysin, pneumolysin, pneumococcal surface protein A (PspA), and pneumococcal surface adhesin A (PsaA) (17-19).

Pneumolysin is a cytolytic toxin produced by all types of Pnc. In mice, immunization with inactivated pneumolysin or recombinant pneumolysin toxoid offers at least partial protection or enhanced survival when challenged with Pnc (20,21). PspA is a surface protein present in all clinically relevant pneumococcal strains. PspAs from different pneumococcal strains vary serologically. However, many PspA antibodies cross-react with PspAs from unrelated strains. Furthermore, active immunization of mice with PspA generates protective immune response against diverse pneumococcal strains (22). Truncated PspAs, expressed as recombinant proteins, are also immunogenic in mice and can elicit cross-protection (18).

Pneumococcal Conjugate Vaccines

Another approach to solving the poor immunogenicity of the capsular PS antigens has already moved to the clinical phase-III trials. This approach is based on the 1929 findings of Goebel and Avery (23), who showed that covalent coupling of haptens to a protein carrier improves the immunogenicity of the hapten. In this way, the anti-PS response gets T-cell dependent characters: there is development of immunologic memory and maturation of the immune response. This is seen as an increase in the antibody concentrations and the antibody affinity and as a switch in the isotype distribution after repeated immunizations. This approach has been used successfully to prepare vaccines against *Haemophilus influenzae* type b (Hib); the incidence of Hib infection has decreased drastically wherever these conjugate vaccines have been used (24).

The PS antigen in a conjugate vaccine seems to benefit at least partly from the immunologic characters of the carrier protein. The protein is presented as peptides in association with the major histocompatibility complex class II molecules on the

surface of the antigen-presenting cells. This stimulates the T-helper cells, which then stimulate adjacent B cells for antibody production and maturation into memory cells. Development of immunologic memory means that the protection does not depend solely on the existing antibody concentration. Instead, the vaccinated persons can respond with a rapid, high, and effective antibody response to colonization or invasion by the respective Pnc type. Studies in Finland suggest that this indeed happens: the efficacy of an Hib conjugate vaccine, PRP-D, was more than 90% in early infancy, even though a large proportion of the infants did not have measurable antibody response after the primary course of immunization (25). A study in the United Kingdom suggests that the carriage of Hib indeed induces a high "booster type" immune response (26).

Conjugation of the PS to a protein carrier has repeatedly been shown to work with Hib; vaccines based on the same principle would also decrease the number of different infections caused by Pnc. Four vaccine manufacturers have prepared pneumococcal conjugate vaccines with basically the same approaches as the Hib conjugates (Table 1). PncOMPC vaccine contains PSs from seven serotypes conjugated to the meningococcal outer membrane protein complex (27). The PncCRM vaccine contains either oligosaccharides (OS) or PSs coupled to a nontoxic mutant diphtheria toxin CRM197. The PS-containing conjugate vaccine is at present heptavalent (28), but it is possible to add types 1 and 5 to the product intended for use in developing countries. The PncT vaccine contains eight PSs coupled to tetanus toxoid, and the PncD product contains the same PSs coupled to diphtheria toxoid (29). Besides these formulations, several other approaches have been tested in animals. These include conjugates using pneumolysoid (30), pertussis toxin (31), and salmonella protein (32) as a carrier. Recently, small peptides selected on the basis of T-cell stimulating properties have also been coupled to pneumococcal PS to form conjugate vaccines (33).

Preclinical Testing

Before human trials, these conjugates were immunogenic and protective in animals, including mice, infant monkeys, and chinchillas (34-37). All these studies indicate that conjugate vaccines have greater immunogenicity than pneumococcal PS vaccines. Even though animal studies can tell if the conjugate vaccine is immunogenic and evokes a T-cell dependent response, the final proof of conjugate vaccines' superior immunogenicity and efficacy over PS vaccines comes only from human studies. So far no animal model can mimic human immunogenicity and efficacy studies.

Table 1. Pneumococcal conjugate vaccines in phase-II and phase-III trials

Vaccine	Serotype	Carrier	Manufacturer
PncCRM	4, 6B, 9V, 14, 18C, 19F, 23F	CRM197	Wyeth-Lederle Vaccines and Pediatrics
PncD	3, 4, 6B, 9V, 14, 18C, 19F, 23	Diphtheria toxoid	Connaught Laboratories
PncT	3, 4, 6B, 9V, 14, 18C, 19F, 23	Tetanus toxoid	Pasteur Merieux Serums & Vaccins
PncOMPC	4, 6B, 9V, 14, 18C, 19F, 23F	Meningococcal OMPC	Merck Research Laboratories

CRM = CRM197, a nontoxic variant of diphtheria toxin; D = diphtheria toxoid; T = tetanus toxoid; OMPC = outer membrane protein complex

Clinical Testing

Pneumococcal conjugates of all the manufacturers mentioned in Table 1 have now been tested in phase-I and phase-II studies. The first human studies were done in adults with mono- or bivalent conjugates and showed that the conjugates were at least as immunogenic as the PS vaccine. Since then, up to eight valent vaccines have been used in human studies, also among infants.

Adults and Toddlers

To show that they are safe and immunogenic, pneumococcal conjugates were first given to small numbers of adults and toddlers. Most of the reported studies have been conducted with mono- to tetravalent vaccines. The PncOMPC studies in adults show that the conjugate vaccine was well tolerated but not more immunogenic than the PS vaccine (38,39). One possible reason might be the low dose (1µg to 5µg of each

conjugate) used in these studies. Different formulations of PncCRM containing either PS or OS linked to CRM197 have been tested in adults. All were well tolerated and evoked a comparable immune response (40). This was confirmed in a study in which heptavalent OS conjugate was immunogenic (28). Results of immunizing adults with PncT or PncD conjugates have been reported in two studies; both vaccines were more immunogenic than the PS vaccine (41,42). The Finnish study with tetravalent PncT and PncD showed that these conjugates can also evoke a mucosal antibody response (42).

PncOMPC vaccine was given to 31 Finnish children at 24 months, and 10 of them also received it at 26 months. The primary response was only slightly higher than to the PS vaccine, but after the second dose a booster type response was seen in most of the vaccinees (43). Studies conducted during the second year of life showed that the heptavalent PncOMPC conjugate was more immunogenic than the PS vaccine (44,45). Different formulations of PncCRM have also been tested in toddlers (46). Conjugates were more immunogenic than the PS vaccine; furthermore, the PS conjugate was more immunogenic than the OS conjugate. One study showed a good booster response to PS vaccine after primary immunization with pentavalent PS-based PncCRM (47). The PncT and PncD conjugates have also proven immunogenic in toddlers. A Finnish study compared 3-µg and 10-µg doses at 24 months, and a U.S. study used 10-µg doses of type 19F conjugates with PS vaccine booster doses (48).

Infants

Keyserling et al. (49) have compared different dosages of type 14 PS containing monovalent PncOMPC vaccine in infants and shown that 2.5µg to 5µg of type 14 PS in the conjugate gave better responses than the lower doses. A Finnish study (50) showed that a primary series of three doses of tetravalent PncOMPC at 2, 4, and 6 months was better than two doses at 4 and 6 months. Furthermore, a booster dose of PncOMPC given at 14 months evoked a secondary response to all PS types. Concomitant administration of PncOMPC with routine infant immunizations does not seem to have an effect on either anti-Hib or anti-Pnc PS antibody responses (51). The heptavalent PncOMPC formulation is as immunogenic as the previous formulations with fewer serotypes (27).

Åhman et al. have shown that the pentavalent PncCRM vaccine containing OS derived from pneumococcal capsule was immunogenic and tolerable in infants (52). The same children developed a good antibody response when boostered with PS vaccine at 24 months, suggesting that the immunologic priming had been good even if the antibody response to the primary series had remained rather low (53). The PS-based PncCRM has been shown to be more immunogenic than OS conjugates also in infancy (54). A Gambian study evaluated the pentavalent PncCRM conjugate (PS-based) in a developing country when given at 2, 3, and 4 or at 2 and 4 months. The vaccine was immunogenic and well tolerated; the schedule of three doses was better than the two-dose schedule (55).

A Finnish study compared three dosages of 1µg to 10µg of each PS in tetravalent PncT and PncD conjugates when administered at 2, 4, and 6 months. These vaccines were immunogenic in infancy, and no difference could be shown between PncT and PncD. The response after a primary series to PncD, but not to PncT, was dose dependent (56). The children immunized with PncD in infancy had a booster response after reimmunization with either PncD or pneumococcal PS vaccine at 14 months (57). All who received PncT were boostered with PS vaccine, and the response was dose dependent; children that had received 10-µg doses of PncT during the primary immunization had the lowest mean booster responses (58). Another Finnish study showed that octavalent (types 3, 4, 6B, 9V, 14, 18C, 19F, and 23F) PncD (3µg of each PS) and PncT (1µg of each PS) induced immune responses similar to the respective tetravalent formulations (29). An Icelandic study showed that the octavalent vaccine was immunogenic in infants when given at 3, 4, and 6 months and that the IgG anti-PS concentrations correlated with the opsonic activity (59).

Because no study has directly compared different pneumococcal conjugate vaccines,

Table 2. Antibody response of Finnish infants to pneumococcal conjugate vaccines administered at 2, 4, and 6 months of age*.

	Geometric Mean of the Anti-PNC PS (µg/ml)								
	Type 6B		Type 14		Type 19F		Type 23F		
Vaccine	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Ref.
PncOMPC	0.17	1.30	0.42	8.27	0.34	9.85	0.28	1.90	(50)
PncCRM	0.25	0.50	0.30	2.49	0.46	1.13	0.18	0.83	(52)
PncT01-4	0.25	0.89	0.24	2.84	0.36	3.73	0.18	0.82	(56)
PncT01-8	0.20	1.28	0.30	2.56	0.56	4.23	0.22	1.03	(29)
PncD03-4	0.26	0.88	0.44	2.20	0.43	5.29	0.21	0.67	(56)
PncD03-8	0.17	1.44	0.31	4.62	0.37	4.94	0.24	1.07	(29)

PncOMP = tetravalent conjugate vaccine with a meningococcal outer membrane protein complex as a carrier
PncCRM = pentavalent oligosaccharide conjugate vaccine with CRM197 protein as a carrier
PncT01-4 = tetravalent conjugate vaccine with tetanus toxoid carrier; 1 µg of each of four polysaccharides
PncT01-8 = tetravalent conjugate vaccine with tetanus toxoid carrier; 1 µg of each of four polysaccharides
PncD03-4 = tetravalent conjugate vaccine with diphtheria toxoid carrier; 3µg of each of four polysaccharides
PncD03-8 = octavalent conjugate vaccine with diphtheria toxoid carrier; 3µg of each of four polysaccharides
*Serum samples are taken before immunization (pre) and at 7 months (post). The data have been gathered from separate studies done in the same population.

the comparison has to rely on data from separate studies. A Finnish group has analyzed the antibody response in adults, toddlers, and infants to all four types of Pnc conjugates. This comparison shows that there are vaccine- and type-specific differences in the antibody responses (Table 2). However, none of the vaccines used in these studies have the composition suggested in the phase-III trials (29,50,52,56).

Comparing the data from different studies is difficult because there can be interlaboratory variation in the enzyme-linked immunosorbent assay results. The Centers for Disease Control and Prevention, Food and Drug Administration, and World Health Organization are working on a standardized anti-Pnc PS assay, which will, if not eliminate, at least reduce the impact of this problem. A standard serum (60) to be used in all laboratories is distributed by Center for Biologics Evaluation and Research/Food and Drug Administration.

Pneumococcal Conjugates and the Carriage of Pnc

Experience with the Hib conjugates (24) suggests that Pnc conjugate vaccines could also reduce the number of carriers of the vaccine types and in this way decrease the spread of bacteria. The results from the only reported study are encouraging. The PncOMPC vaccine decreased the carriage rate among toddlers, while the pneumococcal PS vaccine did not (45). Importantly, the carriage of antibiotic-resistant Pnc also decreased (61).

Efficacy Studies

Phase-III studies with the heptavalent formulations of PncOMPC and PncCRM are ongoing or being started. These studies look at prevention of carriage, acute otitis media, or invasive Pnc infection caused by Pnc of the vaccine serotypes. Furthermore, there are several plans for studying the effect of Pnc conjugates on Pnc invasive infection and pneumonia in developing countries.

Questions to Be Answered in the Future

We do not know if conjugate vaccines can really prevent Pnc infections better than the PS vaccine. We hope that the new vaccines can prevent several types of infections, from symptomless Pnc carriage to serious invasive infections with high death rates. It is quite probable that the protective immune response needed is different for each type of infection. We do not know if parenterally administered vaccine can prevent carriage or mucosal infections such as acute otitis media. It is still unknown whether a mucosal immune response is needed or whether transudation of antibodies from the serum is enough for protection against local infection. Saliva samples of infants immunized with Hib conjugate vaccines contain secretory IgA but also IgG, which has most probably

transudated from serum (62). In an infant rat Hib colonization model, both secretory IgA and serum derived IgG decreased colonization (63). Furthermore, animal experiments suggest that immune response evoked by parenteral administration of a conjugate vaccine would alone protect against acute otitis media (64). In addition, passive immunization of infants with hyperimmune serum pool containing antibodies to pneumococcal PS-induced protection against pneumococcal acute otitis media suggests that protection is offered when high enough serum antibody concentrations are gained (65). At present, there are no data to show which antibody concentrations are needed for protection. Deciding about the protective concentration might be difficult because the development of immunologic memory is an important factor; the protection does not solely depend on the existing antibody concentration.

Most phase-II studies have used a schedule of two or three doses of Pnc conjugate vaccine in infancy (usually at 2, 4, and 6 months) and a booster dose of either conjugate or Pnc PS at the second year of life. The need for a booster dose at the second year is not known; this information would be important especially for planning the vaccination schedules for developing countries, where administering a booster dose can be problematic. The experience from the Hib conjugates suggests that a booster dose might not be needed; the United Kingdom has successfully used a schedule of three doses at 3, 4, and 5 months without a booster dose (66).

Reduction of pneumococcal infections among the elderly would probably increase the quality of their lives. The immunogenicity of pneumococcal PS vaccine in this age group has been satisfactory (67,68). An Hib conjugate (PRP-D) has proven more immunogenic than the Hib PS vaccine in the elderly (69). However, the immune response to PncCRM was not better than to the Pnc PS vaccine, and no booster response was seen (70). Studies with other pneumococcal conjugates in the elderly have not been reported.

Because pneumococcal infections of very young infants are a problem in developing countries, several groups have studied the possibility of maternal immunization with pneumococcal vaccines (71,72). So far only PS vaccines have been used; even though these vaccines are immunogenic in pregnant mothers, the immunity transferred to the neonate is not very long lasting. If the conjugate vaccines induce higher antibody concentrations in mothers, the concentration of passively acquired antibody in the baby would stay high for a longer time. The Hib conjugate vaccines induce good responses in mothers and, consequently, long-lasting protective concentrations in infants born to these mothers (73,74). The effect of simultaneous maternal tetanus immunization, especially if conjugates with a tetanus toxoid carrier are used, and the effect of high maternal antibody level on the antibody responses of the infants have to be determined.

By the year 2000, we may have pneumococcal conjugate vaccines to include in routine childhood immunization programs. The price of the conjugate vaccines has been so high that their use throughout the world has not been possible. An important challenge in developing of pneumococcal conjugate vaccines is to reduce the costs of manufacturing so that all children can benefit from them.

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A Mathematical Model and CD4$^+$ Lymphocyte Dynamics in HIV Infection

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The paper presents a model of CD4$^+$ lymphocyte dynamics in HIV-infected persons. The model incorporates a feedback mechanism regulating the production of T lymphocytes and simulates the dynamics of CD8$^+$ lymphocytes, whose production is assumed to be closely linked to that of CD4$^+$ cells. Because CD4$^+$ lymphocyte counts are a good prognostic indicator of HIV infection, the model was used to simulate such therapeutic interventions as chemotherapy and active and passive immunization. The model also simulated the therapeutic administration of anti-CD8 antibodies; this intervention was assumed to activate T-cell production by activating a feedback mechanism blocked by the high numbers of CD8$^+$ lymphocytes present in HIV-infected persons. The character and implications of the model are discussed in the context of other mathematical models used in HIV infection.

The increased efficiency of modern computer techniques has expanded the possibilities of mathematical modeling in an unprecedented way. However, medical and biologic research has not taken full advantage of these possibilities. Mathematical models are, in fact, working hypotheses that require clear formulation and quantitative definition of factors and relations included in the model. These requirements may discourage biologic and medical research scientists from using mathematical models because quantitative data are often not available to them, or are available only to a limited extent. However, current computer techniques offer the possibility of quickly testing different estimates of a realistic, probable choice. The increased efforts required to construct mathematical models are amply rewarded by the quantitative predictions generated by the models.

In the absence of adequate animal models, mathematical modeling of HIV infection is especially important. Many adequate models of this infection have been formulated (e.g., 1-3), but they have had a relatively small impact on clinical and experimental research. Mathematical modeling, however, was an integral and important part of evaluating recently obtained data on HIV turnover in infected persons (4,5).

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The Mathematical Model

We had simulated the dynamics of lymphocytes in immunologic tolerance (6) for a decade when we became interested in modeling lymphocyte dynamics in HIV-infected persons (7,8). The tolerance model simulates escape from tolerance of a nonreplicating protein antigen and is based on the assumption that lymphocytes specific to the tolerated antigen start to appear when the concentration of the tolerated antigen drops below the threshold level required for tolerance induction in differentiating new lymphocytes. Our model of HIV infection concentrated on CD4$^+$ lymphocytes because the depletion of this T-cell subpopulation, and the parallel decrease in the helper activity of T lymphocytes, seemed to be the major immune system defect caused by HIV infection. When we started to construct the model, the widely held view was that the decrease of this T-cell subpopulation is not caused by the cytopathic effect of the virus

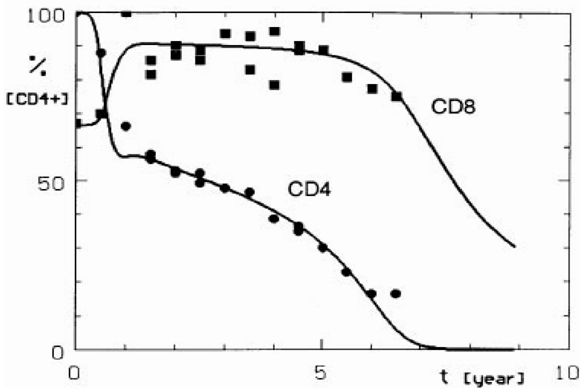

Figure 1.* Simulated CD4⁺ and CD8⁺ lymphocyte dynamics in HIV infection compared with observed mean T-cell values for CD4⁺ lymphocytes (circles) and CD8⁺ lymphocytes (squares).

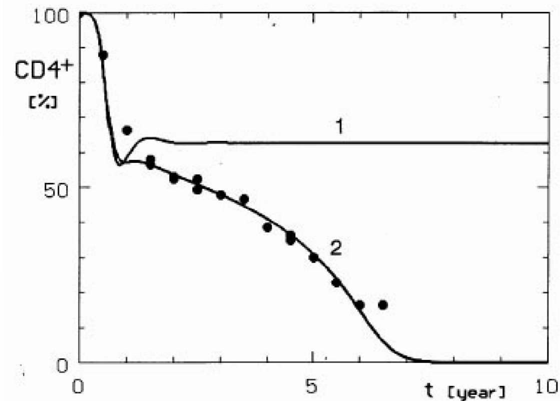

Figure 2.* Comparison of only linear (curve 1, ν =1.0) T-helper activity decrease with nonlinear (standard curve 2, ν = 1.6).

*CD4⁺ cell observed values are depicted as circles and those of CD8⁺ lymphocytes as squares. Both simulated and observed values are depicted as a percentage of normal CD4⁺ lymphocyte numbers (the normal value of CD8⁺ lymphocytes is thus 66.7%).

because in infected persons too few CD4⁺ cells expressed HIV. A direct or indirect effect of HIV products on these cells was, therefore, considered to cause this depletion (9).

In both immunologic tolerance and HIV infection, antigen seemed to eliminate lymphocytes: in immunologic tolerance, lymphocytes carrying the specific antigen receptor were affected, and in HIV infection, the entire CD4⁺ lymphocyte population was the target of HIV products. The dynamics of the affected lymphocyte pools were mutually inverse: in tolerance, the number of specific lymphocytes increased with time because the antigen concentration decreased and in HIV infection, the CD4⁺ lymphocyte count decreased because of the rising level of HIV products as the infection progressed.

In the model, CD4⁺ cell depletion was assumed to result, directly or indirectly, from an effect of HIV products, where HIV proliferated at the same rate during the whole course of the infection; therefore, once a simulated substantial decline of CD4⁺ lymphocytes started, it progressed rapidly to their total depletion. However, previous studies indicated that an early decline in these cells occurs shortly after infection, is followed by a period of slow decline, and then the decline accelerates again about the time AIDS develops (10,11). However, if we assume that HIV proliferation is limited by a helper T-cell-dependent immune reaction, our model could simulate all the observed phases of CD4⁺ cell dynamics well (8,12). In Figure 1, simulation curves of CD4⁺ and CD8⁺ lymphocytes (Appendix) are compared with the mean observed values of these cells in HIV-infected persons (10,12).

The immune reaction limiting HIV proliferation, postulated in the model, was assumed to be a specific cytotoxic activity of T cells that required the cooperation of helper T cells. However, the model is not dependent on this assumption, and any T helper-cell-dependent immune reaction can play this role. Other mechanisms are possible candidates for this function. Recently, interest was focused on cytokines produced by CD8⁺ lymphocytes that inhibit HIV proliferation (13,14), an effect discovered much earlier (15,16). As far as this activity depends on T-helper cells, this situation can be simulated by our model. If T-helper cells did not play any substantial role in this mechanism, our model would not be applicable.

In our model, CD4⁺ lymphocyte dynamics were successfully simulated only if T-helper-cell activity did not decrease linearly with the decline of CD4⁺ lymphocytes but faster than these T cells. This relation is expressed by the power coefficient ν in Equation 6

(Appendix), which must have a value >1.0 (7,8). If the decrease of helper activity was assumed to be directly proportional to the number of $CD4^+$ lymphocytes ($\nu = 1.0$), it was possible to simulate only the initial phase of the $CD4^+$ dynamics—the early drop of these cells. Then a permanent steady state of the $CD4^+$ cell level was established (Figure 2, curve 1). This finding could be of interest for elucidating mechanisms involved in the long-term, and possibly permanent, survival of some HIV-infected persons (17-19) whose condition seems to be characterized by an equilibrium between HIV infection and a protective immune reaction. Although some of these persons have $CD4^+$ lymphocyte numbers in the normal range, their cases do not necessarily contradict the model's prediction of a stabilized HIV infection because with a small HIV load the steady-state $CD4^+$ cell numbers might be indistinguishable from normal ones, given the broad range of normal values.

We are not able to ascribe a definite mechanism to the necessary assumption that the activity of T-helper cells declines faster than the number of $CD4^+$ lymphocytes. The faster decline could be caused by the disruption of the lymphoid tissue structure by HIV infection (20). Another possible cause is the increasing HIV variation as infection progresses (21), if this variation led to decreased sensitivity, or even resistance, to the protective immune reaction of at least a part of the virus population.

From our model of immunologic tolerance (6), two compartments of the studied lymphocytes were retained in the model of HIV infection (7): mature and immature $CD4^+$ cells. We incorporated immature lymphocytes in the model of immunologic tolerance because they were more sensitive to tolerance induction. The assumption of immature $CD4^+$ lymphocyte sensitivity to elimination by HIV did not influence the simulation results substantially (22); therefore, only mature $CD4^+$ cells were considered to be eliminated by the effect of HIV in most simulations we carried out, including those described in this article.

Different mechanisms of $CD4^+$ lymphocyte depletion caused by HIV infection were simulated by various modifications of our model, which assumed either a direct or indirect effect of HIV (22). Because no substantial difference was observed in simulation results, for convenience reasons, most of the work, including the examples presented in this article, assumed that cytotoxic cells limiting HIV proliferation are also instrumental in depleting $CD4^+$ lymphocytes by eliminating those have HIV products. This assumption seems to be supported by recent clinical findings (4,5).

Adleman suggested that the depletion of $CD4^+$ lymphocytes might activate some homeostatic mechanism that increases their production (24). He assumed that this homeostatic mechanism increased production of both $CD4^+$ and $CD8^+$ lymphocytes and did not discriminate between the two T-cell subpopulations.

Adleman also suggested that the substantial and permanent depletion of $CD4^+$ lymphocytes in HIV-infected persons might activate this mechanism. However, because only $CD4^+$ cells are destroyed in HIV infection, the newly produced $CD8^+$ lymphocytes would accumulate. An increase in the number of $CD8^+$ lymphocytes was actually observed in HIV-infected persons, while the total number of T cells remained in the normal range. The increase in the $CD8^+$ cell count might switch off the homeostatic mechanism that increases T-cell production, and as a consequence, cause or at least aggravate the $CD4^+$ lymphocyte depletion (24,25). Because this view was supported by convincing evidence, we incorporated this feedback mechanism in our model (22). When we compared quantitatively the simulated $CD8^+$ cell increase with the observed values, they did not agree well, especially in later phases of the infection when the simulated values continued to increase, while the observed $CD8^+$ lymphocyte counts started to decline. When it was assumed that HIV infection constrained the influx of both $CD4^+$ and $CD8^+$ lymphocytes, satisfactory simulation results were obtained (26) (Figure 1).

Modeling Therapeutic Interventions

Because $CD4^+$ lymphocyte counts are a good prognostic indicator of HIV infection (27), our model is suitable for simulating different therapeutic interventions. The

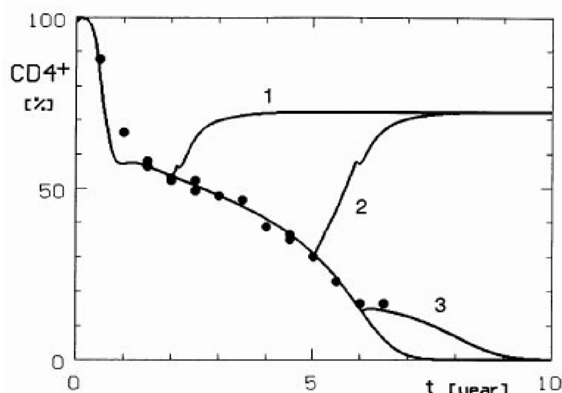

Figure 3.* Simulated effect of permanent AZT treatment (ξ = 0.005) started 2m 5m or 6 years after the acquisition of HIV infection—curves 1, 2, and 3, respectively.

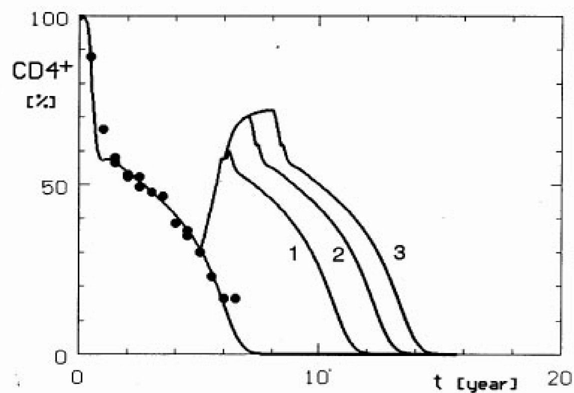

Figure 4.* Simulated effect of temporary AZT treatment (ξ = 0.005) started 5 years after the acquisition of HIV infection and lasted 1, 2, or 3 years—curves 1, 2, and 3, respectively.

*CD4+ cell observed values are depicted as circles and those of CD8+ lymphocytes as squares. Both simulated and observed values are depicted as a percentage of normal CD4+ lymphocyte numbers (the normal value of CD8+ lymphocytes is thus 66.7%).

model has been used to simulate zidovudine (AZT) chemotherapy and specific immunotherapy, both active and passive (28-30). The intensity of most therapies we simulated did not completely eradicate the infection, a situation common to the treatments now available. If a therapy that is assumed not to eradicate HIV infection is simulated to be administered permanently and to retain undiminished effectiveness, the observed overall result (besides slight differences in the dynamics of the changes induced by the various therapeutic measures) is the establishment of a new steady-state level of CD4+ lymphocytes (Figure 3). The height of this level reflects the effectiveness of the therapy: the nearer to normal values, the more effective the interventions. This steady state is always lower than a normal state. Even when the therapy is started at a later stage of HIV infection, the obtained steady state is the same; its value depends only on the effectiveness of the therapy, regardless of the onset of treatment (Figure 3, curves 1 and 2).

However, this result is valid up to a certain point only: when the CD4+ lymphocyte numbers are too low, therapy can no longer reverse the CD4+ cell depletion; it can only slow the decrease and does not establish a steady state (Figure 3, curve 3). The stage of infection in which depletion cannot be reversed varies with different therapies and with different intensities of the same therapy (e.g., AZT doses). It occurs earlier in the course of the infection with less effective treatments than with more effective ones. Actually, there is a point in the intensity of each treatment when it is possible to stop further CD4+ cell decline and establish a steady state with CD4+ cell numbers corresponding to their value at the onset of therapy.

Another aspect of the therapies we simulated was their temporary application. If the therapy eliminated the virus completely, CD4+ lymphocyte counts returned to normal for good. Our simulations concentrated mainly on therapies leading only to limitation of the viral load, and in consequence, to an increase in the numbers of CD4+ cells. When the treatment was stopped, the decline of CD4+ cells started again and essentially proceeded at the same rate as in untreated persons (Figure 4). The maximal increase of CD4+ lymphocytes obtained by such intervention corresponded to that induced by a permanent application of a treatment of the same intensity. Of course, if the applied treatment did not last long enough to allow the CD4+ cells to reach that level, only a lower maximal value was obtained.

According to the results of our simulations temporary therapy only prolonged survival, although extended survival could

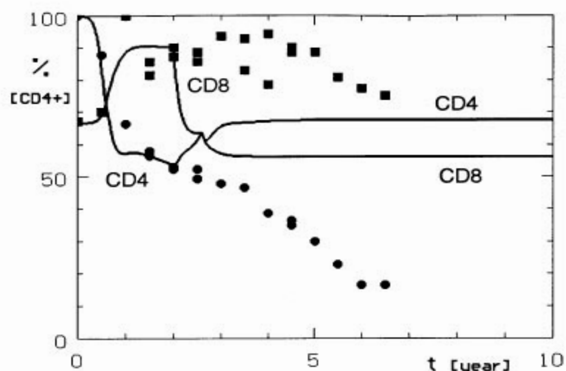

Figure 5.* Simulated CD4+ and CD8+ lymphocyte dynamics after permanent treatment with anti-CD8 antibodies started 2 years after the acquisition of the HIV infection. Cells mediating the protective anti-HIV immune reaction are not affected by this treatment (ρ_R = 0.007, ρ_C = 0.0).

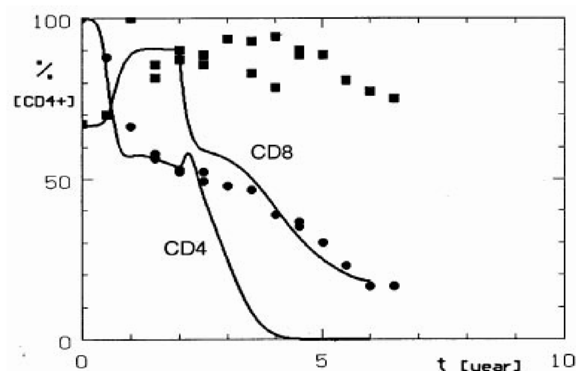

Figure 6.* Simulated CD4+ and CD8+ lymphocyte dynamics after permanent treatment with anti-CD8 antibodies started 2 years after the acquisition of the HIV infection. Cells mediating the protective anti-HIV immune reaction are also affected by this treatment (ρ_R = 0.007, ρ_C = 0.007).

*CD4+ cell observed values are depicted as circles and those of CD8+ lymphocytes as squares. Both simulated and observed values are depicted as a percentage of normal CD4+ lymphocyte numbers (the normal value of CD8+ lymphocytes is thus 66.7%).

exceed the length of treatment. This situation is relevant especially to chemotherapy that loses effectiveness after a relatively short time, probably because of drug resistance acquired by HIV. However, even immunotherapy cannot be expected to retain its undiminished effectiveness for a prolonged period.

Therapeutic Depletion of CD8+ Lymphocytes

It has been suggested that lowering the number of CD8+ lymphocytes in HIV-infected persons by administering anti-CD8 antibodies could activate the homeostatic mechanism, thus increasing production of both CD4+ and CD8+ T cells (24,25). However, this mechanism might be blocked by the high numbers of CD8+ lymphocytes present in HIV infection, as discussed above. Therefore, these authors assumed that a depletion of CD8+ lymphocytes brought about by administering anti-CD8 antibodies might unblock this feedback mechanism and that this could counteract the depletion of CD4+ cells caused by HIV infection.

When this regulatory mechanism was incorporated in our model, we were able to simulate this situation (26,30). Reducing CD8+ lymphocyte values to numbers not much below normal stops the further decline in CD4+ cells and brings them to a steady state, which may be higher than their pretreatment number (Figure 5). Lower doses of anti-CD8 antibodies also stopped further decline of CD4+ cells, but the achieved steady-state level is lower than in the illustrated case. On the contrary, the CD4+ lymphocyte increase is larger with higher antibody doses. As in other cases of temporary treatment, the decrease in CD4+ values starts again after anti-CD8 antibody administration is discontinued.

However, such a therapeutic effect of CD8+ depletion is achieved only if lymphoid cells eliminated by the administered antibody are assumed not to participate in the anti-HIV immune reaction that limits virus proliferation. This is not the case with cytotoxic T cells, or with CD8+ lymphocytes responsible for the production of cytokines that inhibit HIV proliferation. This situation could also be simulated with our model. When these cells assumed to cause a protective immune reaction were also eliminated by the administered anti-CD8 antibody, the CD4+ lymphocyte decrease did not stop; it even accelerated (Figure 6). All doses of anti-CD8 antibody, even very small ones, increased the depletion of CD4+ lymphocytes under this assumption, although the deteriorating effect was not so strong when small doses of antibody were used.

For the sake of brevity, we refrained from presenting examples from other models, although in addition to providing some quantitative differences in simulated results, these examples could be of interest in modeling other aspects of HIV infection (31-33), and the comparison of other results with those of our model would add another dimension to this article. Different models, or some of their parts, can be combined, and in this way the actual situation might be simulated even better; on the other hand, a particular model might simulate a specific situation and help clarify some questions posed by clinical or experimental studies.

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Appendix

The model considers immature and mature CD4+ ($\bar{P}$ and P cells) and CD8+ lymphocytes ($\bar{R}$ and R cells). As normal values of R cells equal about two thirds of those of P cells, it is assumed that normal $\bar{R}$ values correspond in a similar way to 2/3 of $\bar{P}$ cells. The sizes of these cell compartments at time t are described by Eqs. (1)-(4). The amount of HIV products at time t is given by Eq. (5). Finally, Eq. (6) gives the number of cytotoxic T cells specific for HIV (C cells) at time t. In the model used, these cells both limit proliferation of HIV, as indicated in Eq. (5), and effect destruction of CD4+ cells presenting HIV products according to Eqs. (1)-(2).

$$\frac{d\bar{P}(t)}{dt} = \frac{I_P + f\left[(P_0 - P(t)) + (R_0 - R(t))\right]}{d(t)} - \bar{\tau}_P\bar{P}(t) - \bar{c}_P a(t)C(t)\bar{P}(t), \quad \bar{P}(0) = \bar{P}_0 \qquad (1)$$

$$\frac{dP(t)}{dt} = \bar{\tau}_P\bar{P}(t) - \tau_P P(t) - c_P a(t)C(t)P(t), \qquad P(0) = P_0 \qquad (2)$$

$$\frac{d\bar{R}(t)}{dt} = \frac{2}{3}\,\frac{I_P + f\left[(P_0 - P(t)) + (R_0 - R(t))\right]}{d(t)} - \bar{\tau}_R\bar{R}(t), \qquad \bar{R}(0) = \frac{2}{3}\bar{P}_0 \qquad (3)$$

$$\frac{dR(t)}{dt} = \bar{\tau}_R\bar{R}(t) - (\tau_R - \rho_R)R(t), \qquad R(0) = \frac{2}{3}P_0 \qquad (4)$$

$$\frac{da(t)}{dt} = a(t)\left[\theta - \zeta - \gamma C(t)\right], \qquad a(0) = a_0 \qquad (5)$$

$$\frac{dC(t)}{dt} = \lambda a(t)\left[\varepsilon I_C + \alpha C(t)\right]\left(\frac{P(t)}{P_0}\right)^{v} - (\tau_C - \rho_C)C(t), \qquad C(0) = C_0 \qquad (6)$$

where the influx-constraining function was

$$d(t) = \begin{cases} 1 & \text{if} \quad \ln\frac{a(t)}{a_0} < L \\ h \ln\frac{a(t)}{a_0} & \text{if} \quad \ln\frac{a(t)}{a_0} \geq L \end{cases} \qquad (7)$$

Here I_P is the influx of $\bar{P}$ cells, i.e., the rate (all rates are in days^{-1}) of differentiation of $\bar{P}$ cells from stem cells, $\bar{\tau}_P$ is the rate of maturation of $\bar{P}$ cells into P cells, and τ_P is the rate of natural death of P cells; the quantities $\bar{\tau}_R$ and τ_R are defined in a fully analogical way. Further, f is the amplifying coefficient of the linear feedback effect of P and/or R cell decrease on the influx of $\bar{P}$ and $\bar{R}$ cells at time t.

The quantity $\bar{c}_P a(t)C(t)$ is the rate of elimination of $\bar{P}$ cells due to the amount of HIV products $a(t)$ and the number of cytotoxic T cells $C(t)$ at time t. Analogously, $c_P a(t)C(t)$ is the rate of elimination of P cells. The value a_0 is the function of the infectious dose of HIV, θ characterizes the growth rate of HIV, and γ is the rate of inactivation of HIV products mediated by cytotoxic C cells. The maturation of these cells from their precursors is assumed to be dependent on the encounter with HIV products and the effect of HIV specific helper T cells. I_C is the influx of C cell precursors, ε their maturation rate, α the proliferation rate of C cells under the antigenic stimulation by HIV products and helper T cell influence, and τ_C their natural death rate. Helper T cell effect on maturation and proliferation of C cells is expressed by the ratio $P(t)/P_0$; the coefficient v is introduced to characterize the intensity of this helper effect. The value h characterizes HIV-constraining intensity on the $\bar{P}$ and $\bar{R}$ cell influx. Value L defines the level, where such constraining (limiting) effect of $d(t)$ starts. Effects of therapeutic interventions are described by the following parameters: ζ - HIV elimination rate by AZT or passive immunization, λ - immune response-enhancing factor, and ρ_R- and ρ_C-elimination rates of CD8+ and C cells, respectively, by anti-CD8 antibodies.

If not otherwise stated, the model parameters in simulation runs were selected as follows: $\bar{\tau}_P = 0.2$, $\tau_P = 0.01$, $\bar{\tau}_R = 0.2$, $\tau_R = 0.01$, $\tau_C = 0.01$, $I_P = 1.0$, $I_C = 0.2$, $\bar{P}_0 = 5.0$, $P_0 = 100.0$, $\bar{R}_0 = 3.33$, $R_0 = 66.7$, $C_0 = 0.0$, $a_0 = 0.0005$, $f = 0.01$, $\alpha = 0.7$, $\varepsilon = 0.512$, $\gamma = 0.3$, $\theta = 0.02$, $v = 1.6$, $h = 3.5$, $L = 3.0$. Only mature CD4+ lymphocytes were assumed to be susceptible to HIV products, i.e. $\bar{c}_P = 0.0$, $c_P = 20.0$. As a rule, the parameter e was used for final adjustment of the respective simulation run. If no therapeutic interventions are assumed ($\lambda = 1.0$, $\zeta = 0.0$, $\rho_R = 0.0$, $\rho_C = 0.0$), the resulting CD4+ standard curve characterizes best fit of the observed clinical data.

Chlamydiae as Pathogens: New Species and New Issues

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The recognition of genital chlamydial infection as an important public health problem was made first by the recognition of its role in acute clinical syndromes, as well as in serious reproductive and ocular complications, and secondly by our awareness of its prevalence when diagnostic tests became widely accessible. The recent availability of effective single dose oral antimicrobial therapy and sensitive molecular amplification tests that allow the use of noninvasive specimens for diagnosis and screening is expected to have a major impact in reducing the prevalence of disease in the next decade. Clinical manifestations associated with *Chlamydia pneumoniae* infection continue to emerge beyond respiratory illness. In particular, its association with atherosclerosis deserves further investigation. *Chlamydia pecorum*, a pathogen of ruminants, was recently recognized as a new species. The continued application of molecular techniques will likely elucidate an expanding role for chlamydiae in human and animal diseases, delineate the phylogenetic relationships among chlamydial species and within the eubacteria domain, and provide tools for detection and control of chlamydial infections.

Chlamydiae are obligate intracellular bacteria that grow in eukaryotic cells and cause a wide spectrum of human disease (Table). Species were grouped according to their biologic and biochemical properties and a greater than 95% homology in their 16s ribosomal RNA sequences (1). Molecular analyses led to the reclassification of some *Chlamydia psittaci* strains as *Chlamydia pneumoniae,* a human pathogen, and *Chlamydia pecorum,* a pathogen of ruminants. Given the diverse host range of *C. psittaci* strains, more reclassification within this species may be likely.

The oldest reported disease associated with *C. trachomatis* infection is trachoma, a sequela of ocular infection. This disease was described in China and in the Ebers papyrus in Egypt thousands of years ago and continues to be a major cause of preventable blindness, with an estimated 500 million cases of active trachoma worldwide (seven million include blindness from conjunctival scarring and eyelid deformities [2]). In the last two decades, genital chlamydial infection has been identified as a major public health problem because of the recognition that chlamydial infection is associated with disease syndromes such as nongonococcal urethritis, mucopurulent cervicitis, pelvic inflammatory disease (PID), ectopic pregnancy, and tubal infertility. The World Health Organization estimated 89 million new cases of genital chlamydial infections worldwide in 1995 (3). In the United States, each year an estimated four million new cases occur and 50,000 women become infertile as a result of infection (4).

C. psittaci infection, acquired through respiratory droplet transmission of chlamydiae from infected birds, has been considered for many years an occupational hazard for employees of pet shops and poultry processing plants (5). Sources of human *C. psittaci* infection other than infected birds have been identified and may

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Table. Spectrum of human diseases caused by Chlamydiae

Species	Acute Diseases	Sequelae/Chronic Diseases
C. trachomatis		
Serovars A-C	conjunctivitis	trachoma
Serovars D-K	urethritis	proctitis, epididymo-orchitis, Reiter's Syndrome
	cervicitis	pelvic inflammatory disease, ectopic pregnancy, tubal infertility, Fitz-Hugh Curtis Syndrome
	ophthalmia neonatorum	
	neonatal pneumonia	
LGV serovars	lymphogranuloma venereum	
C. pneumoniae	pharyngitis	?cardiovascular disease
	sinusitis	?asthma
	bronchitis	
	community-acquired pneumonia	
C. psittaci		
parrot	atypical pneumonia	
canaries	hepatic and renal	
pigeons	dysfunction	
turkeys	endocarditis	
ducks		
chickens		
cats	conjunctivitis	
ewes	abortion	

be more common than currently recognized. Detection of *C. psittaci* in household cats and breeding catteries illustrates the expanding number of chlamydial diseases in animals that are transmissible to humans (6,7).

C. pneumoniae is a human pathogen recognized as an important cause of respiratory illness (8). Approximately 40% to 60% of adult populations around the world have antibodies to *C. pneumoniae*, which suggests that the infection is extraordinarily prevalent, and reinfection is common. Current interest centers on the emerging role of *C. pneumoniae* infection in the pathogenesis of atherosclerosis and asthma.

Biology of Chlamydiae: An Update

Chlamydiae have a unique biphasic life cycle with dimorphic forms that are functionally and morphologically distinct. An extracellular form, the elementary body (EB), is infectious but metabolically inactive. Once endocytosed, the EB differentiates into a larger pleomorphic form called the reticulate body (RB), which replicates by binary fission. The precise mechanism by which EBs attach and gain entry into the host cell is unknown. Recent work suggests that chlamydiae employ a molecular mimic of heparan sulfate to attach to glycosaminoglycan (GAG) receptors on eukaryotic cell surfaces (9). GAG appears to form a trimolecular complex with the host cell since (EB) infectivity is inhibited by the addition of heparan or heparan sulfate to culture, and pretreatment of EBs with heparan sulfate lyase abolishes EB infectivity. The mechanism of endocytic uptake remains unclear. Once inside the host cell, chlamydiae reside in a membrane-bound vacuole that can evade phagolysosomal fusion. The endosome is transported to the distal region of the Golgi apparatus and incorporates host-derived sphingolipids into the inclusion membrane (10,11). Thus it appears that chlamydiae are able to intercept host vesicular traffic bound for the plasma membrane to sequester lipids and possibly other host substances synthesized in the Golgi. Subversion of host vesicular traffic may represent a dual advantage for chlamydiae in obtaining materials from the host for its metabolism as well as in modifying the inclusion membrane to evade lysosomal fusion and immune detection.

Chlamydiae are considered energy parasites because they lack the enzymes of the electron transport chain and thus require adenosine triphosphate (ATP) and nutrient

resources from the host to fuel their metabolism and replication. Chlamydiae are incapable of de novo nucleotide biosynthesis and are dependent on host nucleotide pools (12). In spite of the successful selection of various metabolic mutants of *C. trachomatis*, progress in elucidating the host-parasite metabolic relationship has been hampered by multiple salvage metabolic pathways in the host and the lack of a genetic shuttle system for chlamydiae.

C. trachomatis

Epidemiology

Genital infections due to *C. trachomatis* are the most common sexually transmitted diseases in many industrialized countries (3). Each year, an estimated four million new cases occur in the United States and three million in Europe. These infections present unique problems for public health control programs because 50 % to 70% of infections in women (and perhaps men) are clinically silent. Unrecognized and untreated, the bacteria may remain infectious in the host for months and be readily transmitted to sex partners. Furthermore, most reported infections occur in the 15- to 24-year-old age group. Young women with cervical chlamydial infections are at risk for pelvic inflammatory disease, which can lead to long-term reproductive sequelae such as chronic pelvic pain, ectopic pregnancy, and tubal infertility. Babies born to infected mothers are also at risk for conjunctivitis and pneumonia. The annual direct and indirect costs of genital chlamydial infections in the United States are estimated at $2.4 billion (4).

Control programs emphasizing early diagnosis, targeted screening, partner notification, and effective treatment have led to a slow decline in the incidence of genital chlamydial infection in countries where these programs have been implemented (13). The true rate of decline may be higher than the reported rate because of increased sensitivity of laboratory testing and more widespread screening. In women, screening of chlamydial infection at the time of Papanicolaou tests, prenatal visits, or attendance at family planning or pregnancy counseling clinics have been effective. In asymptomatic men, who are less likely to access care, asymptomatic infection is not adequately addressed by current public health programs.

In contrast to genital chlamydial infection, trachoma is a household disease that has disappeared in many parts of the world because of improved living conditions and hygiene. In trachoma-endemic areas, severe disease leading to scarring and blindness may be the result of frequent reinfection or persistent infection in those whose immune system does not mount an adequate response to clear the infection. For both ocular and genital chlamydial infections, recent advances in diagnostic and screening technology and single dose antimicrobial therapy will likely have a significant impact on the efficacy of disease control programs and the opportunity for eventual disease eradication.

Laboratory Diagnosis

Since curative antibiotic therapy for chlamydial infections is readily available and inexpensive, early diagnosis is an essential component of public health programs to control these infections. The goals of early identification are to interrupt the chain of transmission in the community and to prevent long-term sequelae. Isolation of the organism in cell culture had been the traditional method for laboratory diagnosis and has remained the method of choice for medicolegal specimens because of its specificity. However, culture requires expensive equipment, technical expertise, and stringent transport conditions to preserve specimen viability; it also has a turnaround time of 2 to 3 days. Hence, in many settings, culture has been replaced by antigen-detection methods, such as enzyme immunoassays (EIA) and direct fluorescence assays (DFA), which have less demanding transport requirements and can provide results on the same day. EIAs are suitable for public health laboratories serving large geographic areas because specimens are stable in transport under ambient conditions and are inexpensive because they allow specimens to be processed in batches by automated equipment. Assays are typically based on the capture of the chlamydial

lipopolysaccharide (LPS) using monoclonal or polyclonal antibodies linked to a solid-phase support. Early problems with low specificity because of cross-reactivity between the chlamydial LPS and that of other gram-negative bacteria have been largely overcome by confirmation with DFA or a blocking antibody assay. With a lower detection limit of 10,000 elementary bodies, EIA lacks sensitivity as a screening assay, especially for asymptomatic men (14,15). Nucleic acid-based hybridization probe tests offer higher specificity but no substantial improvement on sensitivity (15). Nucleic acid amplification tests based on polymerase chain reaction (PCR), ligase chain reaction (LCR), and transcription-mediated amplification technology are now commercially available. The precision of nucleic acid hybridization and the rapid amplification of a single gene target facilitated the design of diagnostic tests with specificities in excess of 99% and lower detection limits of 1-10 EBs. In addition, these tests offer all the advantages of nonculture tests in terms of ambient specimen transport, batching, automation, and rapid processing time of 4 hours. Duplex testing for the simultaneous detection of chlamydial and gonococcal DNA from a single specimen is also commercially available in some countries.

A major advantage of the increased sensitivity of these molecular amplification tests is that noninvasive specimens, such as urine, can be used for testing. The ease of collection and the lack of sampling bias of urine specimens make screening feasible in settings outside physicians' offices. PCR assays on urethral or cervical swabs for the laboratory diagnosis of genital chlamydial infection in symptomatic men and women show sensitivities of 89% to 100% and specificities of 99% to 100% compared with the traditional culture or PCR test, confirmed by a second PCR reaction targeting a different gene (16-18). For urine specimens, PCR assays show sensitivities of 87% to 100% for men and 92% for women and specificities of 96% to 100% for men and 95% for women (18-20). In a study of 447 women with a prevalence of infection of 6%, the sensitivity of urine LCR was 96% compared with 56% for cervical swab culture, 78% for cervical swab EIA, and 37% for urine EIA (21). For men in the same study, the sensitivity of urine LCR was 96% compared with 68% for urine EIA, and 38% for urethral swab culture. In a multicenter study of 2,132 women, cervical swab LCR showed a sensitivity of 87% to 98% compared with a sensitivity of 52% to 92% for culture (22). In LCR studies, a true positive was defined as culture positive or LCR positive confirmed with DFA or another LCR assay with a different DNA target. Thus it appears that molecular amplification techniques for the detection of *C. trachomatis* in urine specimens from both men and women are a substantial improvement over conventional diagnostic and screening methods and will provide an important tool for decreasing the reservoir of infection, especially in asymptomatic men.

In the diagnostic laboratory, molecular techniques present different problems for specimen handling and interpretation of results than cell culture or antigen detection (15). Inherent in the increased sensitivity of these molecular techniques is the potential for false-positive results due to cross contamination between specimens, and run-to-run contamination from equipment, reagents, and supplies. These problems can be overcome by observing stringent rules for specimen preparation (e.g., dedicated equipment) and separating specimen processing and reagent preparation areas to prevent contamination. Enzymatic or photochemical sterilization can be used to eliminate run-to-run contamination. False-negative results may be due to substances in specimens inhibitory to enzymes used for amplification. Known inhibitors include phosphate ions, heparin, heme, crystals in the urine specimens, and detergents used in specimen processing. Internal controls are now commercially available to detect false negatives.

Although molecular tests are more expensive than EIA, cost-effectiveness studies should take into consideration the benefits of averting the enormous costs of long-term reproductive sequelae in women with undetected infections, adverse pregnancy outcomes, and HIV infection. Targeted screening of women to detect cervical chlamydial infection decreases the incidence

of symptomatic PID (23). Patients with genital gonococcal or chlamydial infections are also at increased risk for human immunodeficiency virus (HIV) (24). Although the risk for HIV may be lower in patients with chlamydial infection than in those with genital ulcer disease, the higher prevalence of chlamydial infection in some populations means that the population attributable risk for HIV may be substantially higher for chlamydia. Shortening the duration of infectiousness by early diagnosis and treatment could have a major impact on risk reduction for HIV infection. A recent study showed that strengthening sexually transmitted disease control through education, access to diagnosis, and treatment reduced the incidence of HIV by 42% in study communities in Tanzania over 2 years (25).

Treatment

Azithromycin prescribed as a single oral 1-g dose is equivalent to the traditional 7-day regimen of doxycycline for treating ocular and uncomplicated genital chlamydial infections (26-28). Compared with conventional therapy, azithromycin has excellent pharmacokinetic characteristics, such as increased bioavailability; lower incidence of gastrointestinal tract side effects; and increased concentration in mucus, macrophages, and tissues with a half life of 5 to 7 days (29). These characteristics allow for single dosing, which alleviates the problem of patient noncompliance with multiday regimens. With single-dose therapy, the potential for reinfection due to earlier resumption of sexual activity is a concern. At present, there are limited data on the use of single-dose therapy in adolescents, during pregnancy, and for syndromes such as PID, cervicitis, and nongonococcal urethritis (30-33). Studies are needed to determine if these regimens achieve clinical and microbiologic cure while preserving fertility and preventing further tissue damage to the upper genital tract.

Although the higher cost of azithromycin may be prohibitive for its use in resource-limited settings, selective use in persons at high risk or in those with a history of noncompliance may prove cost-effective. The cost of retreatment as a result of noncompliance and the additional cost of contact tracing can make single dose azithromycin more cost-effective than doxycycline (34).

Pathogenesis

Interesting findings in three areas of *C. trachomatis* pathogenesis further delineate the complex bacteria-host relationship in disease and may have implications for vaccine design. These new observations include the extensive but unexpected polymorphism of the major outer membrane protein (MOMP), the evidence for genetic susceptibility to disease, and the association of antibody response to the 60 kDa heat shock protein (CHSP60) with the development of adverse sequelae following ocular and genital infections.

Polymorphism of MOMP

The ecologic success of a pathogen is determined in part by its ability to evade host defenses. With *C. trachomatis,* MOMP is a major target for protective host immune responses, such as neutralizing antibodies and possibly, protective T-cell responses (35,36). The basis for MOMP antigenic variation is allelic polymorphism at the omp-1 locus, and immune selection appears to be occurring in host populations frequently exposed to *C. trachomatis* (37). Each variant apparently only infects hosts lacking serovar-specific immunity to that variant, and the ecologic success of chlamydiae may be due to their ability, under immune selection pressure, to generate successive allelic variants (36). DNA sequence analyses of isolates from different populations show that most MOMP variants are results of single amino acid substitutions (37-39). Recombination of sequences from MOMP during mixed infections may also have occurred. Recombinant variants with mosaic sequences of MOMP from different strains were especially frequent in persons with high rates of infection. MOMP variants were also more frequently found in women with PID than in those with lower genital tract infections, which suggests a relationship between sequence variation in MOMP and more invasive disease (39). Clearly, the extensive polymorphism of MOMP, the tempo for variation, and the mechanism of immune selection have

important implications for vaccine design (35).

Genetic Susceptibility to Disease

HLA B27 has been associated with Reiter's syndrome following genital chlamydial infection (40). Only a subset of infected persons appear to have long-term complications after acute or repeated chlamydial infections. In a study of 306 persons from trachoma-endemic communities in the Gambia, the HLA class I antigen HLA-A28 was significantly more common in case-patients than in age-, sex-, and location-matched controls (41). In particular, the A*6802 allele was overrepresented among case-patients. It may be that immunopathology is associated with HLA-A*6802 restricted cytotoxic T-lymphocyte responses. The frequency of HLA class II alleles was similar among cases and controls suggesting that, if class II restricted T-cell responses are important in immunopathology, they were not targeted at single epitopes. No individual HLA type was associated with protection from scarring, which suggests that multiple or complex T-cell responses may be involved in protective immunity. Susceptibility to chlamydial PID in a study of sex workers in Nairobi, Kenya, has been associated with a HLA class I allele, HLA A-31 (42). Studies are needed to determine whether susceptibility to silent PID, ectopic pregnancy, and progression to tubal factor infertility are associated with HLA class I restricted immune responses.

Role of CHSP60 in Immunopathology

Antibody response to a 57 kDa chlamydial protein was initially observed more frequently in women with tubal infertility than in controls (43). This protein was subsequently identified as a heat shock protein of the GroEL family of stress proteins. The association between antibody response to CHSP60 and PID, ectopic pregnancy, tubal infertility, and trachoma (44-48) has been documented. The risk factors associated with CHSP60 antibody response are similar to those for chlamydial PID and include older age and chronic or repeated infections. There appears to be genetic restriction for the CHSP60 antibody response. In a study of trachoma in the Gambia, HLA DRB1*0701 was positively correlated with CHSP60 response, while DRB1*0301 and DQB1*0501 were negatively associated (48). However, these alleles were not associated with trachoma and may reflect linkage disequilibrium between HLA class II alleles and polymorphic markers for other immune response genes.

At present, it remains unclear whether antibody to CHSP60 is causally involved in chlamydial immunopathogenesis or is merely a marker of persistent chlamydial infection (35). Both may be true. In cells persistently infected with *C. trachomatis,* the expression of CHSP60 is normal, while other antigens, such as MOMP, are downregulated, thus providing continued antigenic stimulation for the CHSP60 antibody response observed in persons with long-term sequelae (49). T-cell responses to chlamydial antigens, including CHSP60, were more depressed in persons with trachoma than in those who recovered from infection without sequelae (50). Persons with trachoma or reproductive sequelae have high levels of serum antibody response to *C. trachomatis*. In guinea pigs and in gene knock-out mice, both B- and T-cell responses have been important in immunity and resolution of infection (51,52). Therefore, persons with long-term sequelae may have predominantly Th_2 responses, characterized by high levels of B-cell response and inadequate T-cell responses that may not clear the infection thus leading to chronic inflammation. Immunopathology may also be the result of a hit-and-run mechanism in which immune response to CHSP60 breaks self-tolerance to the human HSP60 and leads to an autoimmune reaction that results in tissue damage (35).

C. psittaci

Epidemiology

Human infections with *C. psittaci* are caused by occupational exposure to infected birds or household handling of nasal discharge or fecal material from pet birds. Birds can be healthy carriers of *C. psittaci.* Increased shedding and susceptibility to disease occur under conditions of stress such

as shipping, crowding, starvation, or egg laying. Person-to-person transmission is rare but has been observed in outbreaks. In the *C. psittaci* pandemic of 1929-30, infected birds from Argentina were shipped to different parts of the world causing outbreaks of infection worldwide with death rates of up to 40% (5). Since then *C. psittaci* has been isolated from more than 130 species of birds. Thus, all avian species, including wild birds, should be regarded as potential sources of zoonosis.

Reports of outbreaks of psittacosis in duck and turkey processing plants show that, in spite of availability of medicated feed, diagnostic testing, and screening of poultry, *C. psittaci* infections continue to be a public health concern (53,54). High rates of chlamydial infection in household cats and asymptomatic carriage of *C. psittaci* in cats from breeding catteries raise the possibility that human *C. psittaci* infection from pets other than birds may be underdiagnosed (6,7,55,56). Studies of animal and cellular tropism of various strains within the species may give important clues to the pathogenesis of *C. psittaci* infections.

Clinical Manifestations

Human infection caused by exposure to infected birds or poultry is manifested as a flulike illness characterized by fever, chills, headache, and less frequently, cough, myalgias, rash, arthralgia and joint swelling, and atypical pneumonia in more severe cases. The incubation period is 6 to 19 days. Infections transmitted from ruminants are rare, but placentitis, disseminated intravascular coagulation, and spontaneous abortion in women exposed to infected sheep during lambing have been reported (56). Zoonoses associated with exposure to ruminants are characterized by multiorgan involvement often resulting in hepatic and renal dysfunction and endocarditis. Human conjunctivitis, glomerulonephritis, and endocarditis caused by *C. psittaci* from infected cats and pigeons have been reported (55).

Diagnosis and Treatment

Serodiagnosis has been the method of choice for human *C. psittaci* infections because culture is technically demanding and represents an important biohazard. The complement fixation assay is genus specific. Its interpretations should depend on clinical symptoms and patient history. The microimmunofluorescence (MIF) assay can detect species-specific IgM or IgG antibodies. Antigen detection methods, such as EIA, have been used, but they are based on the capture of the genus-specific LPS. PCR assays are not yet commercially available but can offer lower detection limits of 10 EBs or less (57,58). Molecular techniques not only provide more sensitive and rapid diagnosis than serology, but they also provide the opportunity for fingerprinting strains. This is particularly useful in outbreak investigations and for the confirmation of zoonotic transmission from infected birds or animals.

The recommended treatment for *C. psittaci* infection is 250 mg of tetracycline 4 times daily for 21 days. Although the death rate is low, prolonged hospitalization may be required. Protracted recovery and high incidence of relapse have also been noted.

C. pneumoniae

Epidemiology

C. pneumoniae is a common cause of acute respiratory tract infections and accounts for 6% to 10% of community-acquired pneumonia (8). Infection is usually mild or asymptomatic but can be severe, especially in the elderly, probably as a result of underlying illness, impaired mucociliary clearance, and immune senescence. Unlike *C. psittaci*, *C. pneumoniae* is spread by person-to-person transmission by respiratory droplet and has an incubation period of 7 to 21 days. Outbreaks of infection have been reported in families, schools, military barracks, and nursing homes. Coinfection with viruses (e.g., influenza and respiratory syncytial virus) and with bacteria has been reported frequently. Seroepidemiologic studies show that most primary infections occur during school age and the early teenage years; among adults seroprevalence is 40% to 70%. Reinfections are common, and serum antibodies do not appear to be protective.

Laboratory Diagnosis

Accurate and rapid laboratory diagnostic methods leading to improved patient care, appropriate use of antimicrobial therapy, and better understanding of the epidemiology of this emerging pathogen (59,60) are needed. Culture is highly specific but is technically demanding often requiring multiple passages over a period of weeks to show a positive result. *C. pneumoniae* has been isolated from the nasopharynx of healthy persons, but the rate of asymptomatic carriage in a normal population is unknown (61).

Antigen detection tests, such as EIA and DFA, and molecular detection methods, such as PCR assays, provide a rapid diagnosis without stringent transport requirements. Monoclonal antibodies specific for *C. pneumoniae* are now commercially available for DFA and for culture confirmation (62). PCR assays have lower detection limits of 10 to 100 EBs (57,58,63-65). The protocol developed by Tong and Sillis amplifies a target sequence conserved between *C. pneumoniae* and *C. psittaci* and hence can detect DNA from either pathogen in a single assay (57). A nested PCR procedure is used to differentiate between the *C. pneumoniae* and *C. psittaci* amplicons. The protocol of Rasmussen et al. amplifies a genus-specific target, followed by species differentiation using restriction enzyme digestion (58). The development of multiplex PCR assays containing primers specific for a panel of respiratory pathogens will be useful.

The MIF assay is the standard method used for chlamydia serology today. Ekman compared the performance of the complement fixation (CF), LPS-based EIA, and MIF tests for the serodiagnosis of *C. pneumoniae* and *C. psittaci* infections in an elderly population and found that the CF test has a sensitivity of 10% compared with 88% and 72% for MIF and EIA, respectively (66). IgM antibodies were only detected in 11% of the cases. IgM antibodies are rarely produced in reinfections with *C. pneumoniae.* CF tests may be useful in early initial infections as LPS antibodies are produced early in infection. Serodiagnosis may be made by demonstrating a fourfold rise in CF or EIA titer in paired sera taken a week apart, compared with the 3 weeks or more that it takes by MIF to demonstrate seroconversion. Because reinfections are common and LPS-based serologic tests are not useful in reinfection, the MIF assay remains the most useful and specific tool for the serodiagnosis of respiratory infections due to *C. pneumoniae.*

Treatment

The newer macrolides, clarithromycin and azithromycin, with longer tissue half-life and concentration in mucus and macrophages and improved bioavailability can potentially provide shorter and better-tolerated regimens for the treatment of respiratory infections due to *C. pneumoniae* than doxycycline or erythromycin, which have to be given for 2 to 3 weeks to avoid relapse. They may also be preferred for empiric therapy as they provide broader coverage than erythromycin against etiologic agents in community-acquired pneumonia. The optimal duration of treatment for respiratory infections due to *C. pneumoniae* needs to be determined since studies with documented microbiologic cure are limited, and recurrence of infection is common (67).

Association with Atherosclerosis

The association of *C. pneumoniae* infection with coronary heart disease and acute myocardial infarction was first made on the basis of elevated IgG and IgA antibodies and LPS containing immune complexes in 50% to 60% in patients with coronary heart disease or acute myocardial infarction compared with 7% to 12% in the controls. This study did not take into account risk factors for heart disease such as smoking, hypertension, or serum lipid levels. Subsequently, several cross-sectional studies involving 46 to 461 study participants have shown that a similar association of IgG antibodies against *C. pneumoniae* with coronary artery disease and carotid disease with adjusted odds ratios of 1.6 to 2.6 after controlling for known risk factors (68-72). Electron microscopy, PCR, and immunochemical evidence of *C. pneumoniae* in coronary arterial fatty streaks and atheromatous plaques have also been described (72,73).

Two more recent studies reported equivocal findings. In one, *C. pneumoniae* was detected in 79% of 90 coronary atherectomy specimens from symptomatic patients by direct immunofluorescence and was confirmed by electron microscopy. Only 4% of 24 control nonatherosclerotic coronary specimens were positive for *C. pneumoniae* (74). The 24 control samples included 12 from heart transplant patients whose arteries were damaged, but not by atherosclerosis. The absence of *C. pneumoniae* in these tissue samples argues against its role as a passenger recruited to the site of injury in macrophages. In the other study, *C. pneumoniae* was not detected in 58 coronary atheroma specimens by culture, PCR, or electron microscopy (75). The seroprevalence of *C. pneumoniae* in 65 case-patients was not different from that in 28 asymptomatic controls. In fact, IgG titers were higher in controls than in case-patients. Nonetheless, data suggest that the association of *C. pneumoniae* with atherosclerosis is consistent and biologically plausible. Whether *C. pneumoniae* is causally involved or is a bystander trapped in the atherogenic process is unclear.

The sustained IgA and IgG antibody levels against *C. pneumoniae* in persons with atherosclerosis suggest that chronic infection may be frequent after infection. The site of colonization for a chronic *C. pneumoniae* infection may be in the alveolar macrophages of the lung. Thus the initial event in atherogenesis may be the formation of the fatty streak. Fatty streaks consist of lipid-laden macrophages derived from blood monocytes and T lymphocytes attracted to the arterial subintima. Conversion of the fatty streak to atheroma depends on many factors, e.g., the proliferation and differentiation of smooth muscle cells and fibroblasts. Chronic infection with *C. pneumoniae* may result from organisms harbored in macrophages trapped in the arterial wall. Growth of *C. pneumoniae* in endothelial, smooth muscle cells, and macrophages from peripheral blood monocytes has been reported (76). Injured blood vessels initiate events that promote thrombosis and platelet adhesion at the site of injury. These events in turn promote atherosclerosis. Tissue injury through *C. pneumoniae*-specific circulating immune complexes in patients with chronic heart disease may be an alternate mechanism or compounding atherogenesis. The idea that an infectious agent is involved in the atherogenic process is not new, but the role of *C. pneumoniae* in this process needs to be defined.

Association with Asthma

The prevalence of asthma, an important chronic respiratory disorder, has been steadily increasing. Viral and *Mycoplasma pneumoniae* infections have been implicated in exacerbating the disease. The first observations on the association of *C. pneumoniae* infection with the exacerbation of asthma were made in 1986 when wheezing was associated with acute bronchitis due to *C. pneumoniae* infection (8,77). Subsequent studies showed that exacerbation of asthma due to *C. pneumoniae* infection may occur in 1% to 11% of respiratory infections in adults as well as children. The mechanism underlying the association is unclear. Preliminary results in animal models suggest that *C. pneumoniae* can produce persistent infection and cause pulmonary inflammation, and production of chlamydia-specific IgE antibodies in children with reactive airway disease has been demonstrated (78). A possible scenario for this association is an antigen-specific allergic reaction with the release of pulmonary inflammatory mediators and recruitment of inflammatory cells to the airways, causing airway epithelial damage. Activated T lymphocytes and cytokines appear to play a critical role as mediators of persistent inflammation in asthma. IL-4 is essential for B lymphocytes class switching from IgG to IgE. In vitro human IgE synthesis is reciprocally regulated by IL-4 and interferon-gamma. Thus cytokines from a Th_2 response to infection would facilitate and promote IgE production. Immunotherapy or glucocorticoid therapy targeting $CD4^+$ T cells may decrease the proinflammatory role of these cells and alleviate symptoms of asthma. The role of persistent infection in the pathogenesis of asthma merits further study because, unlike

viral infections, *C. pneumoniae* infections can be eradicated through appropriate antimicrobial therapy.

The hallmark of chlamydial infection is that most persons infected have mild to no apparent clinical disease and some have severe disease. Asymptomatic infection not only creates a problem in detecting cases for disease control programs but also contributes to the development of long-term adverse sequelae, such as scarring trachoma from ocular *C. trachomatis* infection, pelvic inflammatory disease, ectopic pregnancy, and tubal factor infertility from genital *C. trachomatis* infection. The recent availability of effective single dose oral antimicrobial therapy and sensitive molecular amplification tests that allow the use of noninvasive specimens for diagnosis and screening is expected to have a major impact in reducing the prevalence of disease in the next decade. New information from cell biology as well as data from observing the interaction of chlamydiae with the host in terms of metabolic requirements and immune evasion strategies offer clues about the pathogenesis of chlamydia infections and may eventually lead to an effective vaccine. Sporadic outbreaks of psittacosis continue to be reported despite the use of medicated feed and the screening of poultry. Recent reports of *C. psittaci* in cats from breeding catteries illustrate the potential of zoonotic diseases transmissible to humans from pets other than birds. Two new species of chlamydiae, *C. pneumoniae* and *C. pecorum*, were designated in 1989 and 1992, respectively. Clinical manifestations associated with *C. pneumoniae* infection continue to emerge. Possible links to chronic conditions, such as atherosclerosis and asthma remain to be elucidated. With the recent discovery of the involvement of infectious agents in other chronic conditions, it seems reasonable to apply molecular tools for chlamydial detection to identify their potential involvement in other etiologically undefined chronic inflammatory conditions such as inflammatory bowel disease and rheumatoid arthritis.

Dr. Peeling is a research scientist and chief of the Division of Chlamydial and Mycoplasma Diseases at the Laboratory Centre for Disease Control, Health Canada. She is interested in the diagnosis and pathogenesis of chlamydial infections with particular emphasis on the development, risk assessment, and possible prevention of adverse ocular and reproductive sequelae in human chlamydial infections.

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Experimental Inoculation of Plants and Animals with Ebola Virus

Thirty-three varieties of 24 species of plants and 19 species of vertebrates and invertebrates were experimentally inoculated with Ebola Zaire virus. Fruit and insectivorous bats supported replication and circulation of high titers of virus without necessarily becoming ill; deaths occurred only among bats that had not adapted to the diet fed in the laboratory.

The taxonomy of the *Filoviridae* is in a state of flux; the family includes viruses currently designated Marburg, Ebola Zaire, Ebola Sudan, and Ebola Ivory Coast, which are believed to be endemic to Africa, and Ebola Reston, which putatively originates in the Philippines (1,2). The viruses are known particularly for their propensity to cause fatal hemorrhagic disease of humans with person-to-person spread, but their pathogenicity varies from asymptomatic infection (Ebola Reston) to epidemics with death rates of 77% to 88% (Ebola Zaire) (1,3). All of the viruses appear to be highly pathogenic for nonhuman primates. Outbreaks of disease have occurred in Europe and North America in monkeys imported from Africa and the Philippines, sometimes with spread of infection to humans (1). Contact with the tissues of dead nonhuman primates was a source of infection for humans on at least two occasions in Africa (2,4). Nevertheless, the lethality of the viruses for nonhuman primates suggests that, like humans, nonhuman primates are incidental victims of infection and are not true reservoir hosts (1). The source of filoviruses in nature remains unknown, but in some instances, bats roosted in buildings or a cave visited or frequented by people who subsequently were found to have primary cases of infection in outbreaks of disease in Africa; one patient was bitten or stung by what is presumed to have been an arthropod 7 days before coming down with Marburg disease (1). Informal speculation has included the suggestion that filoviruses may be plant viruses, perhaps even involving transmission by arthropod vectors.

The search for the source of the viruses in nature has been hampered by the erratic recognition of outbreaks of filovirus infection in widely separated geographic locations at unpredictable intervals; therefore, comparatively few field investigations have been reported (1). After the 1995 epidemic of Ebola fever in Kikwit, Zaire, teams of scientists coordinated by the Centers for Disease Control and Prevention, Atlanta, and the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, collected large numbers of vertebrate and arthropod specimens during June, July, and August, 1995. Because evidence indicated that the outbreak had actually started in January 1995, it was possible that the virus was no longer circulating in its natural hosts in the vicinity of Kikwit by the time ecologic studies were undertaken. To allow for the possibility that the filoviruses manifest seasonal activity in their natural hosts, a team from the National Institute for Virology (NIV) in South Africa visited Kikwit in January 1996, to trap wild vertebrates and arthropods in the sites investigated by the other teams in mid-1995. Testing of the field material is a lengthy process, and no filoviruses have been detected so far. We decided to narrow the search by performing pathogenicity studies with Ebola virus in representatives of different classes and orders of living things, including vertebrates, invertebrates, and even plants. The underlying assumptions were that if a group of species is either refractory or hypersusceptible to the virus, members of the taxon are unlikely reservoir hosts of the virus, whereas members of taxa capable of circulating virus for prolonged periods without becoming ill are suspected reservoirs.

The strain of Ebola Zaire virus used in the experiments, Zaire-95, had been isolated from the blood of a patient in the 1995

epidemic in Kikwit and designated as the prototype strain of the outbreak. Stocks were prepared from virus at pass level 4 in Vero V76 cell cultures by freeze-thawing infected cultures, clarifying the supernatant culture fluid at 3,000*g*, and storing it in small volumes at -70°C. Virus titers were determined by fluorescent focus assay in 8-chamber slide cultures as described for rabies (5), except that Vero cells were used, and cultures were stained with immune mouse ascitic fluid followed by fluorescein-labeled anti-mouse immunoglobulin and read on day 3 to 5 postinoculation to detect infected foci. Inoculum volumes of 10µl of 10-fold serial dilutions of stock virus or tissue suspensions were adsorbed to cultures, and titers were expressed as fluorescent focus-forming units (FFU) per ml.

One-month-old potted seedlings of 33 varieties of 24 species of weeds and crop plants used in plant virology (Table 1), plus colonized leafhoppers, were obtained from Dr. G. Pietersen of the Plant Protection Research Institute (PPRI) at Rietondale, Pretoria, South Africa. The plants were selected because collectively they could provide culture substrates for a broad spectrum of the known viruses of economically important plants. They were kept under suitable lighting for 10 hours each day and watered as necessary to sustain growth in the laboratory. Pigeons were obtained from the South African Institute for Medical Research in Johannesburg; 1- to 3-month-old hatchling snakes from the Transvaal Snake Park, Midrand; cockroaches from a colony at the Bureau of Standards in Pretoria; and *Mastomys natalensis* and NIH mice from colonies at NIV. All other animals used in the studies (Table 2) were collected in the Kruger National Park, South Africa, with the permission of the National Parks Board as part of a long-standing research project on hemorrhagic fever viruses. The animals were translocated from the park under permit from the Department of Veterinary Services, and the experiments were conducted in a biosafety level 4 containment laboratory at NIV with clearance from the Department of Agriculture, Conservation and Environment of Gauteng Province, and the Animal Ethics Committee of NIV. All animals were fed a diet similar to their natural diets and were provided with fresh drinking water daily.

Table 1. Plants experimentally inoculated with Ebola virus

Scientific name	Common name
Arachis hypogaea	Groundnut
Beta vulgaris	Beetroot
Chenopodium amaranticolor	Goosefoot weed
Chenopodium quinoa	Goosefoot weed
Cucumis sativus	Cucumber
Cucurbita pepo	Pumpkin
Glycine max[a]	Soybean
Gomphrena globosa	Weed
Gossypium hirsutum	Cotton
Lupinus albus	Lupin
Lycopersicon esculentum	Tomato
Macroptilium atropureum	Siratro bean
Nicotiana benthamiana	Wild tobacco
Nicotiana clevelandii	Wild tobacco
Nicotiana glutinosa	Wild tobacco
Nicotiana langsdorfi	Wild tobacco
Nicotiana rustica	Wild tobacco
Nicotiana tabacum	Tobacco
Phaseolus vulgaris[a]	French bean
Pisum sativum	Green pea
Triticum aestivum	Wheat
Vicia faba	Broadbean
Vigna unguiculata[b]	Cowpea
Zea mays	Maize

[a]Five varieties inoculated
[b]Two varieties inoculated

Vertebrates were inoculated subcutaneously with 0.1 ml of stock virus diluted 1:10 in cell culture medium, and back titration of the inoculum indicated that each animal received a dose of 40,000 or $10^{4.6}$ FFU virus. Invertebrates were inoculated with undiluted stock virus and received approximately 1.0µl containing 4,000 or $10^{3.6}$ FFU of virus, except for leafhoppers, which received about 0.3µl inoculum. The arthropods were inoculated intrathoracically (6), except for ants and millipedes, which were inoculated into the hemocoel through the membranous integument between tergites. To simulate mechanical transmission, undiluted stock virus mixed with Carborundum powder was rubbed gently with cotton buds onto two leaves on each of the plant varieties on experiment; to simulate vector-borne transmission, a second plant of each type was inoculated with 1.0µl virus suspension into the phloem of the stem, using the same apparatus as for arthropods. A third plant of

Table 2. Results of experimental infection of various animals with Ebola virus

Species	Common name	Pool size	Day post-infection 1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	28
Columba livia	Domestic pigeon	1	0/1*	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/2	0/2
Hyperolius viridiflavus	Painted reed frog	2	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/2	
Bufo regularis	Common toad	1				0/1		0/1						0/1			0/1	
Chiromantis xerampelina	Grey tree frog	1					0/1		0/1							0/1	0/1	
Hemidactylus mabouia	Tropical house gecko	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		0/1	
Lamprophis fuliginosus	Brown house snake	1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1	0/1	0/2	0/2
Geochelone pardalis	Leopard tortoise	1						0/1		0/1		0/1						0/1
Kinixys belliana	Hinged-back tortoise	1						0/2		0/2		0/2						0/2
Tadarida condylura	Angola free-tailed bat	1				1/2	1/2			2/2	1/1			1/1				
Tadarida pumila	Little free-tailed bat	1	1/1	1/1	1/1		0/1	1/2	0/2	0/1		1/1	1/1	1/1	0/1	1/1	0/4	0/5
Epomophorus wahlbergi	Wahlberg's epauletted fruit bat	1		0/1		1/1		1/1	1/1	1/1		1/1	1/1	1/1			1/1	0/1
Mastomys natalensis ss	Multimammate mouse	1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
Mus musculus	NIH mouse	1		1/1	0/1	0/1			1/1	0/1	1/1	0/1	0/1			0/1	0/2	
Periplaneta americana	American cockroach	2	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1								
Austria agallia	Leafhopper	50																0/1
Messor barbarus capensis	Myrmicine ant	5			0/1		0/1		0/1									
Stegodyphus dumicola	Social spider	2	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1						1/1		
Alloporus sp.	Millipede	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1							
Achatina sp.	African landsnail	1			0/1		0/1											0/1

*Virus isolations/pools tested

each type served as control. Plants were observed daily and those that wilted or developed apparent lesions were harvested; some material was fixed in 2.5% glutaraldehyde in buffer for examination by electron microscopy, and the rest was stored at -70°C for virus assay. The process of embedding, sectioning, and examining the plant tissues by electron microscopy was performed by H.J. van Tonder of PPRI. Animals were sacrificed and assayed for virus content either in pools or individually, before inoculation and at intervals postinoculation (Table 2). Serum and pooled visceral organs were tested separately, and individual organ samples were preserved at -70°C and in Formalin fixative for more detailed study later. Urine and feces samples were tested on some occasions. Materials for virus assay were prepared as 10% suspensions in culture medium, and in parallel with attempts to titrate infectivity, 0.1 ml volumes were injected into Vero cell monolayers in 25 cm^2 flasks, which were subcultured thrice at weekly intervals before specimens were recorded as negative.

Thirteen plants either wilted or developed lesions on the leaves ascribed to mechanical injury during the inoculation process, but no infectivity could be recovered from the tissues, and no evidence of virus infection was observed by electron micros-

copy. All animal experiments (Table 2) were performed in parallel on a single occasion except for a second experiment with insectivorous bats (*Tadarida* spp.). Insectivorous bats had difficulty in adapting to a laboratory diet, and consequently 10 of 18 died in the first experiment. Although virus was recovered from the blood and organs of some, no histopathologic lesions were observed, and no evidence of widespread infection was detected by immunohistochemical technique (7). All of the bats that died had not been eating well, and hence more insectivorous bats (*T. pumila*) were collected from the same colony as before and adapted to a diet of mealworms over a period of 3 weeks before inoculation. The bats in the second experiment received the same dose of virus as the previous group, and no deaths occurred before the 12 animals in the study, including nine that were kept 21 to 28 days, were sacrificed (Table 2). None of the other vertebrates died, although some of the ants, cockroaches, and spiders died, possibly from desiccation as a result of injury during inoculation.

The virus replicated in bats: titers of $10^{4.6}$-$10^{7.0}$ FFU/ml were recorded in sera and titers of $10^{2.0}$-$10^{6.5}$ FFU/ml in pooled viscera of fruit bats. In addition, virus was recovered from the feces of a fruit bat on day 21 postinoculation. Virus was also recovered on a few occasions from snakes, and NIH mice and spiders (Table 2), but this was at a minimal titer of $10^{1.0}$ FFU/ml and could represent residual infectivity from inoculation. Histopathologic and immunohistochemical investigations have thus far been limited to some sets of the bat organs, and the only virus antigen detected was present in the endothelial cells of lung tissue of a bat sacrificed on day 8 postinoculation (Figure). Four insectivorous bat sera collected on day 28 postinoculation and four samples from noninfected bats were tested for Ebola virus antigen or antibody by enzyme-linked immunoassay (8) using conjugated chicken anti-rodent immunoglobulin; antibody was found in only one of the infected bats.

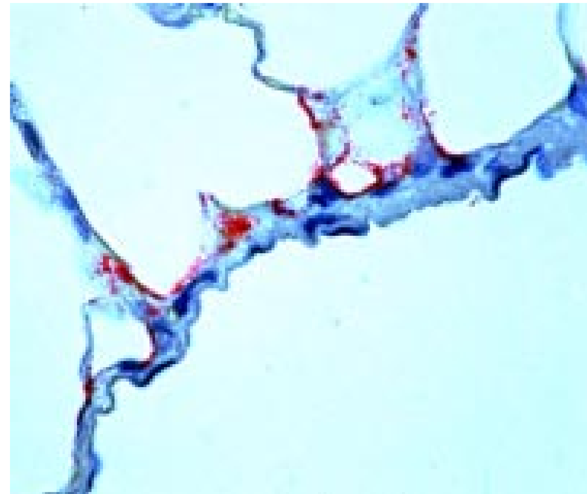

Figure. Ebola virus antigen-positive cells (red) in lung of an insectivorous bat as demonstrated by immunohistochemistry. Note prominent endothelial immunostaining. (Rabbit anti-Ebola virus serum, napthol/fast red with hematoxylin counterstain, original magnification x 250).

Although they do not provide conclusive evidence that bats are potential reservoir hosts of filoviruses or that the other animals are not, the findings demonstrate the validity of the experimental approach to the search for the source of the viruses in nature. If it can be shown, for instance, that a further two to three species of birds of widely divergent orders or families are refractory to the virus, birds can be accorded low priority in field studies, and efforts can be concentrated on animals capable of circulating virus. Even if evidence is obtained that certain animals become infected in the field, it would remain desirable to study the nature of the infection they undergo in the laboratory to determine whether they can harbor virus for prolonged periods and transmit it to other animals. Additionally, materials derived from the experiments can be used to develop, test, and perfect methods for detecting infectious virus, viral antigen, nucleic acid, or antibodies in different species, and to establish whether or not demonstrable immune response develops in ostensibly refractory animals. There are no accepted methods for demonstrating antibody in many wild vertebrates; anti-bat immunoglobulin is being produced as part of the present project.

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body in many wild vertebrates; anti-bat immunoglobulin is being produced as part of the present project.

The two tadarids studied here, and many other bats, have a distribution that overlaps the sites of known filovirus outbreaks in Africa, and the migratory habits of some species would facilitate dissemination of virus (9). The presence of virus in lung tissue implies that respiratory or oral spread of infection could occur in the confined spaces where bats roost, and isolation of virus from feces suggests the existence of mechanisms for transmission of infection to other animals. However, much remains to be learned about the nature of the infection in bats, including the sites of virus replication, persistence, and the behavior of the virus in further genera and species.

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Encephalitis Caused by a Lyssavirus in Fruit Bats in Australia

This report describes the first pathologic and immunohistochemical recognition in Australia of a rabies-like disease in a native mammal, a fruit bat, the black flying fox (*Pteropus alecto*). A virus with close serologic and genetic relationships to members of the *Lyssavirus* genus of the family *Rhabdoviridae* was isolated in mice from the tissue homogenates of a sick juvenile animal.

The *Lyssavirus* genus of the family *Rhabdoviridae* consists of five serotypes: classical rabies virus (serotype 1), Lagos bat virus (LBV) (serotype 2), Mokola virus (serotype 3), Duvenhage virus (DUVV) (serotype 4), and European bat virus (EBV) (serotype 5). The viruses within the genus share serologic relationships, but the serotypes and stable species-associated variants within serotypes can be distinguished by the reactivity profiles of monoclonal antibodies (Mab) directed against nucleoprotein and glycoprotein antigens. Analysis of the nucleotide sequence of the nucleoprotein gene has also shown genetic clusters along the same lines as serologic analysis, except that serotype 5, EBV, has been separated into two genotypes, EBV1 and EBV2 (1). Lyssaviruses have not been isolated in Australia before, although rhabdoviruses in the genus *Ephemerovirus* are present, and viruses with some serologic relationship to the *Lyssavirus* genus, for example Adelaide River virus (2), have been identified but not characterized. All members of the *Lyssavirus* genus can cause rabies or rabies-like diseases in infected animals.

Rabies-like disease has been recorded in bats on all continents except Australia. Classical rabies virus infections are common in insectivorous and hematophagous bats and less common in frugivorous bats in the Americas, while rabies-related viruses (EBV 1 and 2) are found in insectivorous bats in Europe. Two other rabies-related viruses, LBV and DUVV, are found in frugivorous bats and insectivorous bats, respectively, on the African continent. Rabies has been described in a flying fox (*Pteropus poliocephalus*) in India, although the virus causing the disease was not characterized (3). An outbreak of rabies involving several dogs occurred in the Australian island state of Tasmania in 1867 but was quickly eradicated (4). Two cases of rabies in children were reported in Australia (in 1987 and 1990). Both cases were caused by classical rabies virus and were contracted in endemic-disease countries (5). We report for the first time apparent endemic–lyssavirus-induced disease in Australia.

The four largest species of frugivorous bats in Australia are called flying foxes and belong to the genus *Pteropus* (Order Chiroptera, Suborder Megachiroptera, Family *Pteropodidae*). The Australian range of the flying foxes extends from temperate eastern and coastal Australia into the eastern tropics, around the tropical northern coastline, and down as far as the subtropical west coast. The gray-headed flying fox (*Pteropus poliocephalus*) range is the temperate and subtropical east coast, the black flying fox (*P. alecto*) inhabits primarily the subtropical and tropical range, and the little red flying fox (*P. scapulatus*) occupies the entire range except the coolest southern areas. The fourth species, the spectacled flying fox (*P. conspicillatus*) occupies a smaller range in tropical northeast Queensland. Large flying fox "camps," with possibly tens of thousands of foxes, often contain more than one species. Analysis of population genetic markers shows a considerable movement of both *P. alecto* and *P. poliocephalus* across their geographic ranges within Australia (6). The range of *P. alecto* extends to the north of Australia into Papua New Guinea and the eastern islands of Indonesia (7,8). Regular patterns of movement suggest that flying foxes move between northern Australia and Papua New Guinea (L. Hall, pers. comm.). It is, therefore, possible that the virus described in this paper also extends across

the range of these mammals outside Australia.

The flying foxes (*P. alecto*) described in this paper were wild native Australian animals collected near Ballina, in northern New South Wales, Australia. The first case, in 1996, was in a 5-month-old female black flying fox found under a fig tree, unable to fly. It was euthanized by intravenous sodium pentobarbitone injection. Fresh blood, lung, kidney, and spleen were submitted for equine morbillivirus (EMV) isolation; antibody to EMV has been detected in *P. alecto* (9), and it is conjectured that this species may be the reservoir for EMV. Paraffin-embedded formalin-fixed samples, processed by standard techniques, showed a severe nonsuppurative encephalitis. The second case, in 1995, was identified after a retrospective examination of archived paraffin-embedded tissues. The affected animal, a juvenile female of the same species, was reported to be more aggressive than usual, and was euthanized and necropsied in a similar manner to the first. Histologically, although encephalitis was very mild, many eosinophilic, cytoplasmic inclusion bodies were present in various parts of the brain. All tests for EMV were negative.

An indirect immunoperoxidase test for rabies was carried out on tissues from paraffin blocks (10) by using an antirabies Mab (HAM) (Clone 'HAM', c/o Drs. R. Zanoni and E . Peterhans, Institut für Veterinär-Virologie, Länggasstr. 122, CH-3012, Bern Switzerland) that gave good reactions without background staining when used at 1:100. The 1996 bat had positive results over wide areas of the brain, particularly in parts of the hippocampus, the mesenchymal cells of the trigeminal nucleus, and larger motor neurons of the medulla oblongata. The brain of the 1995 bat reacted strongly over all areas. The reactions were either granular, or characteristically, had ring formations in large neurons. In addition, similar reactions were seen in neuronal cytoplasms in nerve plexuses of the gastrointestinal tract from both bats. Electron microscopy examination of ultrathin sections of hippocampus from the 1996 bat showed aggregates of viral nucleocapsids within the cytoplasm of cell bodies. These inclusion bodies were specifically labeled with anti-rabies HAM Mab and gold-labeled rabbit–anti-mouse.

The only fresh samples available were blood, lung, kidney, and spleen from the 1996 bat. The blood was examined for neutralizing antibody to rabies virus (CVS-11) by the rapid fluorescent focus inhibition method (11). No neutralizing antibody was detected. Tissue homogenates (lung, kidney, and spleen) were injected into mouse neuroblastoma cells, individually injected intracerebrally into 3-week-old mice (five mice per sample), and, as a pool of the three tissues, injected into day-old suckling mice (two litters, 14 mice). No virus was isolated from cell culture after two serial passages of 4 days. One weanling mouse injected with kidney homogenate showed hind leg paraplegia 16 days postinoculation. All other mice remained normal until termination (suckling mice at 21 days and weanling mice at 28 days postinoculation). The affected mouse was euthanized, and acetone-fixed smears of brain material were positive for a lyssavirus when tested by the Centocor fluorescein-labeled Mab (Centocor Inc., 244 Great Valley Park, Malvern, PA 19355, USA). Formalin-fixed brain material showed nonsuppurative encephalitis and was positive to the indirect immunoperoxidase test for rabies virus by the HAM Mab.

Polymerase chain reaction (PCR) tests were done on nucleic acids extracted from the brain, lung, kidney and spleen of the 1996 bat and on paraffin-embedded formalin-fixed brain tissues from the 1995 and 1996 bats by using oligomers designed for the amplification of lyssavirus N protein (12) or for nested PCR amplification of the nucleocapsid protein (5). Results from these primers were consistently negative, presumably because of formalin-fixation and/or sequence heterogeneity. Therefore, another nested PCR system was devised for the amplification of N protein. Nucleic acids were extracted (5) and transcribed into cDNA by using a degenerate oligomer NP1087 (5' GAGAAAGAG[A/C]T[G/T]CAAGA[A/C/T]TA. Primary PCR was done with primers NP1087 and NP1279 (5' CAG AGACATATCT[G/C]C[G/T][G/T]ATGTG) with amplification conditions of 94°C for 1 min, 37°C for 2 min, and 72°C for 2 min for 35

Table 1. Amino acid sequences of the virus designated pteropid lyssavirus (PLV) and those of rabies and rabies-like viruses[a,b]

	PLV[c]	PV	4FRA	POL	8FRA	AS	FIN	HOL	NGA	Genotype
PLV	-									
PV	92	-								1
4FRA	93	99	-							1
POL	93	94	94	-						5
8FRA	93	94	94	100	-					5
AS	89	90	91	95	95	-				4
FIN	85	87	88	87	87	84	-			6
HOL	85	88	89	87	87	83	98	-		6
NGA	82	82	83	85	85	85	76	77	-	2
MOK	76	75	76	79	79	80	72	71	86	3

[a] Described in Ref. 13.
[b] Comparisons were made of cognate regions of the N protein (amino acids 298 to 426 inclusive). GenBank accession numbers are given in brackets. PV, PV rabies virus (X03673); 4FRA, fox rabies virus (U22844); POL, European bat virus (U22844, 8615POL, EBV1); 8FRA, European bat virus (U22845, 8918FRA, EBV1); AS, Duvenhage virus (U22848); FIN, European bat virus (U22846, 9007FIN, EBV2); HOL, European bat virus (U22847, 9018HOL, EBV2); NGA, Lagos bat virus (U22842), Mokola virus (U22843).
[c]PLV, the virus now reported, provisionally designated pteropid lyssavirus; PV, PV rabies virus (X03673); 4FRA, fox rabies virus (U22844); POL, European bat virus (U22844, 8615POL, ELB1); 8FRA, European bat virus (U22845, 8918FRA, EBV1); AS, Duvenhage virus (U22848); Fin, European bat virus (U22846, 9007FIN, EBV2); HOL, European bat virus (U22847, 9018HOL, EBV2); NGA, Lagos bat virus (U22842), Mokola virus (U22843).

cycles. Nested PCR was done by using primers NP1087 and NP1227 (5' CTTCA [C/T]C[G/T]ACC[A/T][C/T][C/T]GTTC ATCAT) as above except that the number of cycles was reduced to 25. PCR products were excised and sequenced. Positive PCR amplification signals were derived from the tissue culture virus and paraffin-embedded formalin-fixed brain tissues by using primers NP1087 and NP1227. Sequence analysis of these products showed that they were identifical. Sequence comparisons were done by using the nucleocapsid proteins of known lyssaviruses and the virus reported in this paper, designated pteropid lyssavirus (PLV) (Table 1). Nucleotide sequence comparisons showed that PLV had a 75% homology with LBV, 75% homology with EBV-2, and 79% with Pasteur vaccine rabies virus; at the amino acid level, the virus was 85% homologous with both EBV-2 and LBV (but 92% homologous with the rabies virus), 89% with DUVV, and 93% homologous with EBV-1 viruses. Phylogenetic analysis of both the nucleotide and amino acid sequences (not shown) showed that the virus is closely related to the EBV as well as the classic street rabies strains (12).

Brain material from the affected mouse was repassaged by intracerebral inoculation into 3-week-old mice, in which neurologic signs developed 8 to 11 days postinoculation. Examination of brain homogenate from these mice by negative-contrast electron microscopy showed classical bullet-shaped rhabdoviruses. The isolate was also passaged to mouse neuroblastoma cells, which were acetone-fixed and tested by indirect immunofluorescence using a panel of Mabs against various rabies and rabies-like viruses. The CVS-11 strain of rabies was also tested for comparison. The results (Table 2) confirm that the isolate is a lyssavirus but is different from previously described isolates. Additional nucleocapsid Mab reaction patterns (results not shown) indicated a unique profile that shared the greatest number of positive reactions with serotype 1 rabies (CVS-11) compared with published profile data on other viruses (15). Preliminary testing of the isolate in a modified (incubated 3 days) rapid fluorescent focus inhibition neutralization assay indicated that the virus was neutralized by antisera to rabies virus (mouse anti-Evelyn-Rokitnicki-Abelseth [ERA] virus). The titer of the immune mouse serum against CVS rabies virus was 1,194, and against the bat virus, 1,640.

This is the first evidence of an endemic lyssavirus in Australia. The isolate described has been provisionally called pteropid lyssavirus. The natural history of this virus in bats in Australia needs to be investigated. Further genetic and antigenic analyses are

Table 2. Reactivity patterns of nucleocapsid monoclonal antibodies (Mab) with rabies (CVS-11) and pteropid lyssavirus (PLV)

		IFAT[a]	
MAb[b]	Specificity[a]	CVS-11	PLV
W502-2[c]	lyssavirus	+	+
HAM[d]	lyssavirus	+	+
C15-2[e]	rabies	+	-
62-143-1[f]	rabies	+	+
62-3-1[f]	rabies +, EBV +	+	-
62-146-3[f]	rabies +, DUVV -	+	+
W422[c]	Mokola +, LBV +	-	-

[a]+ indicates a positive reaction; - indicates a negative reaction
[b]Monoclonal antibody were specificities indicated by the following sources:
[c] (14,15); [d] (10); [e] (J Smith, pers. comm.); [f] (15,16)

also needed to fully determine the relationship of the virus to existing *Lyssavirus* serogroups and genogroups and to confirm its separate identity from other as yet uncharacterized rhabdoviruses isolated in Australia. The virus has been submitted to the Rabies Laboratory at the Centers for Disease Control and Prevention, Atlanta, for further Mab profile analysis and crossprotection studies with classical rabies vaccines. Findings will result in a better understanding of the public health implications of this newly emerged lyssavirus.

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Addendum

Since this report was submitted in September, 1996, the host and geographic range of the virus have been extended. The virus has been recognized by immunohistochemical techniques in five bats in three different virus isolations. Some of these bats were from another species, (the little red flying fox [*P. scapulatus*]), and from locations as far apart as 1,700 km along the Australian east coast.

Creutzfeldt-Jakob Disease in the United States, 1979-1994: Using National Mortality Data to Assess the Possible Occurrence of Variant Cases

After a cluster of Creutzfeldt-Jakob disease (CJD) cases among unusually young patients was reported recently from the United Kingdom, we examined trends and the current incidence of CJD in the United States. We found that the age-adjusted CJD death rate in the United States is similar to published estimates of the crude incidence of CJD worldwide and has continued to be stable from 1979 through 1994. The number of CJD deaths in persons <45 years of age remained stable during this period. We found no evidence of the variant form of CJD.

Transmissible spongiform encephalopathies (TSE) are rare forms of progressive neurodegenerative disorders that affect both humans and animals (1). They are distinguished by long incubation periods, characteristic spongiform changes associated with neuronal loss, and a failure to induce inflammatory response (2). The prototype TSE in humans, Creutzfeldt-Jakob disease (CJD), occurs sporadically (approximately 90% of cases), through iatrogenic transmission of the infective agent (<1% of cases), or as an autosomal dominant inheritance (approximately 10% of cases) (3,4). CJD is a rapidly fatal dementing illness that occurs worldwide, with an estimated incidence of approximately one case per million persons. Unconventional agents termed prion proteins (PrPs), which are encoded by genes on chromosome 20, are considered the etiologic agent of CJD. The pathologic properties of these proteins lie in their three-dimensional configuration and their ability to recruit and influence normal PrPs, to undergo similar conformational changes. The transmissibility of CJD has been verified with reports of iatrogenic transmission from a corneal transplant, electroencephalographic depth electrodes, neurosurgical procedures, cadaveric dura mater grafts, and pituitary hormone administration (5-11).

A cluster of CJD cases with a unique neuropathologic picture among unusually young patients in the United Kingdom (12), which was reported during a widespread epizootic of bovine spongiform encephalopathy (BSE), has alerted many countries, including the United States, to update their surveillance for CJD and look for similar cases (13). This increased attention, and the fact that CJD is rapidly and invariably fatal (14), prompted us to analyze the most current CJD deaths and update our analysis of national trends of CJD mortality in the United States. Because of concerns about the theoretical risk for transmission of CJD by blood transfusion, we also examined the mortality records for evidence of diseases associated with increased exposure to blood or blood products.

Multiple cause-of-death data for the United States from 1979 through 1994 were obtained from the National Center for Health Statistics, Centers for Disease Control and Prevention (CDC) (15), with 1994 data reported as provisional. Cause-of-death classifications were based on the Ninth Revision of the International Classification of Diseases (ICD-9) (16-18). CJD deaths were defined as those for which ICD-9 code 046.1 appeared as an entity-axis code. The alternative CJD ICD-9 code 331.5 is not valid in the United States (17,19,20). The case of a 3-year-old child with spongiform degeneration of infancy (ICD-9 code 046.1) was included in the study. We excluded the case of a 5-year-old child with Kawasaki disease (ICD-9 code 446.1) because CJD was not listed on the death certificate and the case of a 17-year-old patient who had had diffuse T-cell proliferative disease.

Annual CJD death rates were calculated as the number of CJD deaths per million persons, on the basis of U.S. resident population estimates, and standardized by the direct method, using the 1990 census population (21). Age-adjusted annual death rates were calculated by sex and race and for the United States overall; age-, sex-, and race-adjusted annual death rates were calculated by standard region (22). Risk ratios (RRs) with 95% confidence intervals (CIs) were calculated by Poisson regression analysis (23). Deaths were examined according to whether autopsy was indicated on the death certificate, as available from 1979 through 1993. We also examined other causes of death listed and searched the records for several diseases associated with increased exposure to blood or blood products, specifically hemophilia A (ICD-9 code 286.0), hemophilia B (ICD-9 code 286.1), thalassemia (ICD-9 code 282.4), and sickle cell disease (ICD-9 code 282.6).

From 1979 through 1994, CJD was recorded as a cause of 3,642 deaths in the United States; 83.4% of these deaths had CJD recorded as the underlying cause. The average annual age-adjusted death rate during the study period was 0.95 deaths per million persons, from 0.78 in 1980 to 1.11 in 1987 (Figure 1). The mean age of death was 67 years (median age = 68 years). Approximately 98% of the deaths were among persons ≥45 years of age. The age-specific death rates were highest for persons in their 70s (Figure 2).

Although 52.9% of the deaths were in female patients, the age-adjusted death rate of male patients was slightly higher than that of female patients (RR = 1.16, 95% CI = 1.09-1.24; Table 1). In contrast to the death rate in age groups <60 years, the CJD death rate in age groups ≥60 years was higher in men than in women (Table 2). Most (95.2%) deaths were among whites. The age-adjusted death rate of whites was higher than that of blacks (RR = 2.66, 95% CI = 2.22-3.18) (Table 1). Other races accounted for only 1.4% of the CJD deaths, with an age-adjusted rate intermediate to that of blacks and whites. The death rate of blacks was lower than that of whites in each age group. Geographically, the age-sex-race-adjusted rate of the South was lower than that of each of the other regions. The previously noted differences by sex and race remained consistent for each region.

Approximately 80% of the CJD deaths were among persons ≥60 years of age. The average annual death rate during the study period of this age group was 4.58 per million persons (3.71 per million in 1983 to 5.52 per million persons in 1987) (Figure 1). Among persons 45 through 59 years of age, the

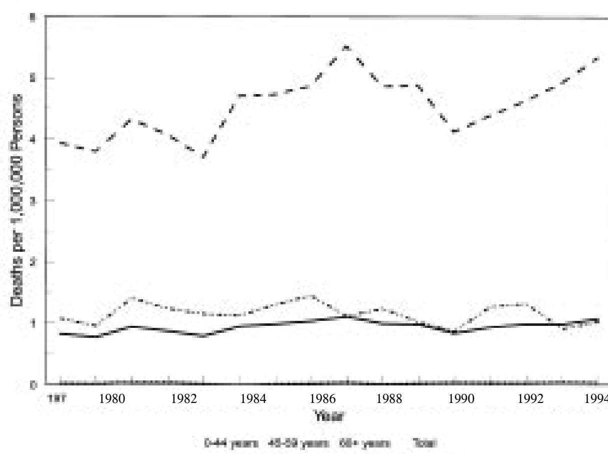

Figure 1. Creutzfeldt-Jakob disease age-adjusted and age-specific death rates, United States, 1979 through 1994.

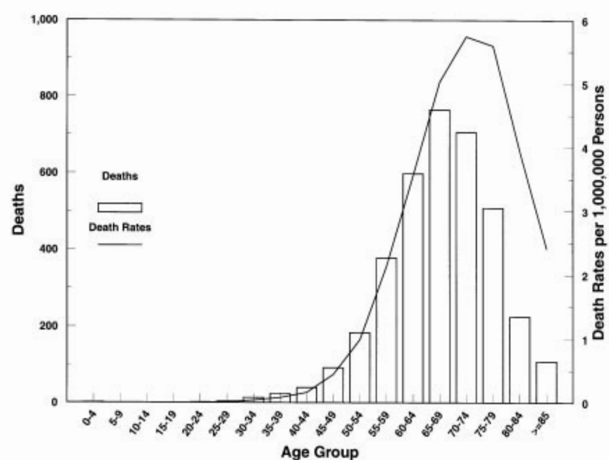

Figure 2. Creutzfeldt-Jakob disease deaths and death rates by age group. United States, 1979 through 1994.

Table 1: Creutzfeldt-Jakob disease deaths and death rates in the United States, 1979-1994

Characteristics		Number of Deaths	Death Rate
Sex[a]	Male	1714	1.04
	Female	1928	0.89
Race[a]	White	3466	1.01
	Black	125	0.37
	Other	51	0.67
Age group, years			
	0-4	1	<0.01
	5-9	0	0
	10-14	0	0
	15-19	0	0
	20-24	1	<0.01
	25-29	3	<0.01
	30-34	13	0.04
	35-39	23	0.08
	40-44	39	0.16
	45-49	91	0.45
	50-54	183	0.99
	55-59	378	2.14
	60-64	598	3.55
	65-69	765	5.03
	70-74	706	5.75
	75-79	508	5.60
	80-84	225	3.94
	85+	108	2.42
Region[b]			
	Northeast	926	1.06
	Midwest	1008	1.04
	South	1068	0.83
	West	640	0.89
U.S.[a]		3642	0.95

[a]Age-adjusted death rates per million persons.
[b]Age-sex-race-adjusted death rates per million persons.

average annual death rate was 1.15 per million persons (0.87 to 1.45 per million). Among persons <45 years of age, the annual death rate was consistently at or below 0.05 per million persons; the number of deaths among such young persons was zero in 1984 and eight in 1981 and 1993. Only five persons who died of CJD during the study period were younger than 30 years of age, and in any single year, there was no more than one such death. In the most recent 5-year period, 1990-1994, the only person with CJD in this young age group had received pituitary-derived human growth hormone.

None of the persons who died of CJD had hemophilia A, hemophilia B, thalassemia, or sickle cell disease mentioned on the death record. During 1979 through 1993, autopsies were performed on 907 persons who died of CJD-related causes (29.7% of those whose autopsy status was known). The proportion of autopsies ranged from 20.7% in 1993 to 37.8% in 1983. Autopsies of persons <45 years of age were indicated in 51.5% of the known deaths, while 30.2% and 29.0% were indicated in the 45- to 59-year-old and ≥60-year-old age groups, respectively.

During 1979 through 1994, the average annual age-adjusted death rate was 0.95 per million persons, which is consistent with published estimates of the crude incidence worldwide of one case per million persons (3,11). The addition of the 4 recent years of data to our previous report did not alter our initial findings (24). The previously reported racial differences in the U.S. CJD death rates were found again and should be further studied. In addition, no CJD-related deaths were found among persons with hemophilia A, hemophilia B, thalassemia, or sickle cell disease.

In 1994 and 1995, a cluster of 10 unusually young (median age at onset 28 years, range 16 to 39 years) CJD patients in the United Kingdom were reported with atypical clinical features (12). These features included behavioral change and dysesthesia when patients sought treatment, followed within weeks or months by a cerebellar syndrome, dementia, and myoclonus in the late stages, a duration of illness of at least 6 months, and electroencephalogram changes that were not diagnostic of CJD. These patients had a characteristic neuropathologic profile that consisted of severe spongiform change, neuronal loss, and astrocytosis in the basal ganglia and thalamus, with abundant kuru-type amyloid plaques surrounded by vacuoles in the cerebrum and cerebellum and PrP accumulation in high density shown by immunocytochemistry. These findings, coupled with the unusually young age of the patients, led to the conclusion that the clinicopathologic features constituted a new variant of CJD. A causal association with the BSE epizootic in British cattle was also hypothesized and recently supported by experimental evidence involving intracerebral inoculation of cyno-

Table 2: Creutzfeldt-Jakob disease deaths and age-specific death rates (per million persons) by sex and race, United States, 1979-1994

Age group (years)	U.S.		Sex				Race					
			male		female		white		black		other	
	deaths	rate	deaths	rate	deaths	rate	deaths	rate	deaths	rate	deaths	rate
0-44	80	0.03	44	0.03	36	0.03	72	0.03	6	0.02	2	0.02
45-59	652	1.15	303	1.11	349	1.19	605	1.23	31	0.54	16	0.98
60-69	1363	4.25	678	4.63	685	3.93	1307	4.58	39	1.38	17	2.58
70-79	1214	5.68	548	6.26	666	5.28	1167	6.05	37	2.15	10	2.87
80+	333	3.27	141	4.33	192	2.77	315	3.38	12	1.62	6	4.79

molgus macaques with brain tissue obtained from cattle with BSE (25).

To help assess the purported uniqueness and distribution of the newly described CJD variant, CDC intensified CJD surveillance activities to seek evidence for the presence of this variant in the United States (13). In analyzing the U.S. multiple cause-of-death data, we did not find any increase in the number of CJD deaths among persons <45 years of age; also, there were no recent CJD deaths in persons <30 years of age, except for the death of the recipient of pituitary-derived human growth hormone (in contrast, five of the eight patients originally reported in the United Kingdom died before the age of 30). To supplement these findings in the United States, CDC also conducted active surveillance in its four Emerging Infections Program sites and in the Metropolitan Atlanta Active Surveillance Program in Georgia (total 1993 population 16.3 million) (13,26); 92% to 100% of neuropathologists and neurologists were contacted, and information was obtained on their patients who died of CJD from 1991 through 1995. Medical records and neuropathologic reports of decedents <55 years of age were also sought for review. Consistent with national mortality data, nine (10%) of the case patients were <55 years of age, only one case patient was <45 years of age, and no case patient was <30 years of age. Review of clinical and neuropathologic records of the nine patients <55 years of age did not show any with the variant form of CJD.

The validity of multiple cause-of-death data is potentially a problem because of possible coding and reporting discrepancies, including misdiagnoses (24). However, past reports have indicated that approximately 80% of histologically confirmed CJD patients were identified through death certificate searches, using national death registries (27). Furthermore, the consistency of data from the active surveillance sites with the national multiple cause-of-death data supports the conclusion that annual review of multiple cause-of-death data provides an efficient and cost-effective method to monitor CJD incidence (24,27). Active follow-up of reported CJD deaths in persons <55 years of age has been initiated in the United States to improve the national surveillance of potential cases of the newly described CJD variant. Access to more current national mortality data makes this data source even more beneficial in monitoring CJD deaths in the United States. Although currently available CJD surveillance data do not provide evidence for the variant CJD in the United States, ongoing CJD surveillance in this country and elsewhere, especially in the United Kingdom, will be critical for ultimately determining the geographic distribution of this illness and its possible relationship to BSE.

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Rapid Increase of Resistance to Erythromycin and Clindamycin in *Streptococcus pyogenes* in Italy, 1993-1995

A survey of antibiotic resistance in *Streptococcus pyogenes* in Italy showed a sharp increase in erythromycin resistance. In 1993, the incidence of erythromycin-resistant strains was on average 5.1%, with marked variations by geographic area. Two years later, the incidence of these strains had registered a 1.5- to roughly 20-fold increase, with a mean value of 25.9%, exceeding 40% in three centers out of 13 and 30% in another four. For all the strains studied, normal levels of susceptibility to penicillin were reported.

Over the past few years, the increased frequency of infections caused by *Streptococcus pyogenes* (group A streptococcus [GAS]) and their sequelae has been reported in several parts of the world (1,2). Even though these reports may reflect an enhanced awareness of and interest in these possibly life-threatening infections on the part of the medical community (3), in at least some areas, an increase in severe infections over time has been documented (4,5).

Meanwhile, the increased clinical use of erythromycin and its derivatives, mostly in upper respiratory tract infections, has been related to an increased resistance of GAS to this antibiotic. Even though fewer than 5% of GAS isolates are reported as resistant to macrolide, lincosamide, and streptogramin (MLS) antibiotics (2,6), local exceptions have been reported, and widespread GAS resistance to erythromycin has so far been reported in Australia (17.6%) (7), Finland (20%) (8), the United Kingdom (22.8%) (9), Japan (60%) (10), and Taiwan (percentage not specified) (11).

Awareness of GAS resistance to erythromycin seems limited. Clinical microbiology laboratories rarely determine erythromycin susceptibility on a routine basis, and only recently have erythromycin breakpoints for streptococci other than *S. pneumoniae* been added in the latest National Committee for Clinical Laboratory Standards (NCCLS) document (12). Since the late 1980s, appreciable incidences of macrolide resistance in cases of pharyngotonsillitis and scarlet fever have also been reported from Italy (13-15).

Temporal trends in GAS resistance to erythromycin and clindamycin were systematically appraised on the basis of data collected over the last 3 years from 15 laboratories that participated in the Italian Surveillance Group for Antimicrobial Resistance (ISGAR). All the strains were isolated from throat swabs collected from symptomatic patients (mostly school-age outpatients) from 1993 through 1995. The number of isolates tested per year and the percentage of resistant ones are represented in the Figure.

GAS were identified by beta-hemolysis production on sheep or horse blood agar plates and by the presence of Lancefield group A antigen tested by commercial latex agglutination techniques (Streptex, Murex Diagnostics Ltd., Dartford, England; Phadebact, Boule Diagnostics AB, Huddinge, Sweden). The susceptibility tests used either the disk diffusion method (according to NCCLS performance standards [16,17]) or semiautomated microdilution tests (ATB, bioMérieux S.A., Marcy-l'Etoile, France; Sceptor, Becton Dickinson Diagnostic Instrument Systems, Sparks, Maryland), which were carried out as recommended by the respective manufacturers. The disk diffusion tests were read by manual measurement of the inhibition diameters or by a semi-automated system equipped with a video camera and image processing software that records the inhibition diameters (Bio-Videobact, Biokit S.A., Barcelona, Spain).

The data came from each automated reader device through data acquisition interfaces (created for that purpose by the respective manufacturers) and were subsequently translated through the *MyMic* software package (18) from individual proprietary formats into a common file format (Xbase) and transmitted to the reference center (Verona) on floppy disks or by electronic mail.

Test results were originally attributed to the different interpretive categories according to the NCCLS documents in force up untillate 1995 (16,17,19). After the data arrived in the reference center, they were reinterpreted on the basis of the new criteria for testing streptococcal species in the latest NCCLS document (12). The zone diameter criteria for resistant and susceptible isolates were 15 and 21, respectively, for erythromycin, and 15 and 19, respectively, for clindamycin. The equivalent minimum inhibitory concentration breakpoints (g/ml) for resistant and susceptible isolates were 4 and 0.5, respectively, for erythromycin, and 1 and 0.25, respectively, for clindamycin.

The survey showed a dramatic increase in the isolation of erythromycin-resistant strains of GAS (Figure). Both the rapid increase in the resistance rate in the areas involved and the subsequent involvement of other geographic sites caused considerable immediate concern since erythromycin had hitherto been effective against most isolates of this species and had been the drug of choice for treating streptococcal infections in patients allergic to penicillin.

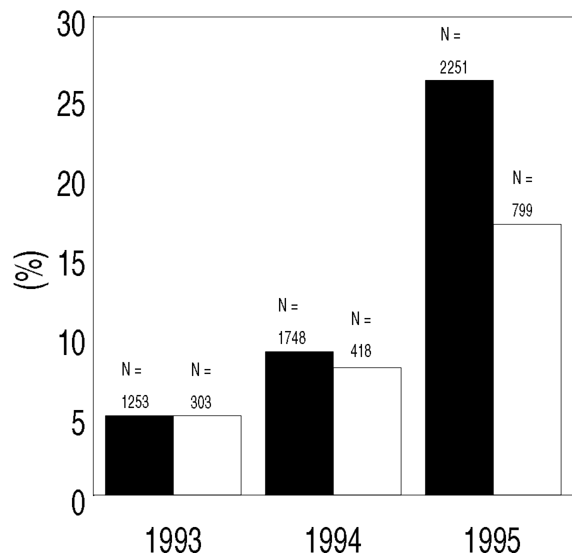

Figure. Number of GAS isolates tested per year and percentage of resistance to erythromycin and clindamycin, Italy, 1993-1995. Black bars represent erythromycin resistance and white bars, clindamycin resistance.

In 1993, the first year surveyed, the incidence of erythromycin-resistant strains was on average 5.1%, with marked variations according to geographic area, from 0% (all 19 strains from Pistoia) to 19.1% (Sassari, 18 strains out of 94). Two years later, in 1995, the incidence of resistant strains had registered a 1.5- to roughly 20-fold increase, with a mean value of 26.8%, from 13% (Palermo area, 3 strains out of 23) to 62% (Venice area, 31 strains out of 50). This incidence again showed geographic variations, but exceeded 40% in three centers out of 13 and 30% in another four. The Palermo area yielded the lowest rate of resistant strains, but its incidence of intermediate strains was exceptionally high (39.1%, 9 strains out of 23, versus a 5% to 10% rate in all other centers).

Resistance to clindamycin was more difficult to evaluate since this antibiotic was tested in only a few centers and on limited numbers of isolates. An increase in clindamycin resistance was recorded in five out of the seven centers that made this kind of data available. The highest rate of clindamycin resistance in 1995 was recorded in Verona (28.9%, 39 strains out of 135), while the lowest was recorded in Sassari (2.2%, one single strain out of 45). For all the strains studied, normal levels of susceptibility to penicillin and ampicillin were reported.

Molecular typing of erythromycin-resistant isolates was performed on strains isolated in the area of Verona in the first 2 months of 1995 (Table). Nine strains out of 14 were resistant to erythromycin, the 16-membered macrolide miokamycin, and the lincosamide clindamycin (the so-called MLS_B phenotype, which has reduced binding of MLS antibiotics to their shared 50S rRNA target site [9,20]); the other five strains were resistant to erythromycin but not to miokamycin or clindamycin (the so-called M phenotype, in which resistance is attributed to an efflux system [21]). All strains of the

Table. Antibiotic susceptibilities,* presence of the *ermAM* gene, pulse-field gel electrophoresis type, and T-protein pattern of *Streptococcus pyogenes* strains isolated in Verona

Strain	ERY†	MIO‡	CLI§	*ermAM*	PFGE type	T-protein pattern
VR1	R	S	S	-	A	8, 25
VR2	R	S	S	-	A	8, 25
VR3	R	S	S	-	A	25
VR4	R	S	S	-	A	8, 25
VR5	R	R	R	+	B	2, 28
VR6	R	R	R	+	C	5, 12, 27
VR7	R	S	S	-	D	4
VR8	R	R	R	+	C	12, 27
VR9	R	R	R	+	E	5, 12, 27
VR10	R	R	R	+	B	2
VR11	R	R	R	+	C	12, 27
VR13	R	R	R	+	C	5, 12, 27
VR14	R	R	R	+	C	12, 27
VR15	R	R	R	+	C	5, 12, 27

*Resistant, susceptible
†Erythromycin
‡Miokamycin
§Clindamycin

MLS_B phenotype carried the *ermAM* gene, which determines resistance to all MLS_B antibiotics, as investigated by polymerase chain reaction (PCR) performed on total DNA (22), by using the following oligonucleotide primers (sequence 5' to 3') derived from the published sequence of the gene (23):

MLS1: AGAAACCGATACCGTTTACGA

MLS2: GGTCAATCGAGAATATCGTCA

The PCR studies used the control strain *Streptococcus sanguis* V736, which carries the *ermAM* gene in plasmid pVA736 (24). In contrast, all strains of the M phenotype were negative to the PCR analysis.

Five different DNA restriction profiles (Table) were found by pulse-field gel electrophoresis (PFGE) of genomic DNA fragments digested with SmaI (Boehringer, Mannheim, Germany), with C predominant. Three profiles were found among MLS_B strains, and two among M strains; no profile was common to both MLS_B and M strains.

Serologic analysis with T-protein-specific antisera (Institute of Sera and Vaccines, Prague, Czech Republic) showed seven T-types. Within each PFGE type, similar but not identical T-types were identified. Again, no T-type was common to both MLS_B and M strains (Table).

Molecular typing results showed a great heterogeneity of erythromycin-resistant isolates in Verona: by combining the PFGE-type and the serotype, at least eight different clones could be identified.

The polyclonality of Verona isolates and the largely different rates of erythromycin- and clindamycin-resistance in most centers seem to confirm that the M phenotype of resistance has become fairly frequent (21,25). The diffusion of GAS strains resistant to erythromycin and susceptible to miokamycin and clindamycin implies that testing of erythromycin alone is no longer sufficient to assess the susceptibility of GAS to all MLS antibiotics, contrary to the claims made by Leclerq and Courvalin (26).

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Assessing the Costs and Benefits of an Oral Vaccine for Raccoon Rabies: A Possible Model

Any cost-benefit analysis of the use of an oral vaccine to control raccoon rabies should include calculating both costs and benefits in terms of $/unit area. Further, cost savings must be adjusted to match the stages of an epizootic: pre-epizootic, epizootic, and post-epizootic. A generic model, which can be adapted to different sites, illustrates the use of threshold analysis to link distribution costs, cost savings, bait density, and vaccine price. Initial results indicate the need to lower the cost of the vaccine, continue research to determine optimal bait densities, and examine distribution plans that do not require continued protection of areas in which raccoon rabies was eliminated through previous vaccination programs.

Over the past 30 years, reported cases of animal rabies in the United States have increased, from fewer than 5,000 per year in the early 1960s to almost 10,000 per year in the mid-1990s (1,2). Most of the increase is attributable to the spread of raccoon rabies from Florida to the northeastern states (1). The impact of this epizootic has been considerable. For example, hospitals in Connecticut reported a 2,000% increase in the annual number of postexposure rabies prophylaxis treatments after raccoon rabies was first detected in the state in 1991 (3). Postexposure prophylaxis costs $2,000 to $3,000, or more, per person (3,4). In addition, a raccoon rabies epizootic increases other costs, including those associated with animal control and laboratory diagnosis (5; Tysmans, J., Costs of rabies in Cumberland County, NC: 1993 and 7/1/94-6/30/95 [MPH thesis], Chapel Hill (NC): Dept. of Health Policy and Admin., Univ. of North Carolina, Chapel Hill, 1996).

Oral vaccines offer a potential solution to rabies in wildlife populations (6,7). The spread of rabies in foxes in an area of Switzerland was halted when an oral vaccine was used to vaccinate 60% of the fox population (8). An oral vaccine to control raccoon rabies (9) is undergoing evaluation in New York and Massachusetts (10,11). Also, large-scale programs are using an oral vaccine against rabies in coyote and gray fox populations in Texas (12).

Uhaa et al. (5) examined the economics of using an oral vaccine in Hunterdon and Warren Counties, New Jersey. However, their study was limited to a 5-year period and only to costs borne by the two counties. To aid the collection of data needed to conduct a cost-benefit analysis of the use of an oral vaccine in large areas (e.g., an entire state) and over prolonged periods (e.g., 30 years), this article outlines some basic premises required for such an analysis and a generic cost-benefit model, which can be modified to fit specific geographic areas. Since many of the necessary data for the model are still being collected, the data used in the model are illustrative rather than definitive.

Any evaluation of the costs and benefits of using an oral vaccine to protect wild raccoon populations must be based on three premises: 1) Oral vaccine is distributed on a per unit area basis, calculated by using estimates of raccoon population density (i.e., a predetermined number of baits per raccoon times the number of raccoons per unit area). Costs of an oral vaccine program, therefore, are expressed in $/unit area. 2) To compare costs and benefits, all benefits that might accrue because of oral vaccine use must also be converted into $/unit area. 3) The cost savings (benefits) must be adjusted to match three broad, time-based categories describing different stages of an epizootic: pre-epizootic, epizootic, and post-epizootic (rabies still present in the population). The greatest cost savings is likely to occur during the epizootic since this stage is typically associated with the highest cost of raccoon rabies control (5; Tysmans, J., MPH thesis, 1996). In New Hampshire, for example, the annual number of requests for Animal Damage Control services involving raccoons

went from 250 per year in pre-epizootic years (1988-1991), to 667 during an epizootic year (1993), and then declined to 165 in the post-epizootic years (13).

The Model

A cost-benefit model with a societal perspective was constructed (on Excel 5.0, Microsoft, Inc.) by using a discount rate of 3% (14) over 30 years to provide a Net Present Value (NPV).[1] The costs of a raccoon rabies vaccination program include purchasing and distributing the oral vaccine in bait form. The benefits of such a program are the direct and indirect cost savings obtained by successfully halting or preventing a raccoon rabies epizootic and subsequent enzooticity. Since the vaccine technology is new, and no data exist on how the technology might change over the 30 years, it is assumed that the real costs and benefits are constant over the 30 years. Parameters and values used in the model are presented in Table 1.

Table 1: Illustrative data used to examine the costs and benefits of controlling raccoon rabies with an oral vaccine

Item	Baseline case	Values used for sensitivity analyses
Discount rate	3%	0%, 5%
Timeline	30 years	-
Oral vaccine density	250 units/sq. mi	100-300 units/sq. mi
Raccoon density	50/sq. mi	threshold analysis
Baits required/raccoon	5	threshold analysis
Cost of oral vaccine bait	$1.50/unit	threshold analysis
Cost of distribution	$100/sq. mi	$260[a]
Benefits: Cost savings		
During epizootic years[b]	$1.52/person/yr	$2.61/person/year[b]
During post-epizootic years[b]	$0.30/person/yr	-
Density human population	103/sq. mi	-
Distribution costs:cost savings		
Ratio of max. $/sq. mi	1:1.57	1:1.03
Sensitivity analysis		
Cost of pet vaccination	-	$16/pet
Extra vaccinations: epizootic	-	11/sq. mi
Extra vaccinations: post-epizootic	-	2.75/sq. mi

[a]Source: New Jersey data (1).
[b]On the basis of the New Jersey data (1), four categories of costs contribute to the cost-savings: animal control, laboratory diagnoses, educational activities, and human pre- and post-exposure treatments.

Two Distribution Scenarios

Since what constitutes an "ideal" plan for large-scale oral vaccine bait distribution is not known, two scenarios were constructed. The area covered and the rate of barrier movement were chosen merely for illustration. Once actual proposals are made, the area covered and the time line may be modified to suit specific geographic areas. The first scenario assumes that bait is distributed in ever-expanding circles, starting with an area covered by a radius of 20 mi (area: 1,257 sq. mi). This area would be baited for 2 years. In the third year, the radius of the circle would be increased to 25 mi. The area between the 20-mi and 25-mi radius would then be baited for 2 years, at which time the radius defining the outer boundary would be increased by another 5 mi. This process would continue for 20 years, at the end of which the radius of the circle would be 65 mi, encompassing 13,273 sq. mi, which is approximately equal to one-third the area of New York State. At the end of the 20 years, a 10-year period would follow during which a 5-mi wide barrier zone would be baited every year. The yearly and cumulative total areas baited are shown in Figure 1. The barrier zone is necessary because the described plan cannot guarantee elimination, and the area covered by a vaccine program is vulnerable to the reintroduction of rabid animals. The second scenario considers the entire 13,273 sq. mi baited during the first 2 years, followed by 28 years of baiting a 5-mi-wide barrier zone.

Costs: Bait

In an economic analysis, opportunity costs should be used (15). However, the vaccine is still experimental,

[1]A cost-benefit analysis uses the following formula (15):

$$NPV = \sum_{t=0}^{t=n} \frac{(Benefits-Costs)_t}{(1-r)^t}$$

where: t = year, from t= 0, . . ., n; r = discount rate.
Threshold costs were calculated by altering the vaccine cost so that benefits minus costs, when discounted and summed up over time, had an NPV of $0.

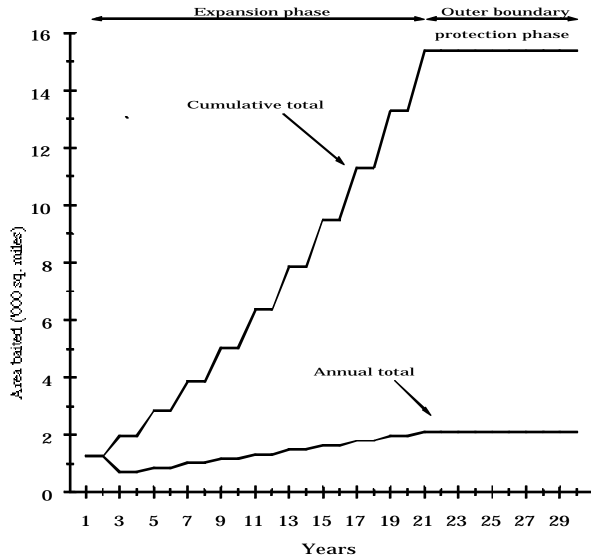

Figure 1. Annual and cumulative total areas baited with oral vaccine using an expanding circle scenario
Notes: Initial circle has a radius of 20 mi, expanded out by 5 mi every 2 years. After 20 years, radius is 65 mi. Thereafter, a 5-mi wide boundary is maintained around the circle for the next 10 years.

and data concerning discount prices could not be obtained. Therefore, the cost of oral vaccine in bait form was calculated at $1.50 per unit. This is the cost reported for trials of the oral vaccine in New York State (C. Hanlon, unpublished report, New York State Department of Health, 1995). In lieu of accurate opportunity costs, a sensitivity analysis evaluates the threshold price (i.e., NPV = $0) of the vaccine. This threshold value can then be compared with actual opportunity costs when more data become available.

Costs: Bait Density

The bait density required to successfully halt or stop raccoon rabies has not been definitively determined (10,11,16,17). Raccoon density can vary enormously with local ecology and weather (16,18-20). On the basis of densities currently under trial, for each of the two scenarios it is assumed that bait is distributed at 250 units per sq. mi, with an assumed raccoon density of approximately 50 per sq. mi (i.e., five baits per raccoon). Bait density is also the subject of sensitivity analysis.

Costs: Distribution

Distribution costs for oral rabies vaccine have yet to be comprehensively documented. In current trials in New York and Massachusetts, bait was dropped by people walking and from aircraft, helicopters, and cars, depending on the terrain and available resources. The use of some vehicles has been donated, as have large amounts of personnel time; therefore, their cost is difficult to determine (an economic analysis must contain such costs). To illustrate the generic model, total distribution costs were assumed to be $100 per sq. mi. This cost is also the subject of a sensitivity analysis.

Benefits: Cost-savings

In the model described here, the benefits from using the oral vaccine are assumed to be the cost savings derived directly from preventing a raccoon-related rabies epizootic and post-epizootic. These cost savings are, therefore, the difference between the pre-epizootic costs of controlling rabies and the costs incurred during the epizootic and post-epizootic periods. It was assumed that a rabies epizootic lasts 2 years (1) and that the years following were defined as post-epizootic. The direct costs associated with the control of animal rabies have been listed (5) and can be broken down into four categories. These categories, with the estimated proportional contribution to the entire cost savings, are animal control costs (41%); laboratory diagnosis costs (13%); education and administration costs (9%); and human costs, such as pre- and post-exposure prophylaxis treatments (37%). Just as opportunity costs should be used for input costs, so potential cost savings should be valued at costs and not charges (15). For example, the average operating cost-to-charge ratio for urban hospitals in New York is 0.635:1 (15).

To illustrate the generic model, data from New York (Harris-Valente et al., unpublished report, 1995) were used to provide a

preliminary estimate of cost savings ($1.52 per person), which will occur during the epizootic phase. The same data source yielded a figure of $0.30 per person during the post-epizootic phase. These savings per person are converted to savings per unit area as follows: On the basis of population estimates (21) from 53 counties in New York State, the median population density was estimated at 103 persons per sq. mi (25th percentile = 67; 75th = 204). Thus, for the areas baited, the savings were calculated at $156.56 per sq. mi for the first 2 epizootic years ($1.52 per person x 103 persons per sq. mi), and $30.90 per sq. mi for the post-epizootic years ($0.30 per person x 103 persons per sq. mi). Cost-savings data from New Jersey (5) are used in the sensitivity analysis.

Distribution Costs: Cost-savings Ratio

The relative importance of distribution costs (excluding costs of vaccine) with respect to the amount of cost savings can be examined by constructing a distribution costs:cost savings ratio on a per-unit-area basis. Thus, the baseline case of $100 per sq. mi distribution costs and maximum cost savings during epizootic years of $156.56 per sq. mi ($1.52 per person x 103 persons per sq. mi) gives a ratio of 1:1.57. This ratio can be altered for sensitivity analyses.

Sensitivity Analyses: Animal Vaccinations

The proportion of household pets and domesticated animals vaccinated before an epizootic can be quite low. For example, in Cumberland County, North Carolina, it was estimated that only 20% of dogs and cats were vaccinated against rabies before the epizootic (Tysmans, J., MPH thesis, 1996). During an epizootic, public health officials often encourage or enforce pet vaccinations, and vaccination rates often increase (5; Tysmans, J., MPH thesis, 1996). Successful elimination of raccoon rabies from an area could be considered a good reason for allowing vaccination rates to remain at lower levels, thus avoiding the costs associated with increased vaccinations. However debatable this proposition is, the economic impact of considering reduced pet vaccinations as a benefit is demonstrated by rerunning the two distribution scenarios and including the potential cost savings associated with reduced pet vaccination levels.

An example of the savings estimate follows: In 1991, there were 52.5 million dogs and 57 million cats in the United States (22), an average of 0.41 pets per person. During pre-epizootic periods, 0.14 pets per person (32% of average pets per person) are vaccinated each year against rabies (5). In the epizootic year this rate increased to 0.24 pets per person (55% of average pets per person) (5). Thus, during an epizootic period, there is an increase of approximately 11 extra pet vaccinations per sq. mi (0.24 - 0.14 pets per person x 103 persons per sq. mi). Pet vaccinations are costed at $16 per pet (Tysmans, J., MPH thesis, 1996). When baseline figures are used for distribution costs and cost savings (Table 1), a total distribution cost:cost savings ratio of 1:3.33 is calculated. No data are available concerning post-epizootic pet vaccination rates, and it is arbitrarily assumed that there are 2.75 extra pet vaccinations in post-epizootic stages (25% of the epizootic increase).

Additional Sensitivity Analyses

The impact of two alternate discount rates (0% and 5%) was evaluated, and the impact of higher distribution costs was assessed. In New Jersey (5), the costs of distribution were calculated at approximately $260 per sq. mi, while cost savings during the epizootic period were calculated at $2.60 per person (without reduced pet vaccinations considered as a benefit). This gives a distribution costs:cost savings ratio of 1:1.03 (compared with 1:1.57 in the baseline scenario) increasing the costs of distribution relative to cost-savings. The impact of removing the cost of having to continuously bait a barrier zone around areas covered by previous vaccination programs was then considered. In the first distribution scenario, costs associated with baiting a barrier for years 21 through 30 were removed from the model. In the second distribution scenario, it was assumed that the barrier need only be maintained for 2 years after the first 2 years of baiting (i.e., no baiting for years 5 through 30). The final sensitivity analysis used the first distribu-

tion scenario to examine the threshold price of oral vaccine for levels of bait density ranging from 100 sq. mi to 300 sq. mi, and using three different distribution costs:cost savings ratios. Threshold price was determined by altering the price of the vaccine until the NPV for a given bait density equaled $0 (see footnote) The first two price ratios were 1:1.57 (baseline, Table 1) and 1:1.96. The latter represents an arbitrary increase of +25% in cost-savings relative to distribution costs. The last price ratio used for the threshold analyses was constructed by assuming that reduced rates of pet vaccination resulted in additional cost-savings of $176 per sq. mi ($16 per pet x 11 extra vaccinations per sq. mi), giving a ratio of 1:3.33.

Results: Baseline Case

The first distribution scenario (expanding circles) provides smaller losses than the second distribution scenario (baiting the entire area in 2 years, followed by 28 years of baiting a 5-mi boundary) (Table 2). Considering the avoidance of increased pet rabies vaccinations as a benefit improves the NPVs of both scenarios and changes the NPV of the expanding circles distribution scenario from negative to positive. For the baseline case, using different discount rates changes the absolute values but not the signs.

Results: Sensitivity Analysis

The smaller distribution costs:cost saving ratio of 1:1.03 increased the losses (larger negative NPVs) and reduced the sole positive NPV to +$0.9 million (3% discount rate). Eliminating the need for baiting a protective barrier saves $4.8 million in the expanding circles scenario ($7.9-$3.1 million, 3% discount rate) and $14.8 million in the entire area scenario ($13.7 million + $1.1 million, 3% discount rate) (Table 2). Figure 2 shows the threshold prices for the oral

Table 2. Net present values (NPV) of different distribution scenarios for using an oral vaccine to control raccoon rabies

Baiting scenario	NPV without pet vaccination ($ millions) Discount rate			NPV with pet vaccinations[a] ($ millions) Discount rate		
	0%	3%	5%	0%	3%	5%
Expanding circles[b]	-10.2	-6.2	-4.7	+5.5	+3.1	+2.2
Entire area at once[b]	-21.3	-15.7	-13.3	-0.5	-1.1	-1.3
Sensitivity analyses						
Reduced distribution:savings[c]						
Expanding circles	-14.3	-8.5	-6.3	+1.4	+0.9	+0.6
Entire area done at once	-30.8	-21.9	-18.2	-12.5	-8.9	-7.3
No baiting of boundary						
Expanding circles	-0.7	-1.8	-2.1	+14.1	+7.9	+4.5
Entire area at once	+2.9	-0.9	-2.3	+23.7	+13.7	+9.7

[a]Cost savings (benefits) of 11 pets/sq. mi during an epizootic period, and 2.75 pets/sq. mi during the post-epizootic period, both at $16/pet.
[b]Expanding circles assumes start with 20-mi radius, expanding by 5 mi every 2 years for 20 years, and then baiting a 5-mi wide boundary for next 10 years. Entire area assumes baiting 65-mi radius for 2 years, followed by 28 years of baiting 5-mi wide radius.
[c]Ratio of distribution costs:cost savings is 1:1.03, based on New Jersey data (1). In baseline scenario, ratio is 1:1.57.

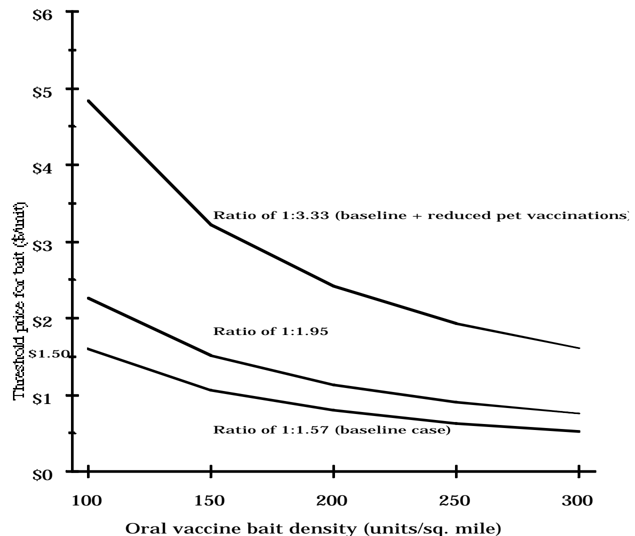

Figure 2. Threshold prices for the oral vaccine at different levels of bait density and three distribution costs:cost-savings ratios for the expanding circles scenario
Notes: Threshold price occurs at the point that the net present value of a vaccination program = $0. The distribution costs:cost-savings ratios are calculated on a per unit area basis, using the maximum cost-savings figures (i.e., savings resulting from avoiding an epizootic).

vaccine in the expanding circles scenario, given different levels of bait density used and three different distribution costs:cost-savings ratios. At the baseline distribution costs:cost-savings ratio of 1:1.57, and at bait density of 250 baits per sq. mi, the threshold price for the oral vaccine is $0.63 per unit. When the distribution costs:cost-savings ratio is increased to 1:1.96, the threshold price for 250 baits per sq. mi is $0.91 per unit (+44% from baseline threshold). When reduced pet vaccinations are considered a benefit and the distribution costs:cost-savings ratio increases to 1:3.33, the threshold prices are always greater than the $1.50 cost of the vaccine (Figure 2).

Many of the data used in the generic model are assumed for illustrative purposes. The results presented here, therefore, should not be used to decide if a large-scale oral vaccine program is economically worthwhile. These results identify at least two priorities: the need to continue research that will determine the optimal level of bait density and the importance of better defining the distribution costs:cost-savings ratio. Both of these priorities are the focus of research efforts in New York and Massachusetts.

The results of the generic model (Table 2) clearly identify the importance of pet vaccinations when considering the costs and benefits of a raccoon vaccination program. It can be argued that avoidance of increased pet vaccinations is a benefit only if the laws requiring small animal vaccinations were passed solely as a result of the raccoon epizootic. It could be counterargued that, because past rabies control programs were so successful, the current laws are unnecessary and an economic burden to society. However, this would suggest that public health officials would accept a repeal of mandatory vaccination requirements except under extenuating circumstances (e.g., a rabies epizootic). Further, there are other sources of rabies exposure (e.g., bats), and the risk for pet exposure from these will most likely not be altered by a raccoon rabies vaccination program.

In evaluating an actual proposed rabies vaccine program, the use of expanding circles, although more economical than baiting entire areas at once (Table 2), may be unrealistic because of natural terrain or political boundaries. The sensitivity analyses demonstrated the costs of having to maintain a barrier (Table 2). The only way to avoid having to maintain a barrier over a long time may be to conduct a large-scale program, involving many contiguous states. However, such a program would pose a financial burden on governments and, at its conclusion, would still not stop rabies from being reintroduced in a manner similar to that which caused the current epizootic.

In addition to the issues related to distribution, two important implicit assumptions in the model affect costs: 1) the vaccine is 100% effective in stopping rabies in baited areas in 2 years; and, 2) rabies is not reintroduced. Removal of these assumptions would effectively increase the cost of the program by requiring increased bait densities, longer baiting periods, or both. Any of these situations would move the optimal bait density in Figure 2 (X-axis) to the right, further reducing the threshold value of the vaccine. Obviously, increased costs would merely increase the negative value of the NPVs presented in Table 2. The threshold analysis used here (Figure 2) provides a basis, even when some data are uncertain, for discussing discount pricing of the vaccine with the manufacturers when bulk purchases for large-scale programs are being planned.

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Traditional Healers and Global Surveillance Strategies for Emerging Diseases: Closing the Gap

A recent position paper by the Centers for Disease Control and Prevention (CDC) stresses that surveillance is critical to an effective defense against new and reemerging infectious diseases and indicates that current international monitoring of such diseases is fragmentary and inadequate (1). Other major studies have also recorded the weaknesses in the present disease reporting system (2-4).

The concept of "global surveillance" implies the coordination of existing networks as well as the addition of state-of-the-art electronic networks to ensure close monitoring of and rapid response to outbreaks, even in the most remote locations (1,2,5-8). Plans for strengthening current surveillance efforts include a global consortium with specialists in epidemiology and infectious diseases working in close collaboration with international agencies, ministries of health, universities, and research laboratories (1,2,6,9-11). Existing programs at the World Health Organization, CDC, the Pan American Health Organization, and elsewhere will be reconfigured to work as a more cohesive system (1). Secure networks will be developed for 1) the transmission of sensitive information; 2) automatic reporting from physicians' offices, hospitals, and laboratories; and 3) the integration of existing and planned information systems. The field application of computer technology, satellite imagery that allows geographically oriented information to be visually and analytically linked to images of the environment, and the development of new statistical and mathematical modeling methods are under discussion (1,3,12).

As medical anthropologists, we note the absence in current plans for global reporting systems of "traditional" or non-Western health care providers, who in communities worldwide are usually the first, and often the only, health specialists to see patients with new or reemerging diseases. These local health specialists, called traditional healers, may have a role to play in the early identification of new or reemerging diseases and could assist in coordinating responses to outbreaks and providing public health education at the local or regional levels.

Most people around the world have little access to modern medical systems (13-15). Even though immunizations and antibiotics increasingly find their way into indigenous systems, healers, midwives, bone setters, herbalists, and other traditional health experts provide most or all medical care. The more remote, indigent, or traditional the population, the greater the likelihood that it will have little access to modern medical care (13,16). If such care is sought, it will be only as a last resort, should traditional healers prove unable to address the illnesses (16) . In many communities, modern medicine is not perceived as better than traditional healing, and it is often more costly. Distance from modern medical resources is another barrier. Medical care that is not sensitive to cultural differences as well as the belief that some types of diseases are not treatable by modern medicine are also prevalent. These beliefs are particularly common in developing countries; however, traditional healers also practice in many ethnic and minority communities in industrialized societies throughout North America, Europe, and Australia (17-19).

A primary dependence on traditional healers continues in areas that, until recently, were considered largely untouched by modern development. It is in just such areas that much of the recent economic development has triggered rapid ecologic change. These once sparsely populated areas, now being pulled into the global economic sphere through logging, mining, and agriculture, are precisely the areas where it is anticipated that many new infectious diseases will originate, as increasing populations come in contact with previously undisturbed vectors of infectious diseases. In such areas, traditional healers are often in a unique position to identify new and reemerging diseases. Whatever their specialty, traditional healers are 1) familiar with diseases commonly found locally; 2) aware of an increase or decrease in the incidence of such diseases in their patient population; 3) among the very first to see

cases of new diseases; and 4) cognizant of the recurrence of a disease they have not seen in some time. If traditional healers are not tied into the global reporting network in a systematic and effective manner, their knowledge of new or reemerging disease information may reach the outside world late or in many cases, not at all. Traditional healers differ not only from country to country, but often from region to region and from one ethnic or minority group to the next. An adequate surveillance system must ensure that in each instance the most appropriate traditional healers are included in some type of timely warning system.

Including traditional healers into a global system does not mean that scientists and clinicians must agree with indigenous explanations of the causes or treatments of infectious diseases. Nor does it require that traditional healers accept modern assumptions about the causes, presence, or treatment of such diseases. However, a complete surveillance system does require that participants cooperate and maintain professional respect and courtesy. The goal is a surveillance system that is sensitive to cultural differences and in which new or unusual medical events can be reported quickly and accurately from the traditional healer to the local medical officials in the hospital or laboratory linked to the global surveillance system.

In recommending the inclusion of traditional healers in a global surveillance network, we do not seek to minimize the differences, or the animosity, between these healers and modern medical practitioners (14). Moreover, the relevant strengths and weakness of traditional healing are not the issue here. Critical time, however, may be lost unless all resources are tied into a disease reporting system.

Lines of communication must be established between traditional healers and local health care systems that serve as the “up-links” to the regional, national, and international early warning systems. A system in which traditional healers know whom to contact and how to establish contact quickly is essential. Traditional healers must be taught why, what, when, and how to report unusual symptoms in their patients to local officials. Training for traditional healers must include explaining, (in terms that are culturally relevant to their understanding of illness and health) why scientists outside their communities need timely medical information from their local practices. What to report is of equal concern. Healers must be briefed in what is reportable. A checklist of specific symptoms, such as new or unusual fevers, rashes, or lesions could be developed for reference. Such a checklist could also include questions on the apparent mode of transmission of the disease, (whether it is appearing in members of the same household; in specific parts of a local area, such as households that share a common water source or are located near a forested area; or in sex partners). The development and circulation of a pictorial reference guide of diseases found in an area might facilitate communication between healers and local officials. Specific guidelines should ensure that reporting is done quickly. Finally, a clear and workable reporting system, with specific information about whom to contact at the local level should be established.

An effective surveillance program must include a systematic educational component for local health officials, with specific discussion about the need to include traditional healers, what information these healers are asked to provide, and how this information, once conveyed to local health officials, must be transmitted to the regional hospital, universities, and ministries of health quickly and effectively. Because many local health officials have heavy demands placed on their time, the more straightforward this transmission link is made, the better for all concerned. The local health official is the key “up-link” between the remote field and the regional or national surveillance centers where a more careful and systematic evaluation of the new or reemerging infectious disease should begin.

Finally, training for both healers and those to whom they report must be comprehensive, and its effectiveness must be evaluated often. A communications bridge must be established and maintained if global warning is to be truly effective.

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Widespread Foodborne Cyclosporiasis Outbreaks Present Major Challenges

To the Editor: The organism now named *Cyclospora cayetanensis* was first recognized as a cause of human illness in 1977. For several years, as its taxonomy was deliberated, it was referred to as "cyanobacterium-, or coccidia-like bodies" (CLBs), or considered to be blue-green algae. In 1993, *C. cayetanensis* was reported to be a protozoan parasite, a coccidian member of the family *Eimeriidae*. To be infectious, the spherical, chlorine-resistant oocyst (8µm to 10µm) found in the feces of infected persons must sporulate in the environment, a process that, depending on conditions, takes at least several days. Upon examination by ultraviolet microscopy, *Cyclospora* oocysts autofluoresce and upon staining, they are variably acid-fast. The incubation period between infection and onset of symptoms averages approximately 1 week. *Cyclospora* infects the small intestine and usually causes watery diarrhea, with frequent stools. It can also cause loss of appetite, weight loss, stomach cramps, nausea, vomiting, fatigue, increased flatus, and low-grade fever. The duration of symptoms is often several weeks, and remitting courses spanning 1 to 2 months, with several relapses, have been reported. Cyclosporiasis is effectively treated with trimethoprim/sulfamethoxazole; however, therapy for patients who are sulfa-intolerant has not been identified.

Before 1996, only three outbreaks of *Cyclospora* infection had been reported in the United States. However, between May 1 and mid-July 1996 almost 1,000 laboratory-confirmed cases were reported to the Centers for Disease Control and Prevention (CDC). A few hospitalizations (<20) were reported, but no *Cyclospora*-related deaths were confirmed. These infections occurred in at least 15 states and Canadian provinces and the District of Columbia. Investigations of approximately 50 event-related outbreaks of diarrheal illness due to *C. cayetanensis*, as well as case-control studies of sporadic, laboratory-confirmed cases by several states, now clearly implicate consumption of fresh raspberries. Complete, high confidence level trace-backs of raspberry shipments related to more than 25 of the events have indicated that the raspberries responsible were imported from Guatemala between early May and mid-June 1996.

On June 17, 1996, CDC began hosting thrice-weekly conference calls to ensure close coordination among CDC, the U.S. Food and Drug Administration (FDA), and the many state and local health agencies investigating these widespread outbreaks and cases. The conference calls provided coordination in tracking and discussing this multifocal problem. In addition, on July 17, 1996, in Atlanta, CDC and FDA held a 1-day work-shop entitled "*cyclospora* - 1996," which was attended by more than 80 persons representing CDC, FDA, the U.S. Department of Agriculture, 16 states, one province, five cities, five universities, the Council of State and Territorial Epidemiologists, the Association of State and Territorial Public Health Laboratory Directors, the Pan American Health Organization, and the government of Canada. The participants in the investigations of *Cyclospora* shared the knowledge gained through their individual investigations of this multistate, multicountry outbreak. The goals of the workshop were to begin to formulate effective prevention strategies for *Cyclospora* infection, to discuss the strength of the evidence implicating Guatemalan raspberries, and to formulate research needs. The workshop allowed for discussions about the epidemiologic and trace-back studies conducted and speculation about where and how the raspberries became contaminated. Representatives from Texas, South Carolina, New York City, Florida, and New Jersey presented data from their respective case-control and cohort studies; CDC representatives provided an overview of the outbreaks and focused on multiple, specific trace-backs from more than 20 of the event-related outbreaks. FDA representatives discussed their roles and regulatory authority in foodborne investigations.

The workshop also addressed the array of scientific challenges concerning *C. cayetanensis*, such as clinical diagnostic techniques, protocols for detection of the organism on produce, and the basic biology of

this protozoon. We do not know the infectious dose, the proportion of infected persons who have diarrhea, the proportion of diarrheal illness caused in various settings by *Cyclospora*, the existence of animal reservoirs, or the viability of the organism in different environmental conditions. It can be transmitted by water and food, and its transmission is seasonal (late spring/early summer), at least where it has been studied (primarily temperate, seasonal climates).

The poor sensitivity and specificity of current methods for diagnosis and detection of *Cyclospora* were discussed. A photomicrographic demonstration convinced the participants that currently the foremost requirement for accurate clinical diagnosis is a skilled microscopist. The status of polymerase chain reaction technologies for detection and diagnosis of *Cyclospora* was presented and discussed, including the inhibitory aspects of berry juices and the difficulty in oocyst recoveries from spiked berry samples. Participants stated the need for a bank of *Cyclospora* organisms and their DNA (molecular libraries) from different locations and outbreaks. Currently, we may not be able to take full advantage of such epidemiologically well-documented specimens; however, the technologies and tools will continue to advance, and these specimens need to be centrally banked now, to be made available when the tools are up to the task. An animal model needs to be developed, or at least explored. The uses for such a model include providing material (oocysts and other life-cycle stages) for reagent development (monoclonal antibodies) to allow studies of the organisms, the disease, immune responses, and potential environmental transmission. Such a model will facilitate the development of prevention and treatment strategies.

Ongoing investigations into how the raspberries were contaminated were discussed. The lack of sensitive and reproducible detection assays for *Cyclospora*, which does not replicate outside the human host, remains the major stumbling block in providing proof of contamination of suspected transmission vehicles. Studies were too preliminary for conclusions. Both the government of Guatemala and the producer/exporter associations were most helpful in the investigations and need to remain involved if we are to better understand what occurred in May and June of this year.

Throughout the workshop, a wider issue than the current situation with *Cyclospora* was discussed: the management of the emerging problem of widespread multistate and international foodborne outbreaks of both infectious and toxic nature. Such outbreaks are increasing and can be expected to worsen as the world moves toward a global food economy. What contaminates a particular food item on a farm, in a herd or crop, at a processing shed, or from a handler, can now cause widely distributed outbreaks, continents away, in a day. More coordination is needed on several fronts in the management of such outbreaks: 1) the development of a structured process for integration and coordination of epidemiologic studies; 2) more aggressive laboratory diagnostic training related to poorly recognized or understood emerging infections; 3) better coordination of press releases related to multistate outbreaks; 4) better understanding and clarification of the legal roles and responsibilities of federal, state, and local agencies; 5) and earlier involvement of industrial partners at all levels, including growing/processing, exporting/importing, transporting, and wholesale/retail sales. Because these types of outbreaks are likely to become international this aspect must be addressed in considering appropriate approaches.

The *Cyclospora* outbreaks of May and June 1996 underlined that without the ability to culture and grow the organisms, without a supply of the organism to develop expedient assays, without an established coordinating body to expedite agreed-upon means for dissemination of information, we, as public health officials, are called upon to provide guidance without the benefit of all the appropriate knowledge. The workshop engendered interchange and discussion on critical issues concerning what is known and unknown about *Cyclospora* and the outbreaks of cyclosporiasis during May and June 1996. The workshop also provided a forum in which it became apparent that public health officials must launch a committed effort to develop an established,

coordinating system among agencies at all levels and deal with the threat of widespread, multistate/international foodborne outbreaks caused by infectious or toxic agents.

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Identification of Cyclospora in Poultry

To the Editor: Human infection with the parasitic protozoa, *Cyclospora*, was first described in 1979 (1), and the organism was only recently categorized as an important gastrointestinal parasite. A single species, *Cyclospora cayetanensis*, has been described in humans (2), while most species in the genus *Cyclospora* have been described only in reptiles and rodents (3). The consumption of undercooked meat and exposure to contaminated water have been considered possible sources of human infection with *C. cayetanensis* (1,4). Coccidia were detected in drinking water in Nepal (5), and the parasite was identified in an animal species (one duck in Peru, by Zerpa et al. [6]) different from those in which it was described earlier. To determine whether a domestic animal is either a host or a reservoir for *C. cayetanensis*, we first examined feces from cats, which are hosts and reservoirs of *Toxoplasma gondii*, a coccidia causing human illness, but got negative results. Because *Cyclospora* were recently phylogenetically linked to *Eimeria mitis* and *E. tenella* (7), coccidial parasites of chickens, we investigated the presence of *Cyclospora* in poultry.

We pooled feces from approximately 600 4- to 6-week-old chickens from a poultry farm near Monterrey, Mexico, and extracted feces from the caecum of 50 6- to 8-week-old chickens from a poultry market at that location. By Percoll discontinuous-gradient centrifugation (Medina-De la Garza et al., submitted), both fecal pools were positive for coccidia, mainly *Eimeria* species and what we regarded as *C. cayetanensis* oocysts. Presence of *Cyclospora* was confirmed by 1) characteristic morphology and size (8µm to 10µm), 2) positive staining with Kinyoun's acid-fast stain, 3) positive autofluorescence under ultraviolet light, and 4) sporulation of oocysts with formation of sporocysts after a 10-day incubation. All these are diagnostic features of *C. cayetanensis* (8) and to our knowledge are not described for any known poultry coccidia.

On the basis of these findings, we suggest that poultry may serve as a possible source for human infection with *Cyclospora*. Consumption of chicken has been reported in one infected patient in the original description by Ashford (1) and in a patient reported recently by Connor and Shlim (9). Moreover, the only existing report of *C. cayetanensis* found in feces from a domestic farm animal concerned a farm duck (6). Zerpa et al. suggest that besides consumption of contaminated water, other modes of transmission involving contact with domestic animals must be considered. So far, however, a possible infection route involving poultry, whether it may be direct consumption of undercooked chicken meat, contamination of food and water sources with chicken feces, or both, remains to be determined. It should be noted that sanitary standards in poultry-breeding facilities in developing countries may not be adequate. This would account for the fact that reports implicating chickens in the transmission of *Cyclospora* (1,9) have occurred in, or in relation to, developing countries. The *Cyclospora* found in the chickens in our study have the diagnostic features of C. *cayetanensis*. Nevertheless, the existence of another, not yet described, *Cyclospora* species infecting poultry, which has similar features but is different from *C. cayetanensis*, cannot be excluded at this stage. In addition, the number of oocysts recovered was not large and because feces were pooled, we could not calculate the number of oocysts passed by each bird. The possibility that oocysts were acquired as a contaminant from food or water sources and were only passing though the gut of the chickens (making the chickens a paratonic host) cannot be ruled out.

The increased recognition of *Cyclospora* as an important cause of diarrhea in both immunocompromised and immunocompetent

persons and the public health relevance of this emerging pathogen as a potential cause of diarrheal outbreaks (3,4) make prompt disclosure of the epidemiologic features and behavior of the parasite necessary. As we propose the possible participation of poultry in the epidemiologic cycle of the coccidia, we invite other *Cyclospora* working groups worldwide to confirm the so far putative reservoir described in this communication and to further study other possible hosts or reservoirs.

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PCR Confirmation of Infection with *Cyclospora cayetanensis*

To the Editor: *Cyclospora cayetanensis*, formerly known as cyanobacterium-like body, is a variably acid-fast microorganism. Recently, it was classified as a coccidian parasite (1) closely related to the genus *Eimeria* (2). Humans infected with *C. cayetanensis* typically have diarrheal illness with a variable number of stools per day and sometimes have nausea and vomiting (3,4). *Cyclospora* infection has been reported in many parts of the world as clustered or sporadic cases (1,3-5).

Variable success in diagnosing infection with this parasite underscores the need for using (as quality control) molecular methods, which do not rely on the level of expertise of laboratory personnel in microscopy. The key features for diagnosis by light microscopy are size (8µm to 10µm in diameter), internal features of stained and unstained oocysts, and autofluorescence of oocysts (1,6). The definitive diagnosis is understood as visualization of characteristic sporulated oocysts, which contain two sporocysts. However, sporulation typically requires incubating oocysts for up to 2 weeks, and this approach cannot be applied to Formalin or polyvinylalcohol-preserved stool smears.

Sporadic and clustered cases of *Cyclospora* infections were reported in the United States and Canada during May and June 1996 (5,7). From these outbreaks, more than 900 cases were diagnosed by examining stool specimens under light microscopy (Barbara Herwaldt, pers. comm.). Epidemiologic studies indicated risk for *Cyclospora* infection from consuming raspberries imported from Guatemala (7). Forty-two stool specimens supplied in 2.5% potassium dichromate from patients with intestinal symptoms were forwarded to the Centers for Disease Control and Prevention to be evaluated by microscopy and by polymerase chain reaction (PCR) amplification. In addition, one well-characterized positive stool specimen from Nepal was provided by John Cross, Armed Forces Research Institute of Medical Sciences,

Bangkok, Thailand, to use as the positive control.

Using techniques we developed for diagnosis of other protozoan parasites in stools, we extracted DNA from all stools. The techniques we used employ glass-bead disruption of oocysts in a buffer containing Laureth-12, purification with the RapidPrep Micro Genomic DNA Isolation Kit for Cells and Tissue (Pharmacia Biotech Inc., Piscataway, N.J.), followed by a final purification step employing the QIAquick PCR purification kit protocol (Qiagen, Inc., Chatsworth, Calif.) (8). The glass-bead disruption of oocysts was far more effective than sonication (2) or freeze-thawing techniques (9). We performed nested PCR in all stool specimens by using Relman et al. (2) primers CYCF1E and CYCR2B for the first step of nested amplification and primers CYCF3E and CYCR4B for the second (nested) step of the PCR. These are the only primers described for amplification of *Cyclospora* DNA. We found optimal conditions for the first step PCR to be denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s, 45 cycles. The same conditions were used for the second step of the nested PCR, but the annealing temperature was 60°C.

By using this approach, we amplified the *Cyclospora*-specific DNA fragment in 16 (38%) of the 26 (62%) specimens reconfirmed as positive by light microscopy. The 10 specimens negative by PCR but positive by microscopy showed either few or moderate numbers of *Cyclospora* oocysts. None of the 16 (38%) specimens negative by microscopy generated positive results in the PCR *Cyclospora* test. Upon further examination by the PCR technique we developed (9), three of these samples were positive for another enteric coccidian, *Cryptosporidium parvum*.

Preliminary evaluation indicates that the sensitivity of PCR is 62%, and the specificity is 100%. Although the sensitivity of the technique should be evaluated further, these results indicate that PCR can be used to detect *Cyclospora*. We assessed the sensitivity of this PCR again by using the Nepalese specimen described above. This specimen, which was used as positive control in all reactions, was amplified even when the extracted DNA was diluted at 10^{-5}.

Lastly, a note of caution. As noted by Relman et al. (2) and confirmed by us through GenBank searches, the nested PCR *Cyclospora* primers cross-amplify other coccidians, especially those belonging to the genus *Eimeria* (because no molecular data exist for another human coccidian enteric parasite, *Isospora belli*, potential cross-amplification remains to be determined). This cross-amplification with *Eimeria* should not present a problem in diagnosing *Cyclospora* in human stool, as no human infections by *Eimeria* are known. However, when analyzing food or environmental specimens, this cross-amplification may complicate precise detection of *Cyclospora*.

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Emerging Infectious Diseases and the Depopulation of French Polynesia in the 19th Century

To the Editor: The same dynamics now considered factors in the emergence of infectious diseases may have been involved in the dramatic depopulation of French Polynesia in the 19th century. Temporal and geographic variation in the frequency and severity of infectious diseases are the result of the encounter and interaction of a population of parasites and a population of hosts. J. Musser reviewed the "bacterial side of the equation" (1). On the host side, there are two historical models that describe the influence of parasitism on human populations (2-4): 1) the South American model, in which new pathogens were introduced into native populations by the European conquistadores, causing the death of 50 million people; and 2) the African model, in which infectious diseases present in native populations protected them from the effects of colonization until modern times when the discovery of quinine and other efficient antipathogenic drugs provided added protection. The second model is well illustrated by the attempted colonization of Madagascar, where the French lost five men to war and 5,000 to malaria (2). This letter intends to illustrate the first model. We suggest that during their first contacts with European navigators in the very late 18th century and the 19th century, Polynesian islanders, much like populations in the South American model, were decimated by newly introduced infectious diseases.

It is difficult to know precisely which infectious diseases were present in Tahiti and the other French Polynesian islands before the arrival of the first Europeans. However, a study of Polynesian languages indicates that Bancroftian filariasis and leprosy were already present, while syphilis and other venereal diseases, influenza, and tuberculosis (TB) were probably unknown. Epidemic diarrhea and dysenteriae could have existed, although first reports mentioned that the oldest Polynesians "never heard of dysenteriae before" (5). In the Marquesian language, names exist for leprosy, bronchitis, abscesses, and impetigo.

The number of inhabitants in Tahiti, as well as in the Marquesas and the Austral Archipelago, was at first only estimated by European explorers. However, a precise census was performed as soon as missionaries and French authorities noted the high death rates in most of the islands (5,7,15,16). Tahiti was annexed by France in 1843; the first census was performed in 1848, and the population size was assessed approximately every 5 years until 1911.

Four major epidemic diseases (TB, typhoid, influenza, and smallpox) devastated the Marquesas from 1791 to 1863/64; approximately 80% of the population died. During that period, exchange of populations between the Marquesas Islands also increased, as a consequence of colonization. Thus, leprosy increased dramatically during the second half of the 19th century, to a prevalence of 4.11% in 1884 (6).

In Rapa, the remote, southern island of the Austral Archipelago, at least three epidemics were reported, resulting in the loss of more than 90% of the population. Although the cause of the first epidemic remained unknown, dysenteriae and smallpox were identified as causes of the second and third epidemics, respectively.

From Rapa, a missionary went to Mangareva in 1831 or 1832, and his visit there was followed by an epidemic that the natives attributed "to his god." He had to flee back to Rapa. The second recorded epidemic disease was "Chinese scabies" in 1865, which decimated the child population. Then, the warship "La Zélée" brought an epidemic of influenza in 1908. In 1910, TB and leprosy were reported "to spread rapidly" (7), and in 1911, the ship "La Gauloise" brought whooping cough to Mangareva.

In Tahiti and the Society Islands, the number and diversity of international and interisland exchanges, involving numerous commercial ships and whalers, make the origin of epidemics more difficult to trace.

However, at least five were reported successively in the Leeward Islands in 1843, 1848, 1854, and 1864 (7), and at least 11 in Tahiti: influenza (1772 to 1774), pulmonary TB (1775), dysentery following the passage of the ship of Vancouver (1790), dysentery after the passage of the whaler "Britania" (1807), disastrous influenza in 1820, whooping cough in 1840, smallpox in 1841, dysentery again in 1843, scarlet fever in 1847, measles in 1852-1854 (800 deaths were recorded) and typhoid fever after the passage of "La Magicienne" in 1877 (8).

Almost without exception, authors attributed the dramatic depopulation of French Polynesia during the 19th century to infectious diseases. Other causes, such as alcohol, opium, local wars, infanticides, and even orgiastic behavior were also mentioned as possible causes. Depopulation occurred to a similar extent in other South Pacific countries (9), e.g., the Cook Islands, Hawaii, Tonga, Samoa, and particularly Fiji, where 50% of the population died. Thus, after limited initial contact with persons exposed to infectious diseases, most of the Polynesian populations died. Why did it happen? Why were epidemics so intense and so severe? It is unlikely that clones of bacteria, viruses, fungi, or parasites with particularly high virulence were introduced into native populations since the long crossing by sailing boats would have selected clones with lower virulence. Moreover, epidemics are also intense and severe in animal populations when new infectious agents are introduced. In Hawaii, the introduction of *Plasmodium* from birds had catastrophic consequences for the local fauna (10).

Host population factors that may influence the spread of an infectious agent (i.e., the intensity of an epidemic) are diverse: 1) social disruption was certainly a major cause for the increase of leprosy and TB in the Marquesas during the 19th century: pacification of the archipelago by Dupetit-Thouars changed traditional behavior and destroyed tribal barriers against leprosy by permitting the development of interisland exchanges, thus contributing to the spread of both leprosy (within the Marquesas) and TB (from Tahiti to one Marquesas island, then between the Marquesas) (11); 2) the absence of most infectious diseases in Polynesia before the 18th century probably slowed the selection of behavioral methods of prevention and the development of traditional medicine; 3) a small population without exposure to infectious diseases would not have selected resistance genes against nonexistent infectious agents; and 4) the lack of population immunity probably had a major role in the spread of new infectious agents.

Host population factors that can influence the virulence of parasites (i.e., the severity of an epidemic) are less frequent. Successive epidemics of closely related viruses or bacteria can enhance the severity of the disease, as in dengue fever (12), or can inversely provide cross-protection, as was suggested between yaws and syphilis (13), whose causative organisms are almost indistinguishable. Reduced genetic polymorphism of 19th century Polynesians who had no immunity to infectious diseases could have contributed to the severity of epidemics in the South Pacific, as it was speculated for South America (4,14).

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Epidemic Zoster and AIDS

To the Editor: Zoster (exogenously reactivated varicella-zoster virus infection) may seem an unlikely candidate for emergence and epidemicity. A recent report, however, describes a zoster outbreak associated with epidemic HIV in injecting drug users in Manipur State, India (1). In addition to underscoring the variety of ways in which "old" diseases may reemerge under complex bio-ecologic conditions, this outbreak may also have implications for anticipating and diagnosing HIV infections and AIDS in developing countries. The Manipur outbreak was associated with a doubling of zoster frequency above background levels, with increased occurrence most notable in males 12-44 years old, who also had the highest HIV prevalence. In a separately studied group of 120 injecting drug users, 20 developed zoster and all were found to be HIV positive (1), a correlation substantially greater than for such other clinical predicters of HIV infection as persistent lymphadenopathy, weight loss, or recurrent dermatoses. Increased zoster occurrence associated with HIV transmission has also been seen in Ho Chi Minh City, Vietnam, and in other Southeast Asian countries, particularly in injecting drug using populations (unpublished). Zoster as a sentinel indicator of community HIV transmission is also suggested by reports from Africa (2).

For over 150 years, it was believed that zoster occurred in local epidemics (3,4). By the 1950s, however, it was generally agreed that zoster represented reactivation of latent gangliar varicella virus either sporadically, or in response to immunosuppression or trauma. Epidemics of "endogenous" immunosuppression, such as those associated with epidemic HIV infection, might thus be expected to produce outbreaks of zoster, as seems to have occurred in Manipur and Vietnam. In the Indian outbreak traumatic zoster seemed unlikely: truncal and facial dermatomes predominated, rather than dermatomes corresponding to drug injection sites (usually the hands or legs). Recognition of zoster outbreaks may be important in developing countries where HIV diagnosis is limited, CD4 cell counts are unavailable, and diagnosis of AIDS is delayed. Zoster is not currently accepted as an AIDS-defining condition (5), and the extent to which it may reflect immune collapse or predict HIV disease progression is uncertain. Nevertheless, greater awareness of zoster as a sentinel indicator of community HIV transmission may be of help not only in clinical diagnosis, but also in public health efforts to recognize epidemic HIV occurrence.

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Ancient Egypt and Today: Enough Scourges to Go Around

To the Editor: In a recent letter (1), Ablin conjectures that translation of the hieroglyphic symbol for *ĀĀĀ* in many ancient Egyptian papyri (Ebers, Berlin, Hearst, London, and Kahum), may be suggesting the existence of human immunodeficiency virus (HIV) or its prototype during the time of the pharaohs. While hieroglyphic interpretations remain challenging, the symbol cited in his letter has most commonly been translated as hematuria (2-4) and has most often been related to schistosomiasis haematobia. This infection, caused by the helminth *Schistosoma haematobium*, has been shown to have occurred in Egypt from early pharaonic times (3200 B.C.), by the demonstration of schistosome eggs (5) and circulating schistosome antigens (6,7) in mummies. Remedies for hematuria were recorded in papyri from many centuries (9 in Hearst, 11 in Berlin, 20 in Ebers), perhaps implying that the condition was serious and widespread. In giving one of the remedies in the Ebers papyrus (circa 1500 B.C.), the text actually mentions worms in the body (although it seems to state that the worms are caused by *ĀĀĀ* disease, perhaps inverting the true order of causality). In the Hearst papyrus one of the remedies cited for hematuria is antimony disulfide. Until only 25 years ago, antimonial compounds were the most effective drugs for schistosomiasis chemotherapy.

It seems likely that, over a period of many centuries in ancient Egypt, *ĀĀĀ* disease was a widespread condition of sufficient severity to require medical attention. I concur with many others in proposing that the translation of *ĀĀĀ* disease is hematuria, and that the relationship drawn between *ĀĀĀ* and worms in the body, antimonial-based remedies, and the knowledge that *S. haematobium* infections were widely present at that time provide strong evidence that *ĀĀĀ* disease refers to schistosomiasis haematobia.

Schistosomiasis is still with us. In fact, through dispersions of both human populations and specific fresh-water snails (the intermediate hosts for schistosomes), this disease now infects some 200 million persons and is responsible for an estimated 800,000 deaths per year (8). While clearly ancient, schistosomiasis can emerge as a new infectious disease in a given location under certain man-made changes in environmental conditions and economic- or war-related migrations of people. For example, in the Senegal River basin, estuarine dams, irrigation systems, and an influx of people to work irrigation-intense crops led, over a period of only 3 years, to an increased prevalence of *S. mansoni* infection from 0% to >95% of the population of >50,000 (9). Even in modern-day Egypt, such interventions as the Aswan High Dam have significantly altered patterns of schistosomiasis (2,10). The Ministry of Health and Population of Egypt and the U.S. Agency for International Development are addressing this ancient scourge through the Schistosomiasis Research Project, a national schistosomiasis research and control program that attacks the disease with available tools, while it presses forward with research on much needed new tools, such as vaccines.

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AIDS and *ĀĀĀ* in Egypt?

To the Editor: A recent letter concerning Egyptian hieroglyphs on the disease *ĀĀĀ* asks if this disease could be AIDS or an HIV-associated condition prevalent in Egypt during the time of the pharaohs (1). We believe this possibility is highly unlikely. Aside from conflicts with current thought on the origin and evolution of lentiviruses, there is a problem of linguistic interpretation. The initial hieroglyph in the series of hieroglyphs comprising the word *ĀĀĀ*, a picture of a discharging phallus, is a "determinative," indicating the class or category to which the word belongs. Although scholars once took this determinative to indicate a phallic connection with disease, even suggesting that *ĀĀĀ* meant hematuria, consistent with schistosomiasis (2,3), it was later proposed that the determinative meant semen or poison, reflecting the Egyptian concept that diseases may be transmitted by an evil spirit in the form of an incubus, impregnating a victim with poisonous semen. This interpretation is now generally accepted (4,5). The phallus-with-discharge thus came to indicate a deadly disease, and *ĀĀĀ* a poisonous disease-causing substance introduced into the body by magic. The word *ĀĀĀ* is used elsewhere in the Egyptian medical papyri in other contexts, such as "*ĀĀĀ* of the heart" and "*ĀĀĀ* of the belly and heart," and is not known to have been used in connection with the bladder or genitalia. While the determinative meaning may not be absolutely established, it is clear from its usage in other contexts that the phallus-with-discharge determinative can indicate fatal or serious illness. The notion that the phallus-with-discharge determinative refers to sexually transmitted disease is not consistent with its usage. To further argue that *ĀĀĀ* represents AIDS or HIV disease is not justified by the linguistic evidence. Without further archaeologic or inscriptional evidence, we would doubt that HIV circulated in ancient Egypt.

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ADDENDUM

Adeleke A, Pruckler J, Benson R, Rowbotham T, Halablab M, Fields B. *Legionella*-like amoebal pathogens—phylogenetic status and possible role in respiratory disease. Emerging Infectious Diseases 1996;3:225-30.

Since the publication of the above article, accession numbers have been allocated to the following sequences:

LLAP -1 U64034
LLAP -8 U64035

Information about the phylogeny of *S. lyticum* and LLAP-3 can be found in references 20 and 21, respectively. The derivation of sequence data (accession numbers: LLAP-4x97357, LLAP-6x97359, LLAP-10x97363, LLAP-11x97362, LLAP-12x97366J) referred to in our publication, can be found in Birtles RJ, Rowbotham TJ, Raoult D, Harrison TG. Phylogenetic diversity of intra-amoebal legionellae as revealed by 16S rRNA gene sequencing. Microbiology 1996; 142:3525-30. In this study, which was carried out concurrently with the study described in the article by Adeleke et al. 16S rRNA sequence data were also obtained for LLAP1 (accession number x97355), LLAP2 (x97356), LLAP7 (x97365), LLAP8 (x97361), LLAP9 (x97360), and the L. lytica strains L2 (x97364) and LLAP3 (x97358).

ABA Sponsors Program on Law and Emerging Infectious Diseases

In August 1996, the International Health Law Committee, International Law and Practice Section, American Bar Association, sponsored a program entitled “Law and Emerging and Re-Emerging Infectious Diseases” to examine how the emergence and reemergence of infectious diseases affects international and U.S. federal, state, and local law. The issues addressed included international legal rules on infectious disease control and the need for their revision in light of emerging and reemerging infectious diseases; emerging infectious diseases and U.S. federal law, especially as it affects the mission of public health agencies; and the importance of state and local law in dealing with emerging infectious diseases.

Common themes included the challenge posed by emerging infectious diseases as law at every level (international, national, and local) is involved and law in various forms (treaties, constitutions, statutes, and regulations) is affected; the need for legal reform at the international and U.S. federal, state, and local levels; the challenges posed by a complex jurisdictional environment (e.g., World Health Organization’s relationship to independent states and U.S. public health agencies’ relationships to state governments); in considering legal reform, the need to balance competing policy objectives, such as the control of infectious diseases versus the freedom of global trade and travel or protecting the community versus privacy rights; the interdependence of legal reform efforts in that local and national implementation of revised international rules will be critical to any global strategy; the need to integrate the efforts of lawyers and public health officials to effectively promote epidemiologic principles and objectives; and the massive scope of the emerging diseases threat stemming from not only its global reach but also the long list of causes behind the emergence and reemergence of infectious diseases (e.g., political and medical complacency, international trade, global travel, war, human behavior, environmental degradation, urbanization, poverty, and inadequate public health infrastructures).

The International Health Law Committee program marks a first step in raising awareness in the legal and public health communities of the many and complex legal issues involved in addressing emerging infectious diseases.

For copies of program presentations and other information, contact David P. Fidler by e-mail at davidfidler@law.indiana.edu.

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A Global Theme Issue: Bibliography of References

Emerging and reemerging infections respect no national boundaries; therefore, they were an appropriate topic for the first “global medical theme issue,” introduced in January 1996. This global theme issue was conceived by three editors, Linda Hawes Clever, *The Western Journal of Medicine*, Magne Nylenna, *Journal of the Norwegian Medical Association*, and George D. Lundberg, *Journal of the American Medical Association*, who in 1995 invited the editors of 78 journals worldwide to participate. A year later, 36 journals in 21 countries on six continents published more than 200 articles pertaining to emerging and reemerging global microbial threats (1). The articles addressed topics ranging from factors contributing to increasing antimicrobial resistance to the impact of global warming on infectious disease. While some solutions were suggested, the global issue primarily served as a call to medical communities and people worldwide to identify contributing factors and begin to develop strategies to control emergent infections.

Following this note is a bibliography of articles published by the 36 journals participating in the global theme issue.

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Conference on Emerging Foodborne Pathogens

The conference on "Emerging Foodborne Pathogens: Implications and Control," March 24-26, 1997, Radisson Plaza Hotel at Mark Center, Alexandria, Virginia, USA, is organized by the International Life Sciences Institute (ILSI), ILSI North America Technical Committee on Food Microbiology, the U.S. Centers for Disease Control and Prevention, Department of Agriculture, and Food and Drug Administration, in cooperation with the Food and Agriculture Organization of the United Nations, and the Pan American Health Organization/World Health Organization. The conference will review the lessons learned and knowledge gained concerning the emergence/reemergence and dissemination of food-related microbial threats to health; identify factors that foster emergence/reemergence and dissemination of these hazards; identify scientific and food safety strategies to address emerging foodborne microbial hazards; and identify future research needs. The conference will be of special interest to food protection and public health professionals, including microbiologists, epidemiologists, physicians, and health policy makers; industry, academic, and government researchers; and others interested in microbial food safety hazards.

For program and registration information, contact

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