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Dispatches

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Competitive Exclusion of *Salmonella* Enteritidis by *Salmonella* Gallinarum in Poultry

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Salmonella Enteritidis emerged as a major egg-associated pathogen in the late 20th century. Epidemiologic data from England, Wales, and the United States indicate that *S.* Enteritidis filled the ecologic niche vacated by eradication of *S.* Gallinarum from poultry, leading to an epidemic increase in human infections. We tested this hypothesis by retrospective analysis of epidemiologic surveys in Germany and demonstrated that the number of human *S.* Enteritidis cases is inversely related to the prevalence of *S.* Gallinarum in poultry. Mathematical models combining epidemiology with population biology suggest that *S.* Gallinarum competitively excluded *S.* Enteritidis from poultry flocks early in the 20th century.

The avian-adapted serovar *Salmonella* Gallinarum, which includes two biovars, Gallinarum and Pullorum, was endemic in poultry flocks in Europe and the Americas in the early 20th century (1). To reduce economic losses to the poultry industry, national surveillance programs were established in the United States (National Poultry Improvement Plan, 1935) and England and Wales (Poultry Stock Improvement Plan, 1939). Since *S.* Gallinarum (antigen formula O9,12:-:-) has no animal reservoir other than domestic and aquatic fowl, the test-and-slaughter method of disease control under these surveillance programs led to its eradication from commercial poultry flocks in the United States, England, and Wales by the 1970s (1,2). At that time, the number of human cases of infection with serovar *S.* Enteritidis (antigen formula O9,12:g,m:1,7) began to increase in these countries (3,4). By the 1980s, *S.* Enteritidis had emerged as a major concern for food safety in Europe and the Americas (5); by 1990 it was the most frequently reported *Salmonella* serovar in the United States (6). Most *S.* Enteritidis outbreaks in Europe and the United States are associated with foods containing undercooked eggs (7-10). Eggs can become contaminated with *S.* Enteritidis through cracks in the shell after contact with chicken feces or by transovarian infection (11). Thus, laying hens were the likely source of the *S.* Enteritidis epidemic in Europe and the Americas.

The inverse relationship between the incidence of *S.* Gallinarum infection in chickens and egg-associated *S.* Enteritidis infections in humans prompted the hypothesis that *S.* Enteritidis filled the ecologic niche vacated by eradication of *S.* Gallinarum from domestic fowl (12). The hypothesis suggests that the epidemic increase in human *S.* Enteritidis cases in several geographic areas can be traced to the same origin, accounting for the simultaneous emergence of *S.* Enteritidis as a major egg-associated pathogen on three continents (5). A connection between the epidemics in Western Europe and the United States was not apparent from analysis of epidemic isolates. Although most human cases from England and Wales result from infection with *S.* Enteritidis phage type 4 (PT4), most cases in the United States are due to infections with PT8 and PT13a (13,14). The PT4 clone is genetically distinct from PT8 and 13a, as shown

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by IS200 profiling, ribotyping, and restriction fragment length polymorphism of genomic DNA fragments separated by pulsed-field gel electrophoresis (15). The reasons for the differing clonal isolates in the United States and Western Europe are unknown. *S.* Enteritidis was likely introduced into poultry flocks from its rodent reservoir (12). The geographic differences in predominant phage types may reflect the fact that at the time of introduction into poultry flocks, different *S.* Enteritidis strains were endemic in rodent populations in Europe and the United States. Subsequently, *S.* Enteritidis strains with the highest transmissibility may have become predominant in poultry flocks on each continent. An alternative explanation for the predominance of PT4 in England and Wales is its introduction into poultry breeding lines in the early 1980s (16), which may have accelerated the epidemic spread of PT4 in laying hens and resulted in its dominance in human isolates from England and Wales. However, factors responsible for the beginning of the *S.* Enteritidis epidemic should be considered separately from those important for its subsequent spread within the poultry industry. These factors were not specific to PT4 but rather allowed different phage types to emerge as egg-associated pathogens on different continents at the same time (5).

One such factor could be the eradication of *S.* Gallinarum from poultry, which would facilitate circulation of *S.* Enteritidis strains within this animal reservoir regardless of phage type. Experimental evidence indicates that immunization with one *Salmonella* serovar can generate cross-immunity against a second serovar if both organisms have the same immunodominant O-antigen on their cell surface (17-19). The immunodominant epitope of the lipopolysaccharide of *S.* Gallinarum and *S.* Enteritidis is the O9-antigen, a tyvelose residue of the O-antigen repeat (20). Immunization of chickens with *S.* Gallinarum protects against colonization with *S.* Enteritidis (21,22) but not *S.* Typhimurium, a serovar expressing a different immunodominant determinant, the O4-antigen (23). Theory indicates that coexistence of *S.* Gallinarum and *S.* Enteritidis in an animal population prompts competition as a result of the shared immunodominant O9-antigen, which generates cross-immunity. Mathematical models predict that the most likely outcome of this competition between serovars is that the serovar with the higher transmission success will competitively exclude the other from the host population (24-26). *S.* Gallinarum may have generated population-wide immunity (flock immunity) against the O9-antigen at the beginning of the 20th century, thereby excluding *S.* Enteritidis strains from circulation in poultry flocks (12). This proposal is based on analysis of epidemiologic data from the United States, England, and Wales. To formally test this hypothesis, we analyzed epidemiologic data from Germany to determine whether the numbers of human *S.* Enteritidis cases are inversely related to those of *S.* Gallinarum cases reported in poultry. We used mathematical models to determine whether our hypothesis is consistent with theoretical considerations regarding transmissibility and flock immunity.

Inverse Relationship of *S.* Enteritidis and *S.* Gallinarum Isolations in Germany

In West Germany, the number of human *S.* Enteritidis cases was monitored by a national surveillance program (Figure) (Zentrales Überwachungsprogram Salmonella, ZÜPSALM)

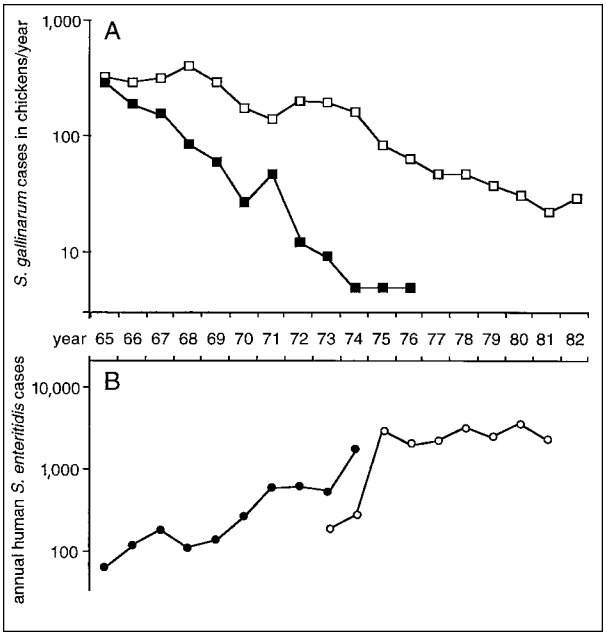

Figure. (A) *S.* Gallinarum infections in chickens in England and Wales (closed squares) (2,27) and the Federal Republic of Germany (open squares) (28). (B) Human cases of *S.* Enteritidis infections per year reported from England and Wales (closed circles) (3,29) and the Federal Republic of Germany (open circles) (Zentrales Überwachungsprogram Salmonella, ZÜPSALM).

from 1973 to 1982. In 1975, the number of human infections began to increase, indicating the beginning of the *S.* Enteritidis epidemic in West Germany. In 1983 the ZÜPSALM program was replaced by a national program for surveillance of foodborne disease outbreaks (Zentrale Erfassung von Ausbrüchen lebensmittelbedingter Infektionen, ZEVALI), implemented by the Department of Public Health (Bundesgesundheitsamt). In the first year of this program, *S.* Enteritidis was responsible for 62 outbreaks, most of which were traced to raw eggs. By 1988, the number of disease outbreaks caused by *S.* Enteritidis had increased to 1,365.

In 1967 in England and Wales, poultry, particularly chickens, became the main human food source of *S.* Enteritidis (3). Before that date, the organism had only sporadically been isolated from poultry (3). A continuous increase in human *S.* Enteritidis cases was recorded from 1968 until the epidemic peaked in 1994 (12,16). Thus, the human *S.* Enteritidis epidemic in England and Wales probably began in 1968 after this organism became associated with a human food source, chickens. The rapid increase in the number of human cases from 1982 to 1988 was probably due to the introduction of PT4 into poultry breeding lines in England and Wales (16). Comparison of data from England and Wales (3,29) showed that *S.* Enteritidis emerged somewhat later in West Germany (Figure).

Eradication of *S.* Gallinarum was among the factors contributing to the emergence of *S.* Enteritidis as a foodborne pathogen (12). To determine whether delayed elimination of avian-adapted *Salmonella* serovars from commercial flocks contributed to the late start of the human epidemic in Germany, we compared the results of surveys performed in poultry flocks in Germany with those from the United Kingdom and the United States. Control programs in the 1930s triggered a steady decline in the incidence of *S.* Gallinarum in poultry flocks in the United States, England, and Wales (1,2,12). By the early 70s, only a few cases of *S.* Gallinarum were reported each year to veterinary investigation centers in England and Wales (27). In Germany, the first national survey performed by the Department of Public Health (Reichsgesundheitsamt) in 1929 showed that 16.3% of birds were seropositive for *S.* Gallinarum (30). Blood-testing performed 20 years later with 6,313 birds in a province (Südbaden) of West Germany still detected 19.5% reactors (31). This high prevalence of *S.* Gallinarum in 1949 likely reflects the fact that after World War II available resources were directed toward rebuilding the poultry industry rather than improving disease control. The comparatively slow decline in the prevalence of *S.* Gallinarum in West Germany is illustrated further by data for cases of disease reported from poultry. The number of *S.* Gallinarum isolations from chicken carcasses received by veterinary laboratories in West Germany was reported by a surveillance program from 1963 to 1981 (28). During this period, the rate of decrease in numbers of *S.* Gallinarum cases in England and Wales was considerably higher than that reported from West Germany (Figure). In each country the numbers of *S.* Gallinarum cases were inversely related to the numbers of human *S.* Enteritidis cases. These data are consistent with the concept that the relative delay in eradicating *S.* Gallinarum from poultry may have contributed to delayed onset of the *S.* Enteritidis epidemic in West Germany.

Competitive exclusion of *S.* Enteritidis by *S.* Gallinarum

To calculate whether the prevalence of *S.* Gallinarum in chickens was high enough to generate flock immunity against *S.* Enteritidis, we analyzed epidemiologic data by mathematical models combining epidemiology with population biology (24-26). The transmission success of a pathogen is measured by the basic case-reproductive number, R_0, which is defined as the average number of secondary cases of infection from a primary case in a susceptible host population (32). In direct transmission, the basic case-reproductive number of a pathogen is directly proportional to the duration, D, for which an infected host can transmit the disease before it is either killed or clear of infection; the probability, ß, by which the disease is transmitted from an infected animal to a susceptible host; and the density of susceptible hosts, X (24).

$$R_0 = ßDX \qquad \text{(equation 1)}$$

After a pathogen is introduced into a susceptible host population, the reproductive rate of the infection declines as a consequence of the removal of a fraction, y, of the susceptible population, X, either by disease-induced death or

acquisition of immunity. That is, the effective case-reproductive number, R, will be smaller than the basic case-reproductive number R_0.

$$R = ßD (X-Xy) = R_0-R_0y \qquad \text{(equation 2)}$$

In an endemic state, each primary case of infection produces, on average, one secondary case. Thus, the effective case-reproductive number in a steady endemic-state situation is R=1. By solving equation 2 for R_0, we obtain (33)

$$R_0=1/(1-y) \qquad \text{(equation 3)}$$

Since *S.* Gallinarum was endemic in poultry populations at the beginning of the 20th century, its basic case-reproductive number, R_0, can be calculated on the basis of epidemiologic data collected before control measures were implemented, by estimating the fraction, y, of birds removed from the susceptible population.

The first method developed for detecting anti-*S.* Gallinarum antibodies was a macroscopic tube agglutination test introduced in 1913 (34). In 1931, the tube agglutination test was partially replaced by the simpler whole-blood test for slide agglutination of stained antigen (35). Initial surveys performed from 1914 to 1929 revealed that on average 9.8% to 23.8% of poultry in Europe and the United States were positive by the tube agglutination test (1,30,36). These data do not provide a direct estimate of the number of immune animals, since both serologic tests are relatively insensitive (37). However, the number of susceptible birds can be estimated by comparing results of serologic surveys with data from vaccination experiments. Immunization with *S.* Gallinarum vaccine strain 9R produces antibody levels high enough to be detected by the whole-blood tube or slide agglutination tests in only a small number of birds (approximately 10%) (20,23). The number of birds protected against challenge with virulent *S.* Gallinarum after a single oral or subcutaneous vaccination is considerably higher (approximately 60%) (23,38). The tube or slide agglutination test results (9.8% and 23.8% of birds, respectively, tested positive) at the beginning of this century suggest that at least 60% were immune to *S.* Gallinarum. In addition to acquired immunity, deaths, which likely occurred in most chicken flocks since *S.* Gallinarum reactors were present on most farms at the time, also reduced the density of susceptible hosts. For instance, only 9 of 144 farms surveyed in Hungary in the 1930s had no *S.* Gallinarum-positive birds (39). The death rates reported from natural outbreaks are 10% to 50%, although higher rates are occasionally reported (40). By the conservative estimate that 90% of birds in a flock will survive an outbreak and approximately 60% of the survivors will have protective immunity, the basic case-reproductive number, R_0, of *S.* Gallinarum is estimated to be 2.8.

S. Enteritidis does not substantially reduce the density of susceptible animals by causing death. Thus, its basic case-reproductive number can be estimated from the number of birds that remained susceptible during the peak of the *S.* Enteritidis epidemic. Antibody titers in *S.* Enteritidis-infected flocks are generally too low to be detected by the tube or the slide agglutination tests (37,41), presumably because this serovar commonly colonizes birds without causing disease and consequently without triggering a marked immune response. Live attenuated *S.* Enteritidis aroA vaccine does not produce antibody titers detectable by the tube or the slide agglutination tests (42), and oral immunization with this vaccine does not protect against organ colonization with wild-type *S.* Enteritidis (43). Hence, exposure to *S.* Enteritidis does not protect at levels found in birds with previous exposure to *S.* Gallinarum. Indeed, in a survey of flocks naturally infected with *S.* Enteritidis, only one of 114 birds tested strongly positive by the slide agglutination test (37). Experimental evidence indicates that birds exposed to *S.* Gallinarum have strong cross-immunity against colonization with S. Enteritidis. For instance, immunization of chickens with a single dose of *S.* Gallinarum vaccine strain 9R causes similar levels of protection against challenge with *S.* Gallinarum (23,38) and *S.* Enteritidis (22,44). The high degree of cross-immunity suggests that the antibody titers detected by the tube agglutination test are predictive of protection against lethal *S.* Gallinarum infection and of immunity to colonization by *S.* Enteritidis. Applying the criteria used to calculate R_0 for *S.* Gallinarum (10% reactors are indicative of 60% protection) to the *S.* Enteritidis data (37) suggests that approximately 5% of birds had protective immunity against this pathogen. From these data, the basic case-reproductive number of *S.* Enteritidis (R_0=1.05) is estimated to be considerably lower than that of *S.* Gallinarum.

Several factors should be considered in interpreting these data. Our estimate of the R_0 value for *S.* Enteritidis is based on epidemiologic data from the late 1980s. The intensive husbandry of chickens in the latter part of the 20th century has increased the density, X, of susceptible hosts and therefore R_0 (equation 1). Furthermore, information on the number of birds in *S.* Enteritidis-infected flocks with positive reactions in the tube agglutination test is sparse, and data from the peak of the epidemic in 1994 are not available. The prevalence of *S.* Enteritidis in poultry has been documented by a survey performed in Lower Saxony, Germany, in 1993, a time when flocks were heavily infected. This study showed that 7.6% of 2,112 laying hens were culture positive at slaughter (45). Although this low prevalence is consistent with a low basic case-reproductive number of *S.* Enteritidis at the peak of the epidemic, these data cannot be used to derive a reliable estimate for the basic case-reproductive number of *S.* Enteritidis at the beginning of the 20th century. Given these limitations, the available epidemiologic evidence appears to be consistent with our hypothesis. From equation 2 ($R=R_0-R_0y$), we estimate that early in the century the number of susceptible birds killed by *S.* Gallinarum (assuming 100% cross-immunity and y = 0.65) reduced the effective case-reproductive number of *S.* Enteritidis to < 1 (R = 0.37). These estimates support the idea that at the beginning of the 20th century *S.* Gallinarum reduced the density of susceptible hosts sufficiently to competitively exclude *S.* Enteritidis from circulation in poultry flocks.

S. Enteritidis is unlikely to be eliminated from poultry by relying solely on the test-and-slaughter method of disease control because, unlike *S.* Gallinarum, *S.* Enteritidis can be reintroduced into flocks from its rodent reservoir. Instead, vaccination would be effective in excluding *S.* Enteritidis from domestic fowl because it would eliminate one of the risk factors (loss of flock immunity against the O9-antigen), which likely contributed to the emergence of *S.* Enteritidis as a foodborne pathogen. In fact, much of the decline in human *S.* Enteritidis cases in England and Wales since 1994 has been attributed to the use of an *S.* Enteritidis vaccine in poultry (16). However, serologic evidence that *S.* Gallinarum is more immunogenic than *S.* Enteritidis suggests that a more effective approach for eliciting protection in chickens would be immunization with a live attenuated *S.* Gallinarum vaccine. This approach would restore the natural balance (exclusion of *S.* Enteritidis by a natural competitor) that existed before human intervention strategies were implemented early in the 20th century.

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Antigenic Variation in Vector-Borne Pathogens

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Several pathogens of humans and domestic animals depend on hematophagous arthropods to transmit them from one vertebrate reservoir host to another and maintain them in an environment. These pathogens use antigenic variation to prolong their circulation in the blood and thus increase the likelihood of transmission. By convergent evolution, bacterial and protozoal vector-borne pathogens have acquired similar genetic mechanisms for successful antigenic variation. *Borrelia* spp. and *Anaplasma marginale* (among bacteria) and African trypanosomes, *Plasmodium falciparum*, and *Babesia bovis* (among parasites) are examples of pathogens using these mechanisms. Antigenic variation poses a challenge in the development of vaccines against vector-borne pathogens.

What Is Antigenic Variation?

Immunodominant antigens are commonly used to distinguish strains of a species of pathogens. These antigens can vary from strain to strain to the extent that the strain-specific immune responses of vertebrate reservoirs determine the population structure of the pathogen. One such strain-defining antigen is the OspC outer membrane protein of the Lyme disease spirochete *Borrelia burgdorferi* in the northeastern United States (1). Different strains express different OspC surface proteins in the rodent reservoirs of *B. burgdorferi*. The single type of *ospC* gene in a cell does not vary during infections of immunocompetent mammals (2). OspC sequences are diverse, and the immune responses to them appear to provide for balancing selection. This diversity between strains in an immunodominant antigen is often called antigenic variation.

True antigenic variation, however, arises in a single clone or genotype in a single host and "involves the loss, gain, or change in a particular antigenic group, usually by loss, gain, or change in one of the polypeptide or polysaccharide antigens…" (3). In most cases, this change is reversible, i.e., the information for producing the original antigen is archived in the cell and can be used in the future. The adaptive immune system of an infected vertebrate selects against the original infecting serotype, but that specific response is ineffective against new variants. One example of antigenic variation occurs in *B. hermsii*, a cause of tickborne relapsing fever (4), which has a protein homologous to the OspC protein of *B. burgdorferi*. However, instead of a single version of this gene, each cell of *B. hermsii* has several copies of silent genes (alleles) that may be expressed during infection. The sequences of these alleles within a single strain of *B. hermsii* vary as widely as the *ospC* alleles of different strains of *B. burgdorferi*.

We review infectious pathogens that undergo clonal antigenic variation and, like *B. hermsii*, depend on arthropod vectors for transmission. These pathogens are not free-living and do not form spores or have equivalent means for survival outside an animal. Vertical transmission in the arthropod or the vertebrate either does not occur or is too rare to maintain the pathogen in nature. Without access to another vertebrate host through an arthropod, the pathogen will die with the host.

We restrict this review to situations in which an immune response against an antigen is synonymous with selection for another allele in the population. Many pathogens have repetitive-gene families. A multimember family may

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resemble a variable antigen gene repertoire in its diversity but may have little or no effect on immunity against infection. An example is the *bdr* family of genes in *B. burgdorferi* (5).

Viruses are also said to have antigenic variation but are excluded from this review because the mechanism they use usually depends either on the accumulation of point mutations in a single genotype (e.g., the antigenic drift of influenza A virus) or on recombination or reassortment between two different genotypes infecting the same host (e.g., antigenic shift of influenza A virus). A possible exception is the African swine fever virus, a poxvirus-like linear DNA virus transmitted by soft ticks. These large viruses have tandem repeated genes at their telomeres that undergo deletions during infection (6).

Common elements

Infection with a vector-borne pathogen that undergoes clonal antigenic variation has several possible outcomes (Figure 1). Acquisition of the pathogen by the vector does not in itself constitute transmission. The vector may become infected by a blood meal, but enough pathogens may not be present in the blood for the vector to transmit the infection to its next host. Pathogen peaks may not be so well delineated, especially during late infection when the growth-and-decline curves for individual variants begin to overlap.

Borrelia spp., *Anaplasma* and related genera, African trypanosomes, *Plasmodium* spp., and *Babesia* spp. are vector-borne pathogens that use antigenic variation to evade the host's immunity. The details of antigenic variation differ, but some features are the same, for example, the use of multiphasic antigenic variation or a change among at least three variable antigens rather than alternating between two. At least 10—and sometimes many more—variants or serotypes may be expressed during a single infection. There is a complete or near-complete gene for each of the variable proteins. Variation is achieved by switching one of the several genes expressed at any one time, rather than by accumulating mutations in a single expressed gene, as commonly occurs in viruses. In the best-studied examples, African trypanosomes and relapsing fever *Borrelia* spp., the rate of antigen switching in the vertebrate host is approximately the same, at 10^{-4} to 10^{-2} per cell per generation.

True antigenic variation has been demonstrated in other human pathogens, including *Neisseria gonorrhoeae*, *Mycoplasma* spp., *Campylobacter fetus*, *Pneumocystis carinii*, and *Giardia lamblia*. In addition, the complete genomic sequences of other pathogenic bacteria, such as *Helicobacter pylori*, *Treponema pallidum*, and

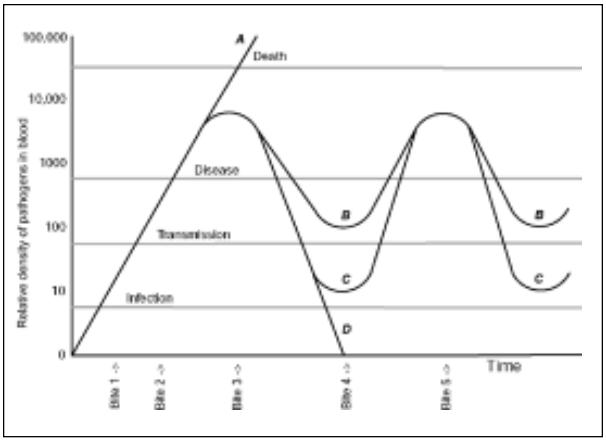

Figure 1. Relative densities of a vector-borne pathogen in the blood of four hosts, A-D. The gray horizontal lines represent the lower thresholds for the persistence of infection, transmission to a new vector, symptomatic disease in the host, and death of the host. In host A, overwhelming infection causes death. In hosts B and C, there is antigenic variation of the pathogen; B differs from C in the continuing likelihood of transmission even during periods of no or little illness. In case C, the host remains infected but is not infectious between relapses of high-density parasitemia and illness. In host D, the pathogen is cleared from the blood by the immune system. The relative density of the pathogens in the blood is on the *y* axis. If the arthropod vector of the infection fed on the four hosts at different times, the following outcomes would be observed: a bite at time 1 would not result in transmission because the density of pathogens in the blood was insufficient for uptake and establishment of the pathogen in the vector. At times 2 or 3, the disease agent is transmitted to the biting arthropod, although the hosts bitten at time 2 are not yet ill. At time 3, the infection worsens, in case A in the absence of an effective immune response. In cases B-D, the infection peaks as neutralizing antibodies to the infecting serotype appear in the blood at time 3. An arthropod taking a blood meal at time 4 could acquire the infection from host B but not from hosts C or D. In case D, the infection has been cleared by time 4. At time 5, both B and C could transmit the pathogens again and both have a relapse of illness as the result of the proliferation of a new serotype. The figure is modified from a figure by Turner (7).

Mycobacterium tuberculosis, encompass large families of repeated genes that are polymorphic in sequence and may be involved in antigenic variation.

The vector-borne pathogens, in particular the African trypanosomes and relapsing fever *Borrelia* spp., offer the least ambiguous models for understanding the biology and evolution of antigenic variation. These pathogens depend on hematophagous arthropods for transmission to new vertebrate hosts, and consequently, the likelihood of transmission is a direct function of the duration and density of the pathogens in the blood. Blood-cell types are comparatively simple, and antibodies alone can clear infection by relapsing fever *Borrelia* spp. and African trypanosomes (8,9). Apparently these two pathogens need to defend only against humoral immunity.

Vector-borne pathogens use one or more genetic mechanisms to circumvent the immune system. Four general mechanisms for antigenic variation have been described (10): modification of transcript levels, gene conversion, DNA rearrangement, and multiple point mutations (Figure 2). An example of the first mechanism is the reversible activation of expression of a variable antigen gene at one locus as expression of a previously active variable antigen gene at another locus in the genome becomes silent, an event that occurs without DNA changes at the loci themselves. In the figure, pathogen X has surface antigen genes *black* and *white* at two loci. At each locus there is a potential promoter, but only at the *black* locus is a gene expressed. After a switch, the *black* locus is silent, but the *white* locus 3 is active.

The second mechanism, gene conversion, is probably the most widespread for replacing expression of one gene with another. The change may be complete, thereby altering all defining epitopes of the antigen, or partial, for example, when a central hypervariable region of a protein is replaced through crossovers in highly similar flanking regions. This process commonly involves genes on separate chromosomes or plasmids in the cell but can also occur within the same replicon. When one gene is displaced at an expression site, the organism uses for that replacement a copy of a gene from a more stable location in the genome. In Figure 2, a *black* gene converts a *white* gene at a site with an active promoter. Gene conversion allows a pathogen to retain a complete repertoire of variable antigen genes.

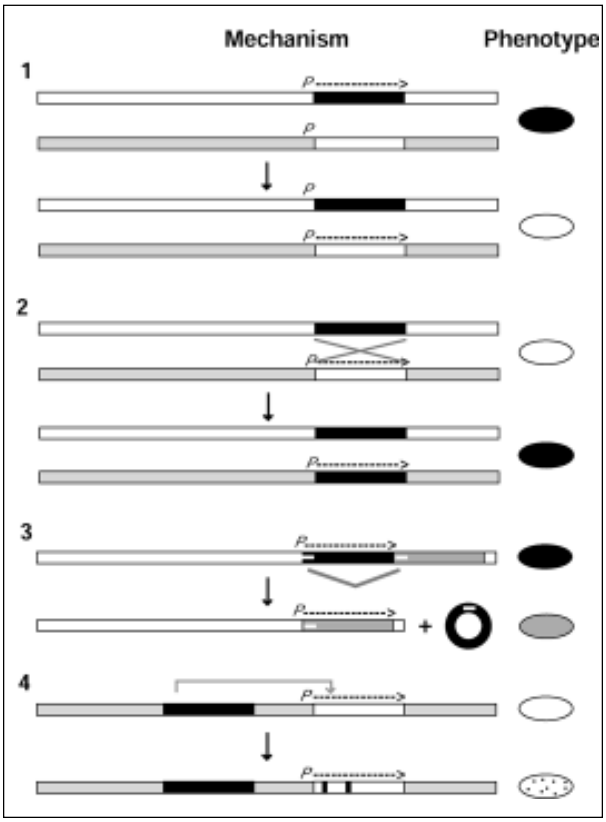

Figure 2. Four genetic mechanisms for antigenic variation in a hypothetical pathogen. 1. Modification of transcript levels. Two loci are shown in the figure for *black* and *white* genes. When the *black* gene promoter (*P*) is silenced and the *white* gene promoter is activated (arrow), the phenotype of the pathogen changes from black to white. No genetic change occurs at either locus. 2. Gene conversion. Two loci are shown for *black* and *white* genes: the *white* gene is at the expression site with a promoter, and the cell phenotype is white. In the switch, the *black* gene sequence is the donor that converts the expression site locus, and as a consequence the phenotype changes to black. 3. DNA rearrangement. In one locus with a tandem pair of variable antigen genes, *black* and *gray*, a recombination between direct repeats (small white boxes) at the 5' ends of the genes results in deletion of the *black* gene as a nonreplicative circle and the rearrangement of *gray* gene to a position immediately downstream of the promoter. The cell phenotype changes from black to gray. 4. Multiple point mutations. A single region of the genome contains an active *white* gene and an archived *black* gene at some distance 5' to it. The *black* gene provides donor sequences for two short conversion patches in the *white* gene. The phenotype of the cell remains white, but there may be several amino acid differences between this mutated *white* gene and the original *white* gene.

More extensive change in the genotype may occur by the third mechanism, DNA rearrangement. In Figure 2 the first gene in a tandem array of two variable antigen genes is deleted, thus moving a previously silent *gray* gene next to a promoter. The recombination is between two short direct repeats common to both the *black* and *gray* genes. Although this results in the loss of that particular allele of the *black* gene as a nonreplicative circle, there usually would be another copy of the *gray* allele in the genome.

In the pathogens discussed here, the fourth general mechanism, multiple point mutations or conversion patches, usually occurs in a gene that has already been activated or moved by one of the other three mechanisms. In Figure 2, the expressed *white* gene undergoes limited gene conversion by the *black* gene, in a process similar to somatic mutations of rearranged immunoglobulin genes.

In any given strain, the repertoire may have considerable sequence diversity, but this does not mean that each strain has achieved a unique solution to the problem of immune avoidance. Other strains and other species in the same genus usually have a repertoire of genes homologous to the set of genes of the pathogen in question (11). The evolutionary distance between two variable antigen genes in the same pathogens may be greater than the distance between two genes in a different species. An example would be the hypothetical variable gene repertoire A, B, C, and D in species 1 and variable gene repertoire A', B', C', and D' in species 2. The sequence identity between A, B, C, and D in species may be no more than 40%, but there may be 80%-90% sequence identity between the B gene in species 1 and the B' gene in species 2.

Relapsing Fever *Borrelia* spp.

The antigenic variation of relapsing fever spirochetes once attracted the attention of the early immunologists, such as Paul Ehrlich, because the infection proved the specificity of the immune response (8,9). In *B. hermsii*, a New World relapsing fever species, approximately 30 serotypes have been derived from a single cell (12). Specific antisera to these serotypes accounted for approximately 80%-90% of the variants that appeared during relapses of infection in mice in prospective experiments (13). The serotype of a *Borrelia* cell depends on its major surface antigen. The 30 or so antigens are divided approximately equally between two families: Variable Large Proteins (Vlp) of approximately 36 kDa and Variable Small Proteins (Vsp) of approximately 20 kDa (14-16). These abundant lipoproteins are anchored in the outer membrane by their lipid moieties. Although the *vlp* and *vsp* genes use the same locus for expression and may have identical signal peptides, no sequence homology can be identified between these two groups of proteins. Information about the variable antigens of other relapsing fever *Borrelia* spp. is less extensive, but *B. recurrentis*, the cause of louse-borne relapsing fever (17), *B. crocidurae* (18), an Old World relapsing fever species, and *B. turicatae* (19), another New World species, have *vlp* and *vsp* genes themselves. The Vsp proteins not only serve as variable antigens that may attract attention as an immune target but also determine tissue tropisms. In a clonal population of *B. turicatae*, expression of one *vsp* gene is associated with invasion of the central nervous system, while expression of another *vsp* gene is associated with high densities of spirochetes in the blood (20, 21).

B. hermsii uses all four general mechanisms for antigenic variation. Gene conversion between a linear plasmid containing a collection of silent *vsp* and *vlp* genes and another linear plasmid with an active *vsp* or *vlp* gene results in the replacement of one variable antigen gene with another downstream from a promoter (22,23). The gene conversion may be less extensive, yielding a chimeric *vlp* gene from a partial gene conversion (24). In *B. turicatae*, conversion may be more extensive, involving 10 or more kilobases downstream of the promoter (19). A DNA rearrangement in which the first member of a tandem pair on a single linear plasmid is deleted also yields a new serotype in the population (25). After the deletion, the formerly distal *vsp* in the pair is now next to the promoter. This type of rearrangement may be followed by a period of hypermutation at the 5' end of the gene (26). These frequent point mutations in the newly expressed gene further diversification of the *vsp* sequences.

These three types of events occur at a single expression site in the genome. Evidence also indicates that variation may also occur through the fourth mechanism: a change in transcription between two separate loci (27). A second locus for *vsp* expression has been found in *B. hersmii* on another linear plasmid (27). When this locus is active, the first expression site is silent (28).

Activation of this second site is associated with infections of ticks, not mammals (29).

B. burgdorferi and related species that cause Lyme disease have, as stated above, only one copy of the *vsp* ortholog, *ospC*, per cell, but it has several copies of sequences called *vlsE* genes, which are homologous to the Vlp proteins of relapsing fever *Borrelia* spp. (30). During infection, but not detectably in vitro, there is variation in expressed VlsE proteins through partial gene conversions from a tandem array of cassettes containing different hypervariable regions of *vlsE* sequences. Variants appeared in both immunodeficient as well as immunocompetent mice, but the rate of accumulation of amino acid changes in VlsE was higher in immunocompetent animals (31).

Anaplasma marginale and Related Bacteria

Anaplasmosis, a persistent intraerythrocytic infection of cattle and goats, has a global distribution. Infected animals have severe anemia and a higher rate of abortion. The infection, which is caused by members of the genus *Anaplasma*, an obligate intracellular rickettsia-like bacterium related to the genus *Ehrlichia*, is characterized by repetitive cycles of rickettsemia at 6- to 8-week intervals. In cattle infected with *Anaplasma marginale*, the number of pathogens in the blood varies between a peak of 10^6 to 10^7 per mL to a low of 10^2 per mL. In each cycle, the number of pathogens in the blood increases over 10 to 14 days and then precipitously declines (32). The cattle are reservoirs for the infection, and ixodid ticks are the vectors. Transmission to the vector ticks depends on the density of the pathogens in the blood (33).

Major surface protein 2 of *A. marginale* is an immunodominant outer membrane protein of approximately 40 kDa. Each strain has a large polymorphic family of *msp2* genes; the variation occurs in the central region of the proteins. Multigene families of MSP2 paralogs have been found in *Cowdria ruminatium* (34), the cause of heartwater disease of ruminants in Africa and Caribbean, and *Ehrlichia granulocytophila*, the agent of human granulocytic ehrlichiosis (35). Each sequential cycle of rickettsemia is associated with a different transcript from at least 17 different *msp2* genes in the family (36). Vaccinating animals with recombinant MSP2 produces antibodies specific for that MSP2. The genetic mechanisms for switches in *msp2* genes are not known but may involve partial gene conversions through homologous recombination.

African Trypanosomes

Trypanosoma brucei is a flagellated protozoon transmitted by tsetse flies to mammalian hosts, including humans and livestock. The infection consists of rising and falling parasitemia resulting from the generation of subpopulations that have antigenically different forms of a major variant-specific glycoprotein (VSG) at the cell surface (37). African trypanosomes switch at rates that are as low as 10^{-7} to 10^{-6} for syringe-passaged lines (38) or as high as 10^{-3} to 10^{-2} for field or fly-transmitted lines (39). VSG proteins, which are 400 to 500 amino acids in length, are anchored to the parasite's membrane at their carboxy terminus by a glycosyl-phospatidyl-inositol linkage. Besides immune evasion, other possible functions of VSGs include shielding other proteins (e.g., permeases) on the surface from immune attack and inhibiting phagocytosis (37).

A parasite can express several VSGs during infection in the mammalian host. Active genes for VSGs are located in one of 20 possible telomeric expression sites on the chromosomes and are transcribed with at least eight other genes (40,41), one of which encodes one of several variable transferrin receptors that confer different binding affinities for the transferrins of different mammals. Therefore, African trypanosomes combine antigenic variation of their surface coats with the ability to take up transferrin from their mammalian hosts (42).

A given VSG coat protein is encoded by a single *vsg* gene. Antigenic variation of VSG coats can occur by all the mechanisms described above, namely, transcriptional control, gene conversions, single crossover events between telomeric genes, and point mutations (37). A complete VSG gene conversion is usual in the early stages of infection, while partial replacement and point mutations that may generate further diversity are observed in the more chronic stages (43,44). Short blocks of sequence homology in the upstream and downstream regions of the donor and acceptor genes may be required for the recombination events, but the precise basis for these switching events remains unknown.

A possible mechanism may involve the unusual DNA base J, which is enriched in

silent telomeric sites but is absent in expressed regions (45). Site-specific nucleases have not been described, but RAD51, an enzyme involved in DNA break repair and genetic exchange in other eukaryotes, may be involved (46). For transcriptional activation and silencing of *vsg* expression in the bloodstream forms of African trypanosomes, the presence of a certain sequence within the promoter may not be critical. When an expression site *vsg* promoter is replaced by ribosomal DNA promoter, *vsg* expression sites may still be silenced or activated (47).

Plasmodium falciparum

During malaria infection, the apicomplexan parasites of the genus *Plasmodium* undergo repeated cycles of growth in erythrocytes. The species *P. falciparum* has strains that differ in several polymorphic proteins, but antigenic variation within a strain also occurs. The best-documented example of true antigenic variation is in the *P. falciparum*-infected erythrocyte membrane protein 1 (PfEMP1) antigens, which are expressed on the surface of the infected erythrocytes. Switching rates between PfEMP1 proteins may be as high as 10^{-2} per generation (Table) (48). By changing which PfEMP1 is expressed, the parasite evades the immune response directed against these immunodominant antigens. The PfEMP1 proteins also inhibit antigen presentation by dendritic cells and provide the means for the infected red cells to adhere to endothelium and extracellular matrix, thus avoiding clearance of the infected erythrocytes by the spleen (48-50).

The variable PfEMP1 proteins range from 200 to 350 kDa. Their extracellular region has variable adhesive domains that confer the parasite-infected erythrocytes with a particular binding specificity that can include the extracellular matrix protein thrombospondin and a variety of endothelial receptors such as CD36, vascular cell adhesion molecule-1 (VCAM-1), E-selectin (ELAM-1), and intercellular cell adhesion molecule type 1 (ICAM-1) (49,50). These adhesive phenotypes lead to the sequestration of infected erythrocytes in the brain, lungs, kidneys, liver, or other organs, thereby determining the clinical manifestations of malaria.

The PfEMP1 proteins are encoded by members of the *var* family of genes (49,51,52). Each parasite devotes approximately 2% to 6% of its genomic DNA to a repertoire of 50 to 150 *var* genes clustered near the ends of chromosomes. Transcription of the *var* genes can occur from expression sites internal on the chromosomes or near a chromosome telomere (53). Changes in *var* expression appear to occur in situ by recombination-independent mechanisms (51,52). Evidence indicates that a single *P. falciparum* simultaneously transcribes multiple *var* genes during its early ring stages, but in trophozoites, tighter transcriptional control results in the expression of a single PfEMP-1 on the surface of the host cell (54,55).

Two additional variant multigene families that, like PfEMP1, are expressed on the surface of infected red blood cells, induce specific antibodies, and undergo clonal variation have been described recently (56,57). These proteins are encoded by the *rif* and *STEVOR* genes, which are located near the telomeres that contain the *var* genes.

Babesia bovis

Members of the genus *Babesia* cause one of the most common parasitic infections worldwide in wild and domestic animals. Some of the species, such as *B. microti*, have been transmitted to humans. Like *Plasmodium*, *Babesia* are intraerythrocytic parasites, but they are transmitted by ticks, not mosquitoes. While several multigene families have been described for various species of *Babesia*, clonal antigenic variation of *B. bovis*, a parasite of cattle, is best

Table. Vector-borne infections with antigenic variation

Disease	Pathogen(s)	Vector	Variable antigens[a]
Relapsing fever	Several species of *Borrelia*, e.g., *B. hermsii*	Soft (argasid) ticks and body lice	Vlp & Vsp
Anaplasmosis	*Anaplasma marginale*	Hard (ixodid) ticks	MSP2
African trypanosomiasis	African *Trypanosoma* spp., e.g., *T. brucei*	Tsetse fly	VSG
Malaria	*Plasmodium falciparum*	Mosquitoes	PfEMP1
Babesiosis	*Babesia bovis*	Hard (ixodid) ticks	VESA1

[a]Vlp = variable large proteins; Vsp = variable small proteins; MSP2 = major surface protein 2; VSG = variant-specific glycoprotein; MfEMP1 = *P. falciparum* erythrocyte membrane protein 1; VESA1 = variant erythrocyte surface antigen 1.

documented (58). The variant erythrocyte surface antigen (VESA1) of *B. bovis* is a heterodimeric protein expressed on the surface of infected red blood cells. The rapid variation of these polymorphic proteins likely contributes to chronic infection in cattle by prolonging the parasite's survival through immune evasion and sequestration of the infected red blood cells in peripheral organs (58). The VESA1 proteins, which have an approximate molecular weight of 128 kDa (59), are expressed on the external tips of the membrane knobs of infected erythrocytes. Their cytoadhesive phenotype depends on the antigenic and structural changes of the VESA1 proteins (60). The gene encoding the VESA1a subunit has been recently shown to belong to the *ves* multigene family (61). The predicted protein does not seem to have cleavable signal sequence, but it does have a predicted transmembrane segment and a cysteine/lysine-rich domain (61). The molecular events that determine the switching mechanism in *B. babesia* are unknown.

Conclusions

The requirement for vector transmission of these infectious pathogens provides a powerful selection for mechanisms that prolong parasitemia. Through convergent evolution, several vector-borne pathogens have arrived at the same strategy of antigenic variation to achieve this goal. The similarity in the genetic mechanisms that such unrelated pathogens as African trypanosomes and relapsing fever *Borrelia* spp. use for antigenic variation is remarkable.

Antigenic variation has important implications for the development of vaccines against these pathogens. If the variable antigen is to be the target of immunoprophylaxis, the vaccine would likely need to be multivalent, perhaps to the point of impracticality. If the infected host animal has not solved the problem of identifying an antigen that is conserved among the variants, thereby neutralizing the infection earlier, how can vaccine developers hope to do this?

A possible way to meet this challenge is to focus on the function domains of the variable proteins. The variable antigens of both the bacterial and parasite pathogens have other roles in pathogenesis besides immune evasion. These include tissue tropism, shielding of adjacent molecules, inhibition of phagocytosis, modulation of antigen presentation, and selective adherence. Certain regions of the variable protein may be irrelevant for these functions of the pathogen, and consequently the encoding DNA sequences could be highly divergent among alleles. On the other hand, the regions conferring these functions would likely be more constrained in structure and thus comparatively more susceptible to cross-reacting antibodies.

Another possible way to meet the challenge of antigenic variation is to focus on the vector-specific surface antigens of these pathogens. The repertoire expressed in the arthropod vector, which lacks an adaptive immune system, is generally more limited than that expressed in the vertebrate host. The Lyme disease vaccine is an example of successful targeting of a vector-specific protein. Although *B. burgdorferi* has not yet been proven to undergo true antigenic variation, there is considerable diversity in the *ospC* sequences that define strain identity within a given area in which transmission to humans occurs. A vaccine based on OspC would likely need to be multivalent. In contrast, *B. burgdorferi*'s OspA protein (62), the sole protein in the vaccine, is natively expressed in the tick's midgut but usually not during infection of mammals (63). Perhaps because of OspA's infrequent encounters with the mammalian adaptive immune system in nature, there is little divergence in *ospA* sequences between strains of *B. burgdorferi* (64). The OspA-based vaccine apparently works by eliciting antibodies that kill or inhibit the spirochetes in the tick, before expression of the more polymorphic *ospC* and *vlsE* genes in the mammalian host (65).

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Toxin Gene Expression by Shiga Toxin-Producing *Escherichia coli*: the Role of Antibiotics and the Bacterial SOS Response

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Toxin synthesis by Shiga toxin-producing *Escherichia coli* (STEC) appears to be coregulated through induction of the integrated bacteriophage that encodes the toxin gene. Phage production is linked to induction of the bacterial SOS response, a ubiquitous response to DNA damage. SOS-inducing antimicrobial agents, particularly the quinolones, trimethoprim, and furazolidone, were shown to induce toxin gene expression in studies of their effects on a reporter STEC strain carrying a chromosome-based *stx2::lacZ* transcriptional fusion. At antimicrobial levels above those required to inhibit bacterial replication, these agents are potent inducers (up to 140-fold) of the transcription of type 2 Shiga toxin genes (*stx2*); therefore, they should be avoided in treating patients with potential or confirmed STEC infections. Other agents (20 studied) and incubation conditions produced significant but less striking effects on *stx2* transcription; positive and negative influences were observed. SOS-mediated induction of toxin synthesis also provides a mechanism that could exacerbate STEC infections and increase dissemination of *stx* genes. These features and the use of SOS-inducing antibiotics in clinical practice and animal husbandry may account for the recent emergence of STEC disease.

The associations between *Escherichia coli* O157:H7 infection, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) were established in the early 1980s (1,2). Shiga toxin-producing *E. coli* (STEC) strains have since been recognized as the cause of both outbreaks and sporadic cases of diarrhea and HUS, involving thousands of cases and numerous deaths (3). Shiga toxins are key virulence factors in the pathogenesis of STEC disease (3). The term Shiga toxin (Stx) refers to two families of related toxins, Stx/Stx1, which includes the classical Shiga toxin produced by *Shigella dysenteriae*, and Stx2 (4). The *stx* genes carried by STEC strains are, with one possible exception (*stx2e*), encoded on bacteriophage genomes integrated into the bacterial chromosome. Stx2-producing STEC strains are more closely associated with HUS than are strains that produce only Stx1 (5,6).

Because antimicrobial agents may play a role in the pathogenesis of severe STEC disease, chemotherapy for STEC infections remains controversial (7,8). The location of *stx* genes (predominantly on λ-like bacteriophage genomes integrated into the chromosome of their host bacterium) has important implications because the induction of the SOS response, an extensively characterized genetic regulatory mechanism, induces high-level expression of previously silent bacteriophage genes (9). *Stx* genes are coexpressed with genes of the bacteriophage (10,11), and certain quinolones (known to be potent SOS inducers) induce increases in toxin (12,13) and bacteriophage production (13) of two to three orders of magnitude within 2 to 4 hours. The potential importance of a link between the SOS response and prophage induction for *Stx1* and *Stx2* expression has been reinforced by recent sequencing and pathogenicity studies (11,14,15).

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We have constructed a genetically modified derivative of a clinical isolate in which the genes encoding both elements of the toxin (*stx2AB*) were partially replaced with a *lacZ* reporter gene. The product of this gene, ß-galactosidase, is easily assayed and detected; its expression reflects the transcriptional activity of the *stx*2 gene and can be visualized in simple agar plate assays and quantified in biochemical assays. We have extended our earlier observations on the effects of quinolones on reporter expression by this strain to include a wider range of antimicrobial agents and the modulating effects of different environmental conditions. Our results show that several agents could increase the amounts of toxin produced and that SOS-inducing agents could play an important role in the epidemiology of STEC infections.

Methods

Bacterial Strain and Growth Conditions

RV31, a strain of *E. coli* O157:H7 isolated locally from a patient with hemorrhagic diarrhea, was used to construct the reporter strain, PK552*(stx2A*::*lacZ*), which contains a copy of the *E. coli lacZ* gene transcriptionally fused to the promoter region of *stx*2 on the chromosome. The reporter strain construction, which involved allelic exchange with a series of suicide plasmid vectors, will be described elsewhere. In the first step, the entire indigenous *lac* operon and adjacent *lacI* gene were deleted to create a Lac^- strain. Then, most of the coding sequence of the *stx2A* gene was replaced with a promoterless *E. coli lacZ* gene, resulting in a transcriptional fusion in which transcription of the *lacZ* reporter gene is controlled by *stx*2 regulatory mechanisms. The structure of the final construct was confirmed by diagnostic polymerase chain reaction, Southern blotting, and nucleotide sequencing across the *lacZ* insertion points.

Bacterial growth in the presence of antibiotics was monitored by the Bioscreen C system and associated Biolink software (Labsystems, Basingstoke, UK). This procedure allowed the simultaneous measurement of growth in up to 200 tests by recording changes in optical density. The *stx2*::*lacZ* reporter strain PK552 was grown overnight at 37°C in Antibiotic Medium No. 3 (Oxoid, Basingstoke, Hampshire, UK). A 2-mL aliquot of overnight culture was added to 100 mL of prewarmed medium and incubated with shaking at 37°C to an optical density at 600 nm (OD_{600nm}) of approximately 0.4. The culture was then divided, and test antibiotics were added to these aliquots from stock solutions. Aliquots of these cultures (200 µL, 4 replicates) were then injected into wells of honeycomb plates (Labsystems). The Biolink software was used to program the Bioscreen C system to specify wavelength, incubation temperature, length of experiment, timing of readings, and the rate of shaking of the cultures. The cultures were incubated in the Bioscreen C system at 37°C with continuous shaking, and OD_{600nm} was recorded every 15 min. MICs for selected antibiotics were determined in the Bioscreen system by using the inoculation pattern described above and by the conventional agar dilution method.

Detection of ß-galactosidase Activity

An agar plate assay was used to screen the responses of the reporter strain, PK552 *(stx2A*::*lacZ*), in producing ß-galactosidase activity in the presence of antibiotics. The assays were performed by using Luria-Bertani (LB) agar plates containing 20 µg/mL of the chromogenic lactose analog X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; Sigma, Poole, Dorset, UK). An agar overlay of the PK552 reporter strain was made by mixing 100 µL of an overnight culture with 3 mL of molten LB top agar, cooled to 48°C, and pouring the mixture over the surface of an LB agar plate. The plate was incubated with antibiotic discs (see below) placed on the surface, under the conditions specified. Expression of ß-galactosidase was indicated by the enzymatic cleavage of X-gal, resulting in a blue color, the intensity of which related to the amount of enzyme produced.

Microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C were produced in a VAIN incubator (Don Whitley Scientific, Shipley, Yorkshire, UK). Microaerobic conditions at 42°C and anaerobic conditions were produced in gas jars by using Oxoid gas-generating kits according to the manufacturer's instructions.

Antibiotic discs (Oxoid/Unipath, UK) were as follows: ofloxacin (5 µg), nalidixic acid (30 µg), cinoxacin (100 µg), enrofloxacin (5 µg): flumequine (30 µg), ciprofloxacin (1 µg), perfloxacin (5 µg), norfloxacin (10 µg), amoxycillin/clavulanic acid (20/10 µg), imipenem (10 µg), aztreonam (30 µg), ceftazidime (30 µg), cefotaxime

(30 µg), cefuroxime (5 µg), piperacillin/tazobactam (10/75 µg), ampicillin (10 µg), cephalexin (30 µg), chloramphenicol (30 µg), doxycycline (30 µg), erythromycin (15 µg), trimethoprim (5 µg), sulphamethoxazole (25 µg), furazolidone (50 µg), amoxicillin (25 µg), novobiocin (30 µg), rifampin (25 µg), gentamicin (10 µg), fosfomycin (200 µg) (oral and systemic salts), polymyxin B (300 IU), and metronidazole (50 µg).

The Bioscreen C system was also used to measure total ß-galactosidase activity in whole cultures of strain PK552 by orthonitrophenyl-ß-D-galactoside (ONPG) assay (16). Aliquots (20 µL, 4 replicates) of culture were removed from wells of the honeycomb plate and added to a fresh plate. To this, 180 µL of Z buffer (60 mM Na_2HPO_4 $7H_2O$, 40 mM NaH_2PO_4 H_2O, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM ß-mercaptoethanol, pH 7.0) containing 2 mg/mL lysozyme, 0.01% sodium dodecyl sulfate (SDS), and 100 µg/mL chloramphenicol was added to the cells. The plate was incubated at 37°C for 30 min to lyse the cells and placed on ice until use. The Bioscreen C system was prewarmed to 28°C, and 40 µL of 4 mg/mL ONPG solution (made up in Z buffer) was added to each well. Plates were incubated in the Bioscreen C system at 28°C for 4 hours, and OD was recorded at both 420 nm and 540 nm every 10 min. ß-galactosidase activity was determined by using the linear portion of the corrected OD_{420} / time relationship by the Miller formula, adjusted for the sample volume (16). Replicate samples (at least four in all assays reported) yielded mean values with coefficients of variation <10% in all cases.

Results

All the quinolones induced reporter expression, while only a few of the other agents had this effect (Figure 1). The induction occurred in three general patterns: a defined zone within the zone of growth inhibition (quinolones), a defined zone at the growth/no growth interface (furazolidone), or a diffuse zone within the zone of inhibition (trimethoprim). In addition, the incubation conditions appeared to produce a background level of induction or suppression. Microaerobic conditions and to a lesser extent incubation at 30°C were associated with background induction, while anaerobic conditions and incubation at 42°C had a suppressive effect as determined by the intensities of the blue zones. However, some quinolone-mediated induction was always detectable, even under the most suppressive condition (42°C). Induction by furazolidone and trimethoprim was, in general, similarly enhanced and

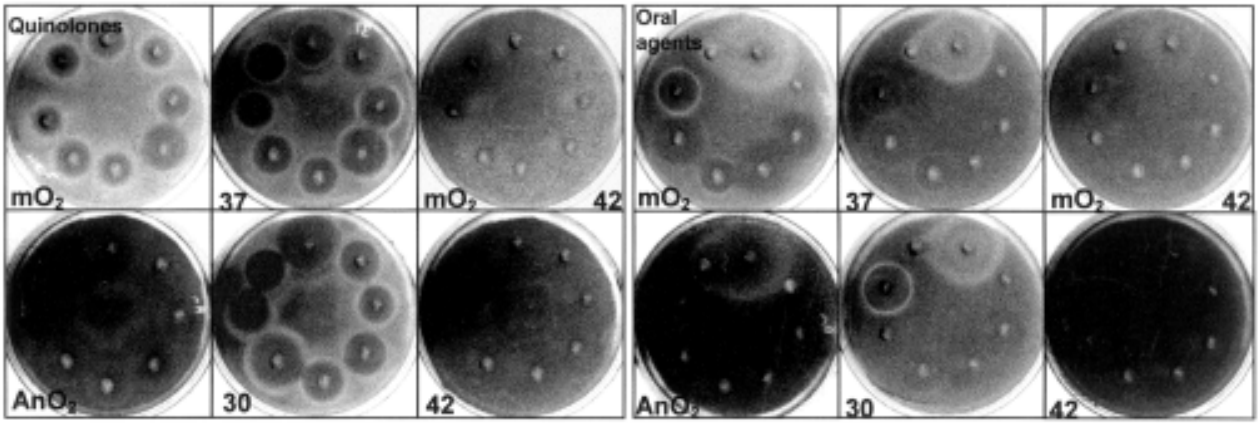

Figure 1. Zones of *stx2* expression induced by various antimicrobial agents under different incubation conditions. The disc-diffusion assay plates demonstrate background levels and zones of blue coloration related to reporter gene β-galactosidase activity produced by our reporter strain. Digital images were acquired and processed identically. Color was encoded by the CMYK system, and only the cyan component is displayed as a gray-scale image. Blue zones appear as lighter areas of the plate, the intensity of which represents the blue intensity as judged by direct comparison. Abbreviations: mO_2, microaerobic and AnO_2, anaerobic incubation conditions; numbers denote incubation temperatures (C). Left panel, quinolones (clockwise from top): ofloxacin, nalidixic acid, cinoxacin, enrofloxacin, flumeqine, ciprofloxacin, perfloxacin, and norfloxacin. Right panel, other oral agents (clockwise from top): trimethoprim, erythromycin, doxycycline, chloramphenicol, cephalexin, amoxycillin, furazolidone, and sulphamethoxazole.

suppressed, although 42°C abolished all induction, and anaerobic conditions did not suppress trimethoprim/sulphamethoxazole induction.

As a group, only the agents that inhibit prokaryotic translation failed to induce reporter expression under any condition tested (Table). Like furazolidone, although less intensely, several ß-lactam agents induced expression at the growth/no growth interface. Imipenem (a carbapenem) not only failed to induce expression but also inhibited induction by the adjacent monobactam, aztreonam (not shown), at subgrowth-inhibitory levels. We subsequently observed that this suppressive effect was confined to ß-lactam-mediated induction (i.e., imipenem showed no suppressive effect against quinolones, trimethoprim, or furazolidone). We also noted an apparent inhibitory effect of clavulanic acid on amoxycillin in the co-amoxyclav combination and a requirement for potentiating conditions (30°C, mAO_2 37°C) for detectable induction by novobiocin, polymyxin B, and rifampin.

We examined some of the features of induction by quantitative assay of ß-galactosidase in cultures induced by exposure to antibiotics in liquid medium (Figures 2 and 3). The relationship of induction to MIC was consistent with that observed in the plate assays. Cefuroxime and furazolidone showed induction at sub-MIC levels, while the highest levels of induction with ofloxacin and trimethoprim were

Table. Induction of *stx2* expression by antibiotics under different incubation conditions[a]

Target of action	Incubation conditions[b]						
Agent (disc content mg/L)	mO_2/37	O_2/37	mO_2/42	AnO_2/37	O_2/30	O_2/42	Zone pattern[c]
Background	+	-	-	-	+	-	NA
DNA Gyrase							
Quinolones[d]	+	+	+	+	+	+/-	≥MIC
Novobiocin (30)	+	-	-	-	+	-	≥MIC
Folate metabolism							
Trimethoprim (5)	+	+	-	+	+	-	≥MIC
Smx (25)	+	+	-	+	-	-	≥MIC
DNA							
Metronidazole (50)	-	-	-	-	-	-	NA
Furazolidone (50)	+	+	-	-	+	-	=MIC
Cell envelope							
Cephalexin (30)	+	+	-	-	+/-	-	=MIC
Amoxycillin (25)	+	+	-	-	-	-	=MIC
Amoxy/Clav (20/10)	+/-	-	-	-	-	-	=MIC
Ampicillin (10)	+	+	-	-	-	-	=MIC
Pip/Taz (10/75)	+	+	-	-	-	-	=MIC
Imipenem (10)	-	-	-	-	-	-	NA
Aztreonam (30)	+	+	-	-	-	-	=MIC
Cefuroxime (5)	+	+	-	-	-	-	=MIC
Ceftazidime (30)	+	+	-	-	-	-	=MIC
Cefotaxime (30)	+	+	-	-	-	-	=MIC
Fosfomycin (200)	-	-	-	-	-	-	NA
Polymyxin B (300 IU)	+/-	-	-	-	+	-	=MIC
Translation							
Gentamicin (10)	-	-	-	-	-	-	NA
Chloramphenicol (30)	-	-	-	-	-	-	NA
Doxycycline (30)	-	-	-	-	-	-	NA
Erythromycin (15)	-	-	-	-	-	-	NA
Transcription							
Rifampin (25)	-	-	+/-	-	-	-	NA

[a] *Stx2* induction effects (zones of blue coloration, Figure 1): -, not detected, +/-, borderline induction, +, definite induction, ≥MIC, zone of induction within the zone of growth inhibition; =MIC, induction on the edge of the zone of inhibition; NA, not applicable; Amoxy/Clav, amoxycillin/clavulanic acid; Pip/Taz, piperacillin/tazobactam; Smx, sulphamethoxazole.

[b] O_2, aerobic; mO_2 microaerobic; AO_2, anaerobic/incubation temperature °C

[c] Note: The patterns indicated were detected in three separate experiments and recorded by two independent observers.

[d] The quinolones tested are listed in the Materials and Methods section.

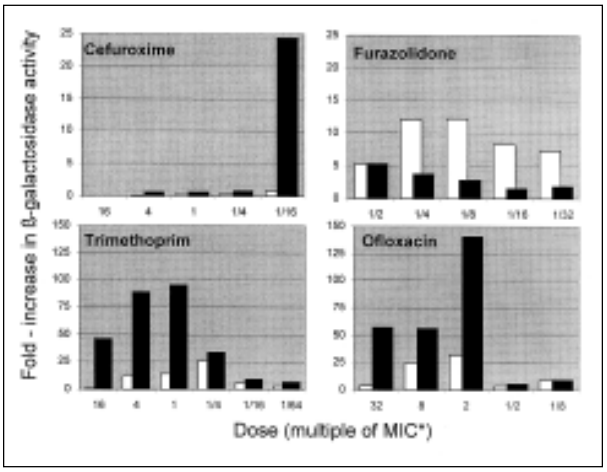

Figure 2. Dose-response characteristics of antibiotic-induced expression of *stx2::lacZ*. Exponential-phase inocula were exposed to antibiotics for 4 h (white bars) or 24 h (black bars), and β-galactosidase activity was determined at the end of these periods. Fold-increase = Test activity (+ antibiotic)/control activity (- antibiotic). Note the difference in scale between upper and lower panels. *MICs refer to the antibiotic concentrations required to inhibit growth in the Bioscreen system, using the same inoculation pattern as in the antibiotic induction experiments. The values were cefuroxime 10 g/L, furazolidone 32 g/L, trimethoprim 0.6 g/L and ofloxacin 0.05 g/L.

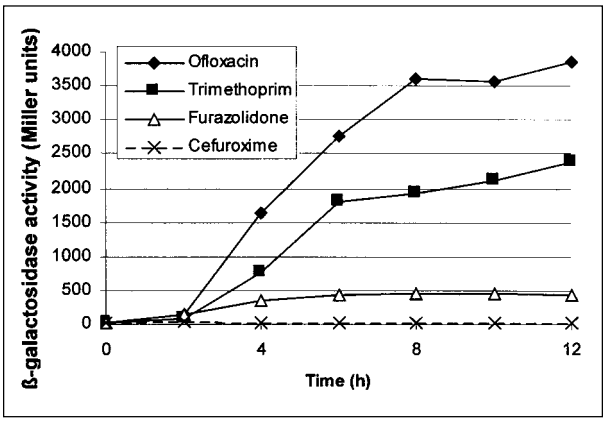

Figure 3. Time course of *stx::lacZ* induction by antibiotics. Exponential-phase cultures were exposed to the antibiotics indicated for up to 12 h, and whole culture β-galactosidase activities were determined. The exposure levels were cefuroxime 0.625 g/L (MICx1/16), furazolidone 8 g/L (MICx1/4), trimethoprim 0.6 g/L (1xMIC) and ofloxacin 0.1 g/L (2xMIC).

seen at supra-MIC exposure levels; the latter two agents were clearly more potent inducers (Figures 2 and 3). We also examined the effects of fosfomycin (0.03-80 g/L) in this assay; no inducing effect was detected.

The time course experiments (Figure 3) confirm the relative inducing potencies (Figure 2) and show that furazolidone-mediated induction is complete by 8 h. Levels of furazolidone-induced enzyme activity were lower at 24 h than at 4 h or 12 h. In contrast, trimethoprim and ofloxacin induction rapidly increased up to 6 h and 8 h, respectively, then continued to increase more slowly until the final measurement at 12 h. Additional expression induced by these two agents continued up to 24 h (Figure 2), while cefuroxime-induced expression occurred only between 12 h and 24 h.

Discussion

These patterns of reporter gene expression demonstrate that several antimicrobial agents have the capacity to increase the amount of Stx2 synthesized by our STEC strain. Levels of ß-galactosidase expression in the reporter strain are closely correlated with biologically active toxin produced by the parental wild type strain (12). The demonstrated link to de novo gene expression distinguishes our study from earlier work in which it could not be determined whether increased toxin levels reflected release of preformed toxin by cell lysis or increased synthesis (17-20). The inductions we observed show how certain agents and environmental conditions may increase the amount of toxin in infected persons and that, in the absence of definitive clinical data, they provide a rational basis for avoiding certain agents in treating patients who may have STEC infections.

Several of the agents tested here—the quinolones (21), trimethoprim (22), furazolidone, and metronidazole (23)—have a recognized capacity to induce bacterial SOS response, which is initiated when damaged bacterial DNA interacts with and activates the multifunctional RecA protein. Activated RecA, in turn, causes the degradation of two key repressor proteins, LexA and CI. The resulting de-repression of genes regulated by LexA leads to the temporary arrest of DNA synthesis and cell division and the activation of error-prone DNA repair. In strains carrying an integrated λ-like bacteriophage, cleavage of the CI phage repressor/activator

protein leads to the induction of previously silent phage-encoded genes (in this case including *stx2A* and *stx2B*), followed by production of phage particles and host bacterial cell lysis (9).

The most potent SOS inducers, the quinolones and trimethoprim, produced the most prominent effects in both agar and liquid culture assays. Metronidazole did not induce reporter expression under the conditions tested; however, this agent would not normally be expected to affect *E. coli*. Another potent SOS inducer, mitomycin C, which is known to increase toxin levels in vitro (24,25), tests positive in agar and broth induction tests with our reporter strain (results not shown). The potential importance of the SOS response to pathogenesis in vivo has recently been underlined by a study in which STEC strains with mutations in *recA*, a critical gene for SOS induction, were rendered avirulent in a mouse 50% lethal dose test (15).

The importance of antibiotic-induced Stx expression would be reinforced if the implicated agents were in some way associated with occurrence or severity of STEC disease. The results of available studies have conflicted with regard to the influence of antibiotics. The age groups studied, the timing of antibiotic therapy, and the range of agents used complicate the analyses. Nonetheless, use of quinolones and trimethoprim/sulphamethoxazole (26,27) has been implicated as a risk factor for HUS. In a recent clinical study, children treated with cephalosporins or trimethoprim-sulphamethoxazole had, respectively, 13.4- and 17.7-fold increases in risk for HUS (28). Our observations suggest that the net effect of one of these agents on the exposure of an infected patient to toxin depends on the stage of the infection, the number of organisms present at the time antibiotics are administered, the immediate environmental conditions of those organisms, and the time-concentration profile and bactericidal effect of the drug. This complex interplay of factors could render an SOS-inducing antibiotic clinically beneficial (e.g., if the numbers of infecting organisms were insufficient to produce substantial amounts of toxin and they were all killed by the first dose), neutral, or disadvantageous in different situations. Moreover, exposure of patients to other potential SOS-inducing agents (cf. 9,23) could further complicate the relationship between antibiotic use and severe STEC disease. A strong association between mitomycin C administration and HUS was detected in a study of "cancer-associated" HUS (29).

Grif and colleagues reported on the effects of sub-MIC levels of 13 antibiotics on release of biologically active toxin (as distinct from toxin gene expression) by three STEC strains into culture supernatants after overnight incubation (20). These authors observed substantial interstrain differences in the responses, as well as increased levels of toxin after exposure to several agents that had not previously been associated with SOS-inducing activity. Grif et al. did not distinguish between enhanced release of preformed toxin and increased toxin production, and the most potent inducing effects we have observed were at suprainhibitory levels of exposure. Nonetheless, we cannot rule out interstrain differences in susceptibility to SOS activation and the possibility that other induction mechanisms may be involved. Our observations on SOS induction by several ß-lactam agents and the inhibitory effects of imipenem appear pertinent. Suppression of toxin expression by imipenem supports previous observations on toxin release (30). Paired disc-diffusion assays involving imipenem and other recognized SOS-inducing antibiotics indicated that this effect was confined to induction by ß-lactam agents (Kimmitt, Harwood, and Barer, unpubl data). This finding, combined with the different time course of induction with cefuroxime, reinforces the view that there may be induction pathways distinct from the SOS response. Although the nature of the putative alternate induction pathway(s) remains obscure, the clear evidence for ß-lactam-mediated induction may contraindicate use of the implicated agents in treating patients with STEC infection.

Although any increase in exposure of STEC-infected persons to Shiga toxins seems undesirable, SOS-mediated induction seems particularly hazardous because of its potential rapid effects. We report kinetics (Figure 3) somewhat slower than those we observed previously in the shaken conical flask incubations (12). The rapid buildup in toxin levels associated with SOS induction could facilitate entry of toxin into the bloodstream and subsequent disseminated effects on the kidneys and other organs. SOS-mediated induction also leads to dissemination of the toxin-encoding bacteriophage.

Matsushiro et al. observed parallel increases in toxin and bacteriophage counts in response to

norfloxacin (13), and we have made similar observations with ofloxacin and trimethoprim (Kimmit, Harwood and Barer, unpubl data). Moreover, sequencing studies clearly indicate that expression of *stx2* and bacteriophage genes is coordinately regulated (11,14). Hemorrhagic colitis and HUS attributable to STEC were recognized after trimethoprim and the 4-quinolones were introduced into human and veterinary clinical practice, and the substantial recent increase in reports of STEC disease follows expanded use of fluoroquinolones.

Fluoroquinolones and trimethoprim have been recommended for prophylaxis and treatment of travelers' diarrhea (31), and the former are often used to treat severe bacterial enteric infections. Our findings indicate that this approach may be inappropriate if STEC infection is a possibility. Furthermore, reports that certain antimicrobial agents may ameliorate or reduce symptoms or the frequency of life-threatening complications in STEC infections provide an incentive to find a rational basis for selection (8,27). Our results suggest that agents with SOS-inducing activity, antimicrobial or otherwise, should be avoided (Table).

We conclude that SOS-mediated induction of Shiga toxins and toxin–encoding bacteriophages may contribute to the emerging epidemiologic pattern of STEC disease. Many other bacterial virulence determinants are encoded on lysogenic bacteriophage genomes (32), and the issues raised here may have public health and clinical implications beyond the understanding of STEC disease.

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Dr. Kimmitt is trained in medical microbiology and molecular biology. He is currently studying the molecular biology of chlamydia infection at the London School of Hygiene and Tropical Medicine.

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Imported Lassa Fever in Germany: Molecular Characterization of a New Lassa Virus Strain

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We describe the isolation and characterization of a new Lassa virus strain imported into Germany by a traveler who had visited Ghana, Côte D'Ivoire, and Burkina Faso. This strain, designated "AV," originated from a region in West Africa where Lassa fever has not been reported. Viral S RNA isolated from the patient's serum was amplified and sequenced. A long-range reverse transcription polymerase chain reaction allowed amplification of the full-length (3.4 kb) S RNA. The coding sequences of strain AV differed from those of all known Lassa prototype strains (Josiah, Nigeria, and LP) by approximately 20%, mainly at third codon positions. Phylogenetically, strain AV appears to be most closely related to strain Josiah from Sierra Leone. Lassa viruses comprise a group of genetically highly diverse strains, which has implications for vaccine development. The new method for full-length S RNA amplification may facilitate identification and molecular analysis of new arenaviruses or arenavirus strains.

Transmission of Lassa virus (family *Arenaviridae*) from its natural rodent reservoir to humans can cause hemorrhagic fever, a clinical syndrome associated with high death rates. Lassa fever is endemic in West Africa and has been reported from Sierra Leone, Guinea, Liberia, and Nigeria (1-4). The geographically restricted occurrence of the disease is not well understood as its rodent host (*Mastomys* species) is prevalent in much larger areas of sub-Saharan Africa. The importation of Lassa virus into other regions, for example by travelers, is rare, with only a few cases documented (5-9). Although imported disease often raises public concern because of the possibility of human-to-human transmission; the highly pathogenic nature of the virus; and the lack of an effective, safe therapy, the actual risk for infection from an imported case appears to be low (5,7), and adequate guidelines have been published for disease management in patients and contacts (5,10).

Arenaviruses can be divided phylogenetically, serologically, and geographically into two major complexes: the Old World complex (e.g., Lassa virus, lymphocytic choriomeningitis virus [LCMV]) and the New World complex (e.g., Tacaribe virus, Junin virus, Machupo virus) (11). Isolates of Lassa virus also differ in their genetic, serologic, and pathogenic characteristics (11-13). This variability is evidenced by the poor cross-complement fixation and cross-neutralization among Lassa virus isolates of different geographic origins (3,14). Serologic differences were demonstrated by testing a panel of Lassa virus-specific monoclonal antibodies against many Lassa virus isolates (13).

The single-stranded arenavirus genome consists of a small (S) and a large (L) RNA fragment, sizes 3.4 kb and 7 kb, respectively. The S RNA encodes the viral glycoprotein precursor protein (GPC) and the nucleoprotein (NP). The L RNA encodes the viral polymerase and a small, zinc-binding (Z) protein. Sequencing of the complete S RNA of two Lassa virus strains, originating from Sierra Leone (strain Josiah) (15) and Nigeria (strain Nigeria) (16), as well as sequencing of short S RNA fragments of additional isolates (e.g., strain LP from Nigeria) (11,17,18) showed considerable genetic

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differences. Sequence analysis of the full-length S RNA of a large number of isolates has been complicated by technical problems such as the necessity to produce enough virus in cell culture for direct cloning (15,16) or localization of conserved regions within the S RNA for polymerase chain reaction (PCR) primers (18).

We report the isolation and sequence characterization of a new Lassa virus strain from a traveler who imported the virus into Germany. This novel strain originates from an area of West Africa where Lassa fever has not yet been reported. To facilitate molecular analysis of new Lassa virus isolates, a long-range reverse transcription (RT)-PCR was established. The primers bind to highly conserved RNA termini and allow amplification of full-length S RNA directly from serum.

Methods

The Patient

A 23-year-old German woman became ill with fever and flulike symptoms after traveling through three West African countries (Figure 1). In Abidjan, Côte D'Ivoire, she visited the outpatient department of Centre Hospitalier Universitaire de Cocody, where her illness was diagnosed as malaria. She returned to Germany on day 6 of illness and was hospitalized at the Diakonie Hospital at Schwäbisch Hall. After the diagnoses of malaria and bacterial infection were ruled out, the patient was transferred on day 9 of illness to the department of tropical diseases at the Missionsärztliche Klinik in Würzburg, where she was noted to have fever, pharyngitis, diarrhea, and pleural effusion. Lassa fever was suspected, and serum was sent to the Bernhard-Nocht-Institut, Hamburg, where Lassa virus infection was diagnosed by PCR and virus isolation. Despite immediate ribavirin treatment and intensive care, the patient's clinical condition deteriorated, and she died on day 14 of illness with hemorrhage, organ failure, and encephalopathy (19).

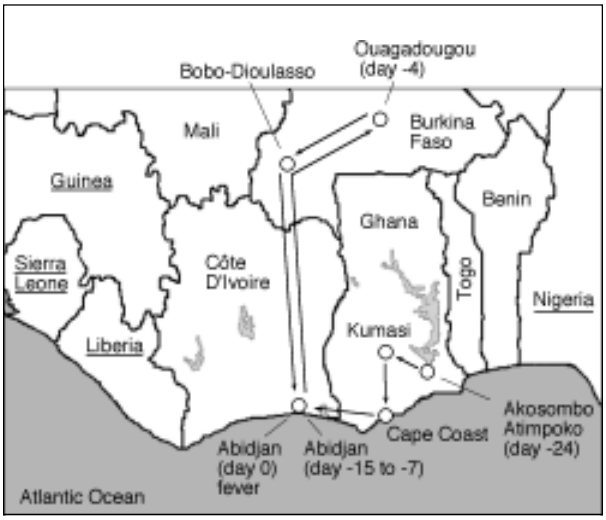

Figure 1. Map of West Africa and travel history of the patient before the onset of febrile illness (day 0). Countries in which Lassa fever is endemic (1-4) are underlined.

Virus Isolation and Detection by Immunofluorescence and Immunoblot

In the biosafety level 4 facility, Vero cells grown in 10 mL of Leibowitz medium were injected with 1 mL, 0.1 mL, 0.01 mL, and 0.001 mL of serum. The cell culture was examined daily by immunofluorescence for Lassa virus infection as well as morphologic changes. Cells were harvested, spread onto immunofluorescence slides, air-dried, and acetone-fixed. Immunofluorescence was performed by using Lassa virus NP-specific monoclonal antibody L2F1 (20) (dilution of 1:50) and fluorescein isothiocyanate-labeled anti-mouse immunoglobulin (Ig)G diluted 1:60 (Dianova, Hamburg, Germany).

For immunoblot analysis, cells were harvested and pelleted by centrifugation. The cell pellet was lysed in SDS loading buffer and boiled for 5 min. Total cell lysate was separated in an sodium dodecyl sulfate (SDS)-15% polyacrylamide gel, and proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Germany). Lassa virus Z protein was detected by chemiluminescence with polyclonal chicken anti-Z serum (dilution 1:5,000) and peroxidase-labeled anti-chicken IgY (dilution 1:2,000) (Dianova) as secondary antibody.

RT-PCR of S RNA

Virus RNA was isolated from 140 µL of serum or cell culture supernatant of Lassa virus and LCMV-infected Vero and L cells, respectively, by using the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (elution of RNA in 50 µL of buffer). For reverse transcription of the full-length S RNA, purified RNA (3-6 µL) was incubated with 20 pmol of RT primer (CGCACCGDGGATCCTAG GC) in an 8-µL assay at 70°C for 15 minutes. The

mixture was quickly chilled on ice and then centrifuged. A 19-µL reaction premix containing 8 µL RNA-primer mix, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 500 µM dNTP was incubated at 50°C for 2 minutes. Then 200 units (1 µL) Superscript II reverse transcriptase (Life Technologies, Karlsruhe, Germany) and a drop of mineral oil were added. The reaction was run with the following temperature profile: 50°C for 30 minutes, 55°C for 5 minutes, 50°C for 20 minutes, 60°C for 1 minute, and 50°C for 10 minutes. The enzyme was inactivated at 70°C for 15 minutes. RNA was removed by adding 2 units (1 µL) RNase H (Life Technologies) and incubating at 37°C for 20 minutes. cDNA derived from full-length S RNA was amplified by using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany) with a hot start. A 45-µL reaction premix containing 1 µL of cDNA, 1X reaction buffer with 1.5 mM $MgCl_2$, 200 µM dNTP, and 0.3 µM primers PCR1 to PCR4 in different combinations (PCR1, tatggcgcgcCGCAC CGDGGATCCTAGGC; PCR2, tatggcgcgcCGCAC CGAGGATCCTAGGCATT; PCR3, tatggcgcgcCG CACCGGGGATCCTAGGCAAT; PCR4, tatggcgc gcCGCACCGGGGATCCTAGGCTT; PCR5, tatgg cgcgcCGCACCGDGGATCCTAGGCWWT; heterologous sequences to facilitate cloning via *Asc*I in lower case) was overlaid with 2 drops of oil and heated to 55°C. Subsequently, 5 µl of enzyme mixture containing 2.6 units *Taq* and *Pwo* polymerase in 1X reaction buffer with 1.5 mM $MgCl_2$ was added. PCR was run for 40 cycles with 94° for 1 minute, 55°C for 1.5 minutes, and 72°C for 3 minutes with an increment of 2 minutes after every 10 cycles in a Robocycler (Stratagene, La Jolla, California).

In a separate PCR, a 340-bp fragment of the S RNA was amplified by using Superscript One-Step RT-PCR System (Life Technologies) and primers 36E2 (ACCGGGGATCCTAGGCATTT) and 80F2 (ATATAATGATGACTGTTGTTCTTT GTGCA) as described previously (18).

Sequence Determination

PCR products were purified by using the QIAquick PCR purification kit (Qiagen) and were directly sequenced with the BigDye Terminator AmpliTaq kit (Applied Biosystems, Weiterstadt, Germany). Extension products were separated on an ABI 377 automated sequencer (Applied Biosystems). The 340-bp fragment was sequenced by using primers 36E2 and 80F2. The full-length S RNA amplification product of independent RT-PCRs was pooled, and plus and minus strands were sequenced by the following primers (numbers denote the position of the 5'-nucleotide of the primer in the genomic sequence of Lassa virus S RNA, strain Josiah; the sequences of LV-S^J and LV-S^{AV} primers are derived from strain Josiah and strain AV, respectively): LV-S^J 1-plus (GCACCGGGGATCCTAGGCATTTTGGTTGC); LV-S^J 359-plus (GGACTAGAACTGACCTTGACC AACAC); LV-S^{AV} 834-plus (GCACATTCACGTGG ACACTGTCAGA); LV-S^{AV} 1032-plus (TGAAAT CTGAAGCACAAATGAGCAT); LV-S^{AV} 1883-plus (GTGATTCAAGAAGCTTCTTTATGTC); LV-S^{AV} 2372-plus (AGATTTTGTAGAGTATGTTT CATA); LV-S^J 2937-plus (TGCACTTAATGGCCTTTCTG TTCT); LV-S^{AV} 479-minus (GGTGGAAAGTTGA GATTAT GCTCAT); LV-S^J 991-minus (CATGTC ACAAAATTCCTCATCATG); LV-S^{AV} 1906-minus (ACATAAAGAAGCTTCTTGAATCACA); LV-S^{AV} 1955-minus (ATTGAGGCGCTCCCCCGGAATA TGG); LV-S^J 2618-minus (CTAAATATGATTGAC ACCAAGAAAAG); LV-S^J 3092-minus (AATCAA GCGGTCAACAATCTTGTTGA); and LV-S^J 3402-minus (CGCACAGTGGATCCTAGGCTATTGGA TTGC). Each nucleotide position was sequenced by at least two different primers. The overlapping sequences were identical, and no sequence ambiguities were observed.

Phylogenetic Analysis

Phylogenetic analysis was performed with the NP gene fragment for which the largest set of arenavirus sequence data exists, at position 1724-2349 of the genomic S RNA of Lassa Josiah (11). NP gene sequences were aligned for Lassa AV and the following arenavirus strains (virus, GenBank/EMBL accession number): Tacaribe, M20304; Ippy, U80003; Mopeia AN20410, U80005; Mopeia AN21366, M33879; Mobala 3076, AF012530; Mobala 3051, U80006; Mobala 3099, U80007; Lassa LP, U80004; Lassa Nigeria, X52400; Lassa Josiah, J04324; LCMV Armstrong, M20869; LCMV WE; M22138; and LCMV MaTu-MX, Y16308. Full-length NP and GPC gene sequences were analyzed for Lassa strains Josiah, Nigeria, and AV, and for Mopeia AN21366. Phylogenetic analysis was performed by using programs of the PHYLIP 3.57c package (21). A gap was treated as a single mutation event. Distance matrix calculation and neighbor-joining (NJ) analysis were conducted with the

programs DNADIST and NEIGHBOR, and maximum likelihood (ML) analysis was conducted with the DNAML program. Analyses were performed with default settings on a bootstrapped dataset (100 replicates).

Results

Origin and Isolation of the New Lassa Virus Strain

The exact geographic origin of the virus and the mode and date of transmission could not be determined. During the incubation period, which can last up to 3 weeks (22), the patient visited Ghana, Côte D'Ivoire, and Burkina Faso (Figure 1). Therefore, the virus originated from one of these West African countries, where Lassa fever has not been reported.

The virus grew rapidly in Vero cells. Fifteen hours after inoculation, few cells were positive, and after 40 hours virtually all cells were infected in cultures injected with 0.1 mL serum as tested by immunofluorescence. In contrast to previous Lassa virus isolations in Vero cells (23), no substantial cytopathic effects were seen. Whether this observation is due to technical variables (such as inoculation dose or culture duration) or reflects a biologic feature of the isolate is unclear. Immunofluorescence with an NP-specific monoclonal antibody showed the speckled, cytoplasmic pattern typical of Lassa virus NP (13,20) (Figure 2A). Isolation of a Lassa virus was further confirmed by immunoblot analysis of strain AV-infected cells using Lassa virus Z protein-specific antibody. An 11-kDa Z protein was demonstrated (Figure 2B) as has recently been detected in Lassa Josiah-infected cells (24).

RT-PCR for Amplification of Full-Length S RNA

To improve and simplify molecular analysis of new Lassa virus isolates, a protocol for reverse transcription and PCR amplification of the full-length S RNA was established. The RT primer used for reverse transcription binds to the extreme termini of genomic and antigenomic S and L RNA (Figure 3A). These termini are highly conserved among all arenaviruses. Reverse transcription was performed at a baseline temperature of 50°C with short rises to 55°C and 60°C to resolve the stable RNA secondary structure of the intergenic region. A mixture of *Taq* and *Pwo* polymerases, the latter of which has 3'-5'-exonuclease (proofreading) activity, was used in PCR. This enzyme combination can

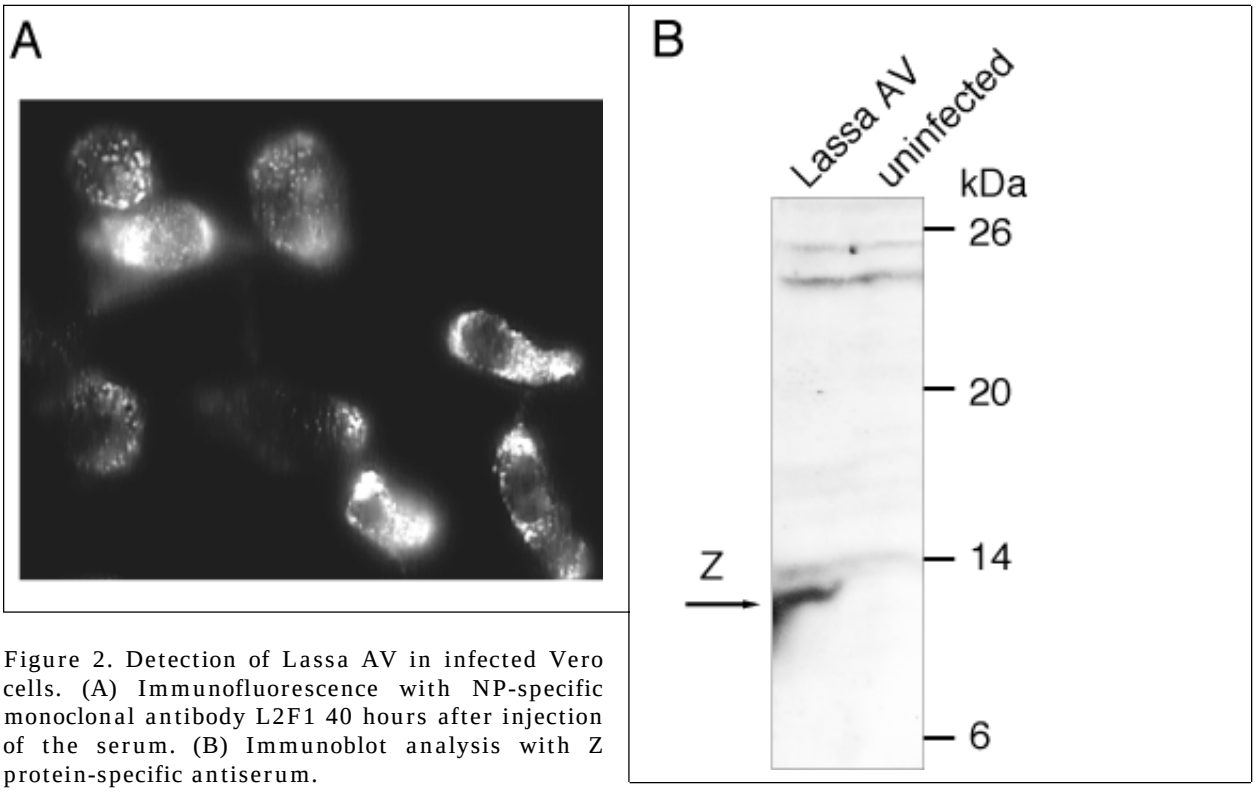

Figure 2. Detection of Lassa AV in infected Vero cells. (A) Immunofluorescence with NP-specific monoclonal antibody L2F1 40 hours after injection of the serum. (B) Immunoblot analysis with Z protein-specific antiserum.

amplify long templates with high fidelity and sensitivity (25). Various primers and primer combinations tested were found suitable for efficient amplification of full-length S RNA of Lassa virus and/or LCMV: PCR1; PCR2; PCR3; PCR2 and PCR3; PCR2, PCR3, and PCR4; and PCR5 (Figure 3B and data not shown). The PCR primers were largely identical to the RT primer but contained heterologous 5'-sequences that allow cloning of the amplification product through the restriction enzyme *Asc*I. The 3'-end of primer PCR1 exactly corresponded to that of the RT primer, but primers PCR2 to PCR5 contained additional two or three nucleotides at their 3'-end. These nucleotides were added to reduce or prevent amplification of shorter products generated by mispriming during reverse transcription. Although the 3'-nucleotides of primers PCR2 and PCR3 did not perfectly fit onto both termini of Lassa virus S RNA, each primer alone was able to amplify the full-length fragment (data not shown). This feature, which may facilitate amplification of strains with mutations in the primer binding site, can be explained by the 3'-5'-exonuclease activity of *Pwo* polymerase, which degrades primers and corrects 3'-mismatches (26). In conclusion, we developed an RT-PCR protocol allowing rapid molecular characterization of S RNA of Lassa virus and LCMV isolates. Because of the high conservation of the primer binding sites, the protocol may also be applied to other arenaviruses.

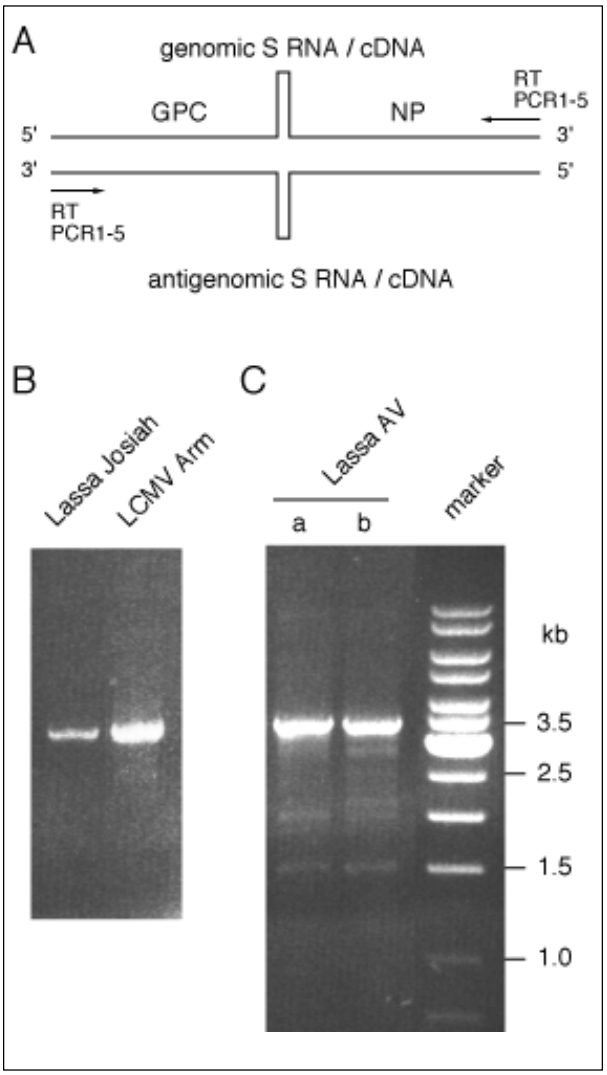

Figure 3. Reverse transcription (RT) and polymerase chain reaction (PCR) amplification of full-length S RNA. (A) Position of the RT and PCR primers at the termini of S RNA. The stem-loop structure in the intergenic region is schematically shown. (B) Amplified S RNA of Lassa Josiah and LCMV Armstrong virus separated in ethidium bromide-stained agarose gel. S RNA was isolated from supernatant of infected cells, and PCR was done with primers PCR2-4. (C) S RNA of Lassa AV was amplified in two RT-PCRs (a and b) and separated in ethidium bromide-stained agarose gel. RNA was isolated from serum, and PCR was performed with primers PCR2-4. Quantification of Lassa virus RNA in the specimen by endpoint titration with the 340-bp PCR assay showed $>10^6$ S RNA molecules/mL serum.

Sequence Determination of S RNA of Lassa AV and Comparison with Lassa Josiah and Nigeria

S RNA was isolated from the patient's serum and amplified in two RT-PCRs with the primer combination PCR2, PCR3, and PCR4. Both reactions showed a major product at the 3.5-kb position (Figure 3C), indicating that the predominant virus population contained full-length S RNA. Some minor species in the 1.5- to 3-kb size range may represent naturally occurring RNA, with deletion as occasionally seen in arenavirus-infected cell cultures (27), or artifact fragments generated during RT-PCR. The PCR products were purified, pooled, and sequenced. The S RNA sequence was confirmed by sequencing the 340-bp PCR fragment produced by primers 36E2 and 80F2. The overlapping sequences were completely identical. The sequence was sent to GenBank and was assigned accession number AF246121.

Alignment of the S RNA sequence of strain Josiah with that of strains AV and Nigeria showed considerable variability among the three

strains (Figure 4). The highest frequency of nucleotide changes, deletions, and insertions was observed at the 3'- and 5'-noncoding regions just upstream of the GPC and NP start codons on the genomic and antigenomic strands, respectively (position 25-55 and 3303-3365). Essentially no

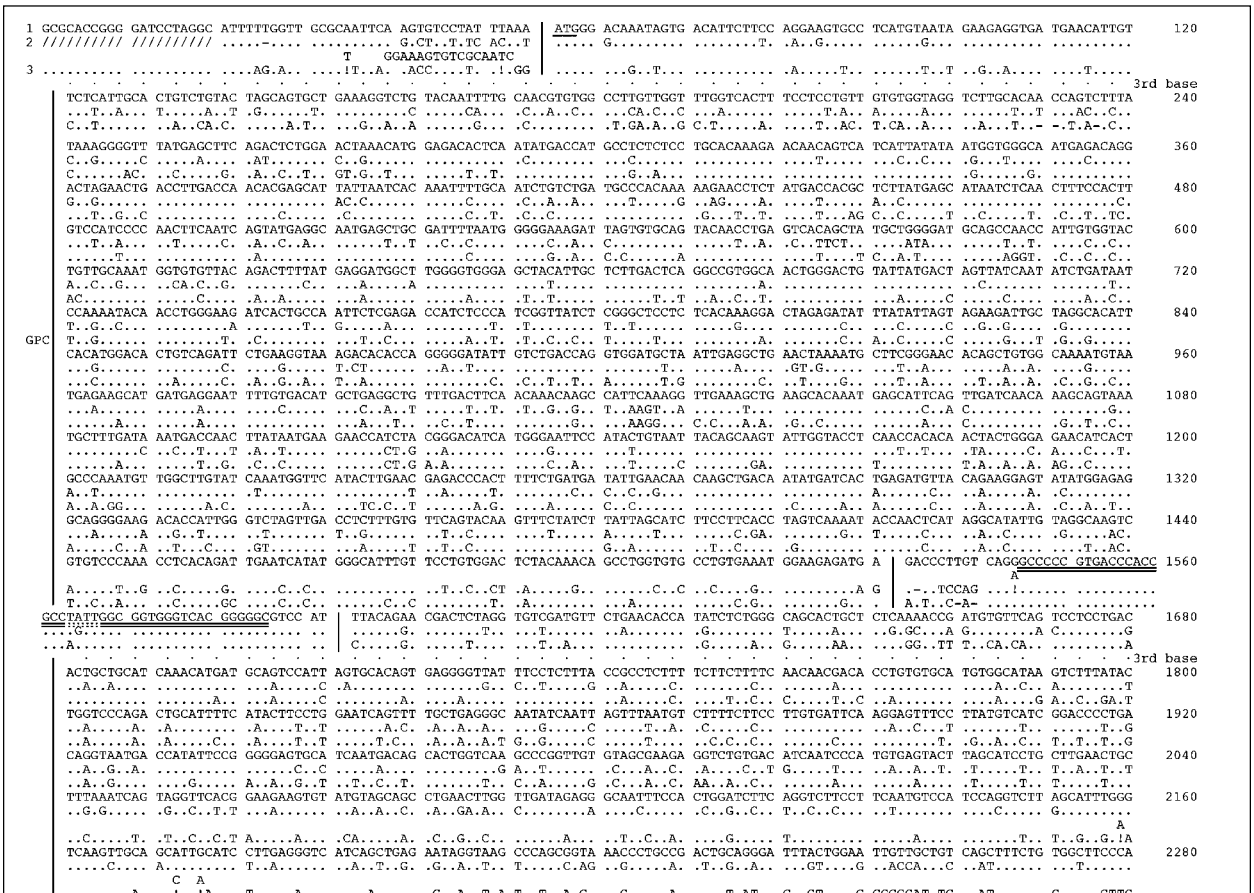

Figure 4. Alignment of the genomic S RNA sequences of Lassa Josiah, AV, and Nigeria (sequences 1, 2, and 3, respectively). The 3'- and 5'-noncoding regions and the intergenic region are separated from the coding regions by vertical bars. Long vertical lines on the left mark the GPC and NP coding regions. Third base positions are marked by a line of dots above each coding region. The GPC and NP start codons are underlined. The stem of the stem-loop structure is underlined by a double line, while the loop is underlined by a dotted line. The RT primer binding sites are indicated by slashes. Inserted nucleotides are shown above the sequence with the position of insertion indicated by a vertical line. The S RNA sequence of strain AV was sent to GenBank and was assigned the accession number AF246121.

nucleotide was conserved in these regions or in a short sequence in the intergenic region between the GPC stop codon and the beginning of the RNA stem-loop structure (position 1532-1540). In contrast to these regions, the RNA stem-loop structure (position 1545-1586) was highly conserved, with no changes in the stem and little variability in the loop. The NP and GPC coding regions differed among the three strains by approximately 20%, almost exclusively because of nucleotide exchanges (Table). The partial NP gene sequence of strain LP differed by 25% from that of strain AV. The mutations were scattered over entire coding regions except for short conserved stretches. The most prominent feature of this variability was the high number of changes at third codon positions, which accounted for approximately 80% of all nucleotide differences (Table). The amino acid variability was considerably lower (5%-9%) than the variability at the nucleotide level (Table). The degree of nucleotide and amino acid sequence divergence was slightly higher in NP than in GPC. Alignment of the GPC amino acid sequences showed differences at the N-terminus and within as well as in the vicinity of the B-cell epitopes (Figure 5) (28,29). The putative GP1/GP2

Table. Nucleotide and amino acid differences between Lassa strains in S RNA coding regions

	% Nucleotide difference in:		% Changes at 3rd codon positions per total changes in:		% Amino acid difference in:	
Strains[a]	GPC	NP	GPC	NP	GPC	NP
AV vs. JOS	16.5	19.6	84.4	81.5	5.1	6.0
AV vs. NIG	20.3	21.3	83.7	78.3	6.7	8.1
NIG vs. JOS	19.2	22.4	83.7	77.1	6.9	8.9

[a]AV, strain AV; JOS, strain Josiah; NIG, strain Nigeria; GPC = glycoprotein precursor protein; NP = nucleoprotein.

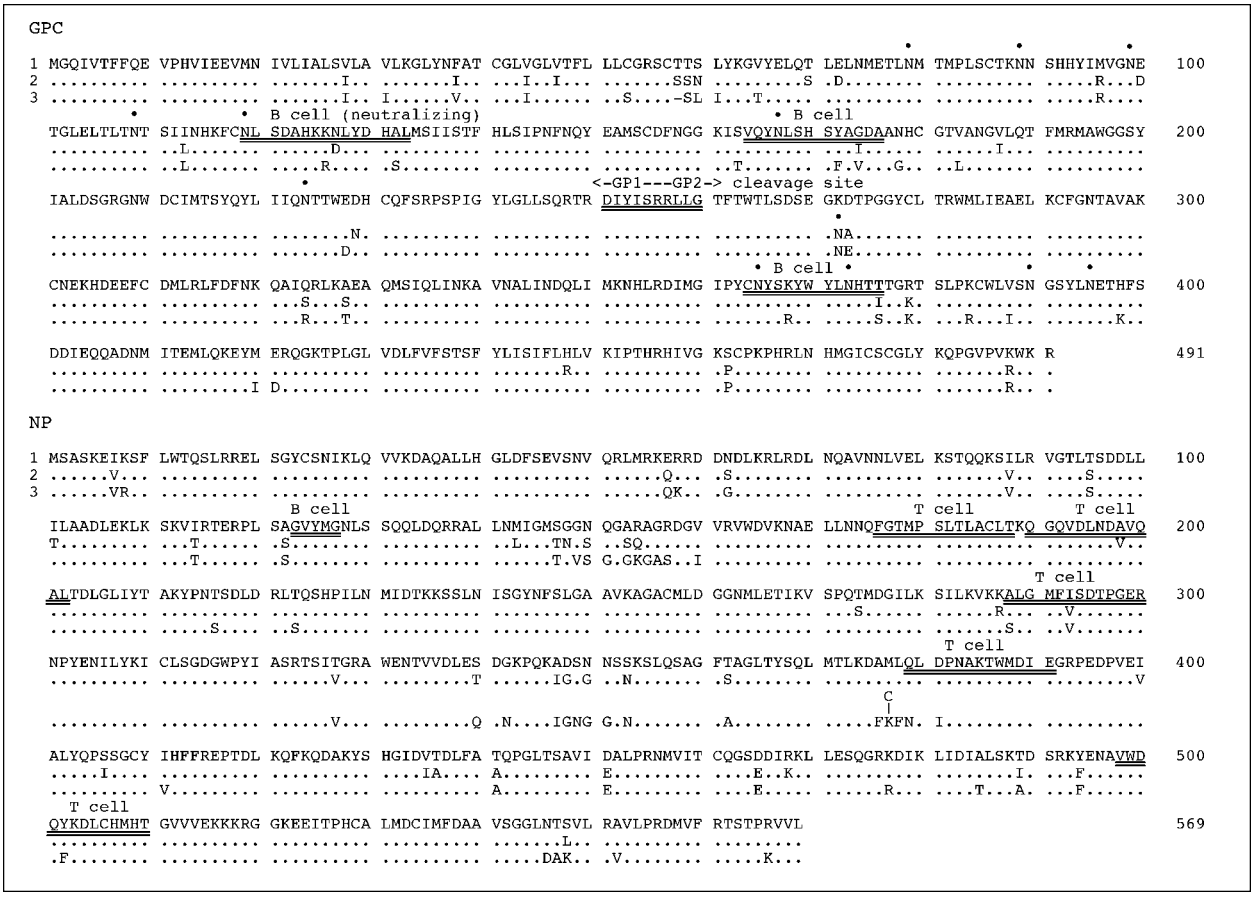

Figure 5. Alignment of the GPC and NP amino acid sequences of Lassa Josiah, AV, and Nigeria (sequences 1, 2, and 3, respectively). B-cell epitopes [GPC 119-133 (28), GPC 124-176 (28), GPC 364-376 (29), NP 123-127 (31)], T-cell epitopes (17), and the putative GPC cleavage site (30) are doubly underlined. Dots above the GPC sequence mark potential N-linked glycosylation sites. Inserted amino acids are shown above the sequence with the position of insertion indicated by a vertical line.

cleavage site (30) was completely conserved, as were potential N-linked glycosylation sites, with the exception of an additional site in Lassa AV and Nigeria at position 272. In NP, two clusters of amino acid variability (position 43-60 and 340-353) were both characterized by a high number of glycine residues at different positions in the three strains (Figure 5). The mapped NP B-cell epitope (31) was conserved, and only a few changes occurred in the T-cell epitopes recently identified in NP (32).

The phylogenetic relationship of Lassa AV to known Old World arenaviruses as well as to the other Lassa strains was analyzed by using partial NP gene sequences. Strain AV segregated with all Lassa strains into a single Lassa group with 100% bootstrap support and was placed in sister relationship with strain Josiah (Figure 6). The latter relationship was confirmed in analyzing the full-length coding regions with 65%/61% (NJ/ML analysis) bootstrap support for the GPC gene and 94%/97% (NJ/ML analysis) bootstrap support for the NP gene.

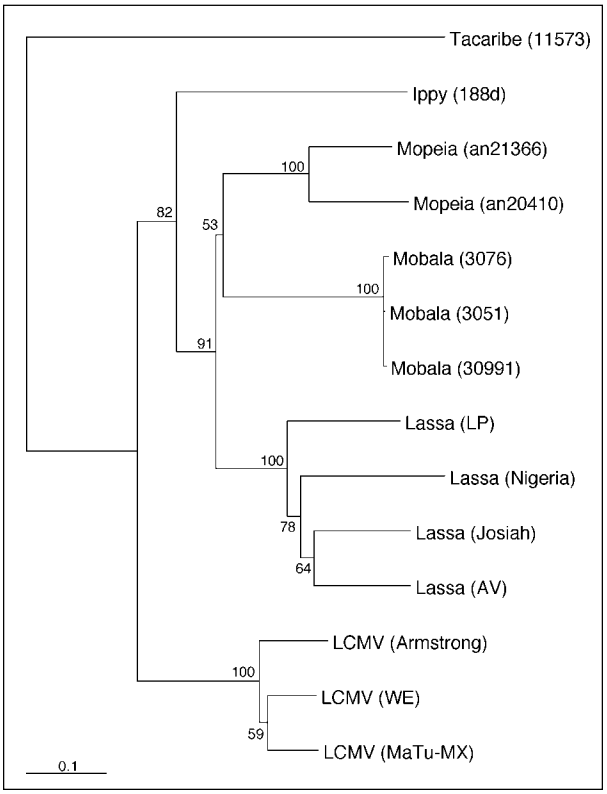

Figure 6. Phylogenetic analysis of Old World arenaviruses, including Lassa AV. The tree was computed for an NP gene fragment by using the neighbor-joining method. Bootstrap support (in %) is indicated at the respective branch. The same topology and similar bootstrap values, except for the terminal lymphocytic choriomeningitis virus branches, were obtained by using the maximum likelihood method. Tacaribe virus belonging to the New World arenaviruses was used as outgroup to root the tree.

Conclusions

The complete S RNA sequences of three Lassa virus strains—Nigeria (16), Josiah (15), and AV—are now known. All three full-length sequences, as well as the partial S RNA sequence of strain LP (11), differ considerably, suggesting that Lassa viruses comprise a monophyletic yet genetically diverse group. Strain AV appears to be phylogenetically most closely related to strain Josiah. Prominent features of this variability are a high number of substitutions at third-base positions, a high degree of divergence at the 3'- and 5'-noncoding regions just upstream of the NP and GPC start codons, but conservation of the intergenic stem-loop as well as the 19-nucleotide termini, which are conserved among all arenaviruses. Conservation of these termini in strain AV was not directly demonstrated in our study but was suggested by the efficient reverse transcription and amplification with primers binding to these ends. The divergence of the 3'- and 5'-noncoding regions (excluding the conserved termini) indicates either that their function does not depend on a specific primary sequence or that the functional variability of these elements has no major impact on the Lassa virus life cycle. These sequences correspond to the 5'-untranslated regions on the NP and GPC transcripts. Variability in these regions, especially in the so-called KOZAK sequence around the start codon (33), may influence efficiency of translation initiation and, thus, protein expression and virus production. Mutations in noncoding regions may eventually explain pathogenic differences among Lassa virus strains (12), as they have in other viruses (34-36). In contrast to the 3'- and 5'-noncoding regions, the RNA stem-loop structure was highly conserved, suggesting that this element does not allow modification without seriously affecting Lassa virus replication. Of the other arenaviruses, only Mopeia virus has stem-loop sequences in common with Lassa virus (37), which may be one reason that both viruses can form stable reassortants (38). The diverse geographic origins of three of the four prototype strains (LP and Nigeria are both of Nigerian origin) and the relatedness of isolates circulating within an area

(13) suggest geographic clustering of Lassa virus strains. Genetic differences among *Mastomys* species of several regions of West Africa may have led to selection of subspecies-specific Lassa strains. Alternatively, different Lassa strains may have evolved in genetically identical *Mastomys* populations, which are geographically separated because of lack of migration.

The high degree of variability poses problems for the design of diagnostic PCR and sequencing primers. Most of our sequencing primers that were designed on the basis of sequences of strain Josiah and Nigeria failed to anneal to the new strain as a result of several mutations in their binding sites. In addition, the binding site of primer 80F2 (18), which had been designed for diagnostics on the basis of nine Lassa sequences, contained three mutations. As they affected only the 5' half of the primer, performance of the PCR was not seriously reduced, confirming its usefulness for diagnostic purposes. The full-length S RNA RT-PCR may be an alternative for diagnostics because of its highly conserved primer binding sites, although its sensitivity may be somewhat lower.

Phylogenetic analysis showed minor differences in the tree topology of the Old World arenaviruses in comparison to previous analysis (11). In our analysis, Mobala and Mopeia viruses were placed in close relationship, while the previous study indicated that Mobala is most closely related with Lassa virus. However, in both studies, bootstrap support was low and the tree topology depended on the inclusion of changes at the third codon position (11). Placement of Lassa Nigeria, Josiah, and LP differed in both studies in a similar manner. Analysis of additional sequences may be required to elucidate the exact phylogenetic relationship among Mopeia, Mobala, and Lassa viruses, as well as between Lassa virus strains Nigeria, Josiah, and LP.

Development of a vaccine against Lassa virus is a main goal of research (39). Protective immunity is achieved in animals by vaccination with Lassa NP or GPC-expressing vaccinia virus and seems to be mediated by the T-cell response (40-42). However, whether a recombinant vaccine based on a single Lassa protein of a specific strain cross-protects against heterologous Lassa strains has not yet been studied. Recently, several epitopes recognized by Lassa NP-specific CD4+ T-cell clones of one person were mapped (32). Most of them are conserved in at least two of three Lassa strains (Josiah, Nigeria, and AV). The relatively large number of T-cell epitopes recognized, as well as their partial conservation, suggests a level of T-cell cross-reactivity that might be sufficient for cross-protection against heterologous strains after immunization with NP-based vaccines. This view is supported by experiments with Lassa GPC-based vaccines, which indicate CD4+ T-cell-mediated cross-protection even against LCMV (43). Use of the new Lassa virus strain as heterologous challenge virus after immunization with recombinant vaccines, as well as use of its proteins in in-vitro assays to study T-cell cross-reactivity, may enhance our understanding of Lassa virus-specific cross-protective immunity.

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Naturally Occurring *Ehrlichia chaffeensis* Infection in Coyotes from Oklahoma

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A nested polymerase chain reaction assay was used to determine the presence of *Ehrlichia chaffeensis, E. canis,* and *E. ewingii* DNA in blood samples of free-ranging coyotes from central and northcentral Oklahoma. Of the 21 coyotes examined, 15 (71%) were positive for *E. chaffeensis* DNA; none was positive for *E. canis* or *E. ewingii*. Results suggest that *E. chaffeensis* infections are common in free-ranging coyotes in Oklahoma and that these wild canids could play a role in the epidemiology of human monocytotropic ehrlichiosis.

Human monocytotropic ehrlichiosis, a tick-borne zoonosis caused by the rickettsial pathogen *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae), occurs primarily in the southern, southcentral, and mid-Atlantic regions of the United States (1,2). The principal vector is the lone star tick, *Amblyomma americanum* (L.), and associations between the presence of the tick and the occurrence of human ehrlichiosis have been documented (1). The principal wildlife reservoir is the white-tailed deer *(Odocoileus virginianus)* (3,4). Indeed, site-specific geographic and temporal associations have been made between the presence of *A. americanum* and *E. chaffeensis* antibodies in deer (5,6). No other wildlife species has been incriminated in the epidemiology of this disease, although serologic reactivity was detected in free-ranging raccoons *(Procyon lotor*) and opossums (*Didelphis virginianus*) from Georgia (5) and white-footed mice (*Peromyscus leucopus*) from Connecticut (7). Additionally, red foxes (*Vulpes vulpes*) have been shown to be susceptible to infection under experimental conditions (8). Although some rodents have been experimentally infected with this pathogen (9), research findings about natural infections in wild rodent populations have been inconsistent (7,10). Domestic dogs are susceptible to both natural and experimental *E. chaffeensis* infections (11-13).

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Methods and Study Design

To determine whether free-ranging coyotes (*Canis latrans*) serve as a reservoir host for *E. chaffeensis, E. canis*, or *E. ewingii*, we used a nested polymerase chain (PCR) assay to survey for the presence of DNA of these organisms in blood samples from 21 free-ranging coyotes from central and northcentral Oklahoma. Coyotes were obtained as part of animal damage control (U.S. Department of Agriculture) from an area in the established range of *A. americanum* (14,15), in which *E. chaffeensis* was endemic in deer and *E. chaffeensis, E. canis,* and *E. ewingii* had been found in dogs (13,16). Immediately after the coyotes were shot, EDTA-anticoagulated whole blood was collected for isolation of DNA for PCR assay. Blood samples were stored at 4ºC until processing. DNA was isolated from whole blood (200 µl) with the QIAamp Blood Kit (Qiagen, Santa Clarita, CA), according to the manufacturer's instructions.

Purified DNA from each blood sample was tested in four PCR amplifications by using primers HE1, HE3, EE5, and ECAN5 (12,13,17); reaction conditions are described in Figure 1. For DNA sequencing, PCR reactions were performed, and products were separated by agarose gel electrophoresis. Bands were stabbed multiple times with sterile pipet tips, which were placed into PCR reaction mix as template (19). PCR reactions were pooled and purified by using Qiagen Qiaquick PCR purification kit, according to manufacturer's instructions. DNA was sequenced at the Oklahoma State University

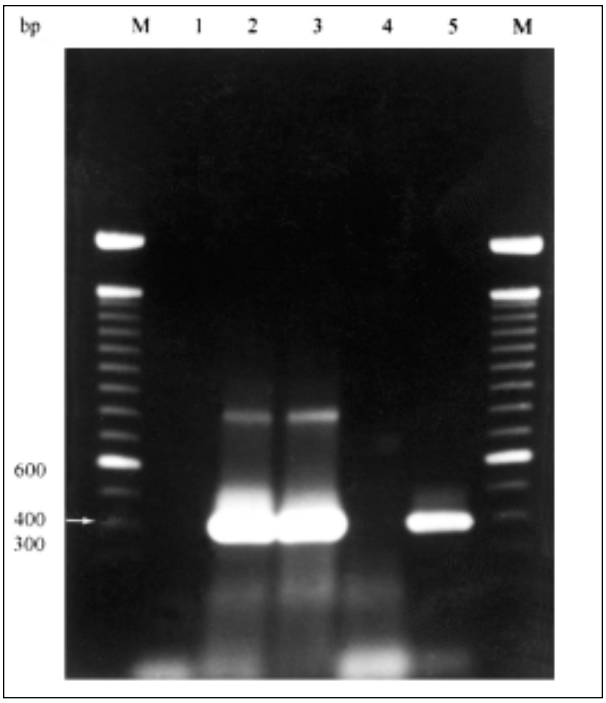

Figure 1. Agarose gel electrophoresis of results of PCR amplification of *Ehrlichia chaffeensis* nss rRNA gene from whole blood samples of coyotes numbers 9-11.* Lane 1= negative control (no DNA); Lane 2= coyote 9 (+); Lane 3= coyote 10 (+); Lane 4= coyote 11 (-); Lane 5 = positive control (*E. chaffeensis*-infected DH82 cells). M = 100-bp DNA ladder (Life Technologies, Rockville, MD).*

**Ehrlichia* forward primer ECC (5'-AGAACGAACGCTG-GCG GCAAGC-3') and *Ehrlichia* reverse primer ECB (5'-CGT ATTACCGCGGCTGCTGGCA-3') amplified all *Ehrlichia* spp (12,18). These reactions (50 µl) contained 10 µl of template DNA in 10 mM Tris-Cl (pH 8.3), 0.2 mM each deoxynucleoside triphosphate (dNTP), 2 mM MgCl2, 50 mM KCl, 0.5 µm each primer, and 1.25 U of Taq DNA polymerase (Promega Corporation, Madison, WI). A hot-start PCR was used in which each enzyme was added to reactions after an initial 3-min denaturation step at 94°C. Reactions consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, and extension at 72°C for 2 min. Products of this reaction were used as template with species-specific primer sets for three nested reactions. Primers HE1 (5'-CA ATTGCTTATAACCTTTTGGTTATAAAT-3') and HE3 (5'-TA TAGGTACCGTCATTATCTTCCCTAT-3') (17) were used for *E. chaffeensis*-specific amplifications. Primers ECAN5 (5'-C AATTATTTATAGCCTCTGGCTATAGGA-3') (12,13) and HE3 were used for *E. canis*-specific amplifications, and primers EE5 (5'-(CAATTCCTAAATAGTCTCTGACTATT-TAG-3') (this study) and HE3 were used for *E. ewingii*-specific amplifications. Reactions (50 µl) contained 10 µl of the reaction product with ECC and ECB primers as template, and the remaining reaction components as above. A hot-start PCR was used in which the enzyme was added to reactions after an initial 3-min denaturation step at 94°C. Reactions with species-specific primers were in two stages. The first consisted of three cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1.5 min. The second consisted of 37 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1.5 min. Distilled, deionized water served as a negative control. Positive control DNA samples were purified from *E. chaffeensis*-infected DH82 cells, blood from a dog experimentally infected with *E. canis*, and diluted general primer PCR reactions of synovial fluid from a dog experimentally infected with *E. ewingii*. To prevent contamination of samples, DNA purification, PCR master mix assembly, and amplifications were performed in separate rooms. Positive displacement pipetters and aerosol-free pipette tips were also used as further precautions.

Recombinant/DNA Protein Research Facility (Stillwater, OK) using an Applied Biosystems (Foster City, CA) 373A automated DNA sequencer. Sequences were analyzed with MacVector software (Oxford Molecular Group, Inc., Campbell, CA). A partial sequence (300 bp) from each end of the 390-bp amplified fragment was determined for both the *E. chaffeensis*-positive control and one positive coyote. The sequences obtained here were compared to those previously deposited in GenBank (13) to verify that *E. chaffeensis* DNA was amplified.

Results and Discussion

Of the 21 coyotes tested, 15 were positive by PCR assay for *E. chaffeensis* (Figure 1); none was positive for *E. canis* or *E. ewingii*. To our knowledge, this is the first reported evidence of natural *E. chaffeensis* infection in a coyote and the first PCR-based evidence in a free-ranging mammal other than white-tailed deer. Although these findings do not question the importance of white-tailed deer in the endemic maintenance of *E. chaffeensis*, they do point to coyotes as potential reservoir hosts.

All stages of *A. americanum* feed readily on coyotes (14,20). Moreover, white-tailed deer and coyote populations overlap in much of the *E. chaffeensis–A. americanum* disease-endemic regions of the United States (1,21-24). Movement of these deer, as indicated by their home range (usually not exceeding 1.6 km [25]) is more restricted than that of coyotes (whose range may exceed 31 km [22]). These behavioral factors, and coyotes' apparent susceptibility to infection with *E. chaffeensis*, make them an ideal bridge species for the spread of this tickborne pathogen among wild species as well as a source of infection for ticks that may subsequently feed on other hosts, including humans and domestic animals.

The results of this study, although based on a limited number of free-ranging coyotes, suggest that in the geographic range of the study, coyotes likely play little or no role in the endemic

maintenance or spread of other species of *Ehrlichia* that commonly parasitize domestic dogs or humans. Coyotes are susceptible to experimental infection with *E. canis* (26), and domestic dogs and ticks from Oklahoma have been shown to be naturally infected with both *E. canis* and *E. ewingii* as well as *E. chaffeensis* (13). In fact, *E. ewingii* DNA was recently identified from patients in Missouri, which expands the known host range of this organism, making it a newly emerging zoonosis of public health concern (27).

The occurrence of *E. ewingii* in domestic dogs and ticks in Oklahoma (13), the broad host range of *A. americanum* (its natural vector [14,23,28]), and the documented occurrence of *A. americanum* in both wild and domestic canids (13,20,24) suggest a potential for future cross-species transmission of this organism from domestic to wild and human hosts.

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Atypical *Chryseobacterium meningosepticum* and Meningitis and Sepsis in Newborns and the Immunocompromised, Taiwan

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From 1996 to 1999, 17 culture-documented systemic infections due to novel, atypical strains of *Chryseobacterium meningosepticum* occurred in two newborns and 15 immunocompromised patients in a medical center in Taiwan. All clinical isolates, which were initially misidentified as *Aeromonas salmonicida* by an automated bacterial identification system, were resistant to a number of antimicrobial agents. The isolates were characterized as atypical strains of *C. meningosepticum* by complete biochemical investigation, 16S rRNA gene sequence analysis, cellular fatty acid analysis, and random amplified polymorphic DNA fingerprinting (RAPD). This is the first report of a cluster of atypically variant strains of *C. meningosepticum*, which may be an emerging pathogen in newborns and the immunocompromised.

Chryseobacterium meningosepticum, formerly known as *Flavobacterium meningosepticum* and CDC II-a, is a gram-negative rod widely distributed in nature. The pathogen causes meningitis in premature and newborn infants (1-3) and pneumonia, endocarditis, postoperative bacteremia, and meningitis usually associated with severe underlying illness in adults (4-7). Although *C. meningosepticum* infections are rare, accurate diagnosis is important because the species is usually resistant to multiple antibiotics, especially to those (including extended-spectrum ß-lactam agents and aminoglycosides) typically prescribed for treatment of aerobic, gram-negative bacterial infections. Moreover, epidemics may occur, and a death rate as high as 55% has been reported in a nursery outbreak (8,9).

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Recent studies indicate that the species *C. meningosepticum* is highly heterogeneous and composed of many subgroups, which may be reclassified as separate species (10,11). Genetically defined subgroups within *C. meningosepticum* also differ in their pathogenicity (11). From September 1996 to March 1999, 17 culture-documented systemic infections due to a group of atypical *C. meningosepticum* strains occurred in two newborns and 15 immunocompromised patients in our institution. All isolates, which were initially misidentified as *Aeromonas salmonicida*, were resistant to a number of antimicrobial agents. The isolates were definitively identified as atypical strains of *C. meningosepticum* by full biochemical investigation, 16S rRNA gene sequence analysis, and cellular fatty acid analysis. Random amplified polymorphic DNA fingerprinting (RAPD) studies showed that these strains are distinct from bacteria of other genera tested, as well as from *C. meningosepticum* isolates from other geographic areas.

Methods

Patients and Bacterial Isolates

From September 1996 to March 1999, 17 patients admitted to Chang Gung Memorial Hospital and Children's Hospital had clinical samples positive for *A. salmonicida*. Primary cultures from these patients showed pure growth of nonfastidious, glucose-nonfermenting bacilli that were oxidase positive and nonmotile. The isolates were initially identified as *A. salmonicida* (%I.D. = 0.70) by the ID 32 GN Automatic Identification System (Biomerieux, St. Louis, MO), software version 3.2.2. Because this organism is a fish pathogen and has not been isolated in humans (12), we reviewed the charts of these patients. Clinical data for age, immunocompromising diseases, type of infection, and disease outcome were collected and analyzed. Infections were considered community acquired if the patient came to medical attention acutely ill, with an initial positive culture. Infections were considered nosocomial if cultures were negative at the time of admission or if symptomatic disease developed after the first 72 hours of hospitalization. All 17 isolates were tested biochemically and identified as *C. meningosepticum* with atypical reactions. These conventional biochemical tests were incubated at 30°C, and reactions were recorded after 2 days and 7 days of incubation in ambient air. Interpretation and identification were based on standard schema (13).

Clinical isolates from four index cases (strains 96, 97-1, 97-2, and 97-3) were sent to the University of Maryland and the University of Illinois at Chicago Medical Center for confirmation with conventional biochemical methods; they were again identified as *C. meningosepticum*.

Broth Microdilution Susceptibility Tests

Broth microdilution MIC tests were performed and interpreted by National Committee for Clinical Laboratory Standards protocols (14), including cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, MI). A final inoculum of 5×10^5 CFU/mL and incubation for 16 to 20 h at 35°C in ambient air were used.

16S rRNA Gene Sequence Analysis

The MicroSeq™ 500 rDNA Bacterial Sequencing Kit (PE Biosystems, Foster City, CA) was used to amplify and sequence approximately the first 500 bp of the 16S rRNA gene, according to manufacturer's instructions. DNA sequencing with dRhodamine-labeled dye-terminators provided two overlapping strands of sequence data, with two sequencing primers. The sequence data were analyzed and assembled with AutoAssembler Software (PE Biosystems, Foster City, CA). Bacterial identification based on 16S rRNA gene sequence data, generation of the dendogram, and calculation of the percentage difference or genetic distance between sequences were performed with the MicroSeq™ Microbial Identification and Analysis Software (PE Biosystems, Foster City, CA).

Fatty Acid Analysis

Whole-cell fatty acids were extracted and analyzed (15). Analysis was performed by using an automated Hewlett-Packard HP 5890 II Microbial Identification System (MIDI, Inc., Newark, DE). Fatty acid profiles were compared with a library of cell profiles of clinically relevant bacteria making up a similarity index.

Random Amplified Polymorphic DNA Fingerprinting

This polymerase chain reaction (PCR)-based assay was performed as described (16). All bacterial strains were grown on L agar and incubated at 37°C overnight. Three loops of bacterial colony were mixed with 250 µL of Tween 20 and TE buffer and incubated at 94°C for 20 min. After addition of 250 µL of chloroform and centrifugation at 14,000 rpm for 4 min, the supernatant was collected, and the DNA was quantified by UV absorbance at A_{260}. The primer sequence used to produce discriminatory fingerprinting profiles of bacterial strains was 5' GTCGATGTCG 3'. Each RAPD PCR reaction mixture (25 µL) contained 15 ng of genomic DNA, 40 pmol of oligonucleotides, 1 unit of *Taq* polymerase (GIBCO-BRL, Gaithersburg, MD), 250 µL deoxynucleoside triphosphate (Pharmacia, Laval, Quebec, Canada), 10 mM Tris-Cl (pH 8.0), 50 mM KCL, 0.001% gelatin, and 3 mM $MgCl_2$. Each reaction mixture was overlaid with 25 µL of mineral oil and amplified with a Perkin-Elmer Cetus DNA Thermal Cycler model TC-1 according to the following profile: 4 cycles, each consisting of 5 min at 94°C, 5 min at 36°C, and 5 min at 72°C; 30 cycles, each consisting of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C; and a final extension step at 72°C for 10 min. PCR-amplified products were separated by 1.5% agarose gel

electrophoresis and, after staining, were visualized and photographed under UV illumination. RAPD was performed twice on each strain, and fingerprinting was also analyzed twice. Final comparison was done by visual analysis, as well as Molecular Analyst Fingerprinting Plus Software (Bio-Rad, Ontario, Canada). We also used RAPD to examine four clinical isolates of *C. meningosepticum*, one *Klebsiella pneumoniae*, and two *Burkholderia cepacia*, along with the *Pseudomonas aeruginosa* strain P1 (16) as controls.

Results

The first patient was a 3-day-old, full-term neonate who was transferred to our center for the treatment of early-onset neonatal sepsis and meningitis in September 1996. The infections of this index patient and of another newborn were presumed to be perinatally acquired, although culture of the cervix of the mothers was negative for this organism. Most of the other infected patients were immunocompromised (Table 1). Age >60 years and long-term hospital stay also appear to be risk factors for the infection. All infected patients <60 years of age had substantial immunosuppressive underlying diseases (Table 1). Three of the 15 nonneonatal patients died of the infection; the two newborns survived, but with severe neurologic sequelae, despite antibiotic treatment. After the first case in 1996 and three consecutive infections due to this unusual organism in early 1997, we initiated a surveillance system to monitor all infections caused by the organism in our hospital.

Biochemical properties of the isolates were nearly identical to those of typical *C. meningosepticum* (17), except that 8 of the 17 isolates (47%) were positive for nitrite reduction and none of them grew on MacConkey agar. Colonies of these isolates were pale yellow on blood agar plates. All the isolates were nonmotile and oxidase-positive, hydrolyzed esculin and gelatin, were positive for *o*-nitrophenyl-ß-D-galactopyranoside, and produced indole. However, as described (17), the indole reaction was only weakly positive after a 48-h incubation at 30°C, and a more robust reaction was observed with inoculation into heart infusion broth rather than tryptophan broth.

The four index strains were also tested with other automated systems, including the API 20E, API 20NE, and Vitek GNI+ rapid identification systems (Biomerieux, St. Louis, MO). On API 20E, all four isolates gave a profile number of 106300400, which was interpreted as an "unacceptable profile" in the 20E database. On API 20NE, four different profile numbers were generated for the four strains (2476305, strain 96; 2476304, strain 97-1; 3476306, strain 97-2; and 2456304, strain 97-3). Profiles for three of the four strains were interpreted as "excellent

Table 1. Clinical features of patients

Infection	Age	Underlying disease(s)	Source of cultures	Date of isolation
Neonatal diseases				
Sepsis, meningitis	3D	None	Blood, CSF	09/13/1996
Sepsis, meningitis	2D	None	Blood, CSF	01/24/1999
Community-acquired infection				
Sepsis	65Y	Cirrhosis of the liver	Blood	12/04/1997
Sepsis	68Y	Congestive heart failure	Blood	08/09/1997
Sepsis	7Y	IgA nephropathy	Blood	02/11/1998
Sepsis, cellulitis	84Y	Hypertension	Blood	04/06/1997
Sepsis	24Y	Aplastic anemia	Blood	03/11/1999
Sepsis	43Y	Chronic renal failure	Blood	04/10/1997
Hospital-acquired infection				
Sepsis	18Y	Acute lymphocytic leukemia	Blood	06/01/1997
Sepsis	62Y	Cirrhosis of the liver	Blood	02/05/1999
Sepsis, wound infection	49Y	Burn	Blood, wound	06/29/1997
Sepsis, wound infection	37Y	Burn	Blood, wound	11/13/1997
Sepsis	42Y	Common bile duct cancer	Blood	10/10/1997
Sepsis	84Y	Brain infarction	Blood	05/24/1997
Sepsis	72Y	Myocardial infarction	Blood	08/17/1998
Sepsis	19Y	Pelvic fracture	Blood	03/10/1998
Sepsis	68Y	Tuberculosis	Blood	03/20/1998

Y= years; D = days; CSF = cerebrospinal fluid.

identification" at 99.9% for *C. meningosepticum*. The profile number of 3476306 for strain 97-2 was interpreted as "doubtful" at 99.1%, but the only possible choice listed for organism identification was *C. meningosepticum*. By using the Vitek GNI+ card, we generated a profile number of 62022000040 for three of the four isolates, giving an interpretation of *C. meningosepticum* at 99%. Strain 97-2 gave a slightly different profile number of 60022000040, but with a 97% match for *C. meningosepticum*.

An old version of software was installed for use with the ID 32 GN Automatic Identification system (Biomerieux, St. Louis, MO) in the original identification of these isolates; this software was unable to correctly identify them as *C. meningosepticum*. We updated the database with a newer version 3.6.8 in May 1999. When the originally misidentified isolates were rerun through the updated database, *C. meningosepticum* was the first choice (%I.D. = 1.00), with *A. salmonicida* as the second choice (%I.D. = 0.70). No cases of *A. salmonicida* infection have been identified in our laboratory since May 1999.

MIC results for the 17 clinical strains from Taiwan were determined by the broth microdilution method (Table 2). All the agents tested except piperacillin and ciprofloxacin had poor in-vitro activities against these organisms. Comparative sequence analysis with the MicroSeq system revealed that the four index isolates share the same sequence, which clustered most closely with the type strain of *C. meningosepticum* (Figure 1); however, the genetic distance of 2.7% suggests that these isolates may represent either an atypical biovar of *C. meningosepticum*

Table 2. Comparative in vitro activity of various antibiotics against 17 strains of atypical *Chryseobacterium meningosepticum*

	MIC (µg/mL)		
Antibiotic	Range	MIC_{50}	MIC_{90}
Ampicillin	16-64	32	32
Ticarcillin	32->128	128	>128
Piperacillin	1-128	8	32
Cephalothin	32->32	32	32
Cefamandole	16->32	>32	>32
Cefotaxime	8->32	32	>32
Ceftriaxone	8->32	16	32
Imipenem	1->32	32	32
Ceftazidime	2-64	16	>32
Gentamicin	16-64	32	64
Amikacin	2-64	64	64
Ciprofloxacin	0.25-16	1	4

or a novel species within the genus (18). Cellular fatty acid analysis indicated that these isolates were close to *C. meningosepticum*, with a similarity index of 0.94. The major peaks were i-15:0 (38.5%), i-3-OH 17:0 (15.9%), and i-17:1 (7.8%).

Eight RAPD fingerprints were obtained from the 17 *C. meningosepticum* isolates, which differed genetically, based on RAPDs, from the other four clinical *C. meningosepticum* strains from Canadian patients with bacteremia. The RAPD typing results of the four index isolates, 96, 97-1, 97-2, and 97-3, were compared with those of other closely related gram-negative bacteria (Figure 2). Using this typing method, we identified an apparently conserved 0.4-kb

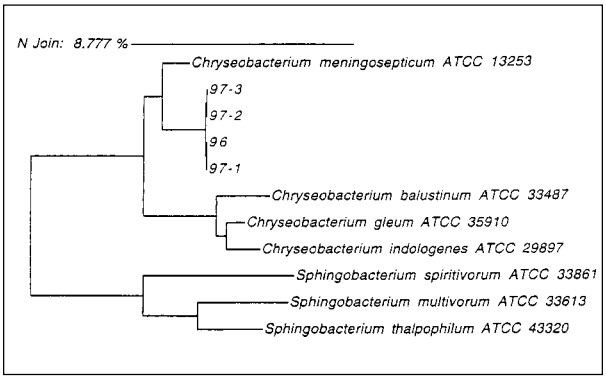

Figure 1. Dendogram derived from the 16S rRNA gene sequence analysis, showing the phylogenetic relationships among the four atypical strains of *Chryseobacterium meningosepticum* (96, 97-1, 97-2, 97-3), *C. meningosepticum* strain ATCC 13253^T, and several closely related bacteria.

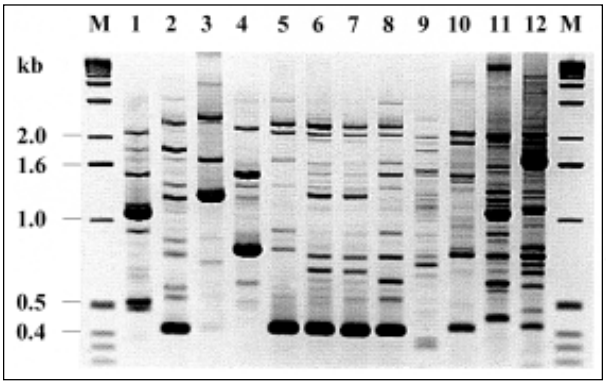

Figure 2. Random amplified polymorphic DNA fingerprinting (RAPD) types generated by arbitrarily primed PCR. Lanes 5-8 show results for the four index strains of the atypical *Chryseobacterium meningosepticum*, 96, 97-1, 97-2, and 97-3, respectively; lanes 1-4, four clinical isolates of *C. meningosepticum* from Canada; lanes 9-10, two clinical isolates of *B. cepacia*; lane 11, *P. aeruginosa* strain P1; and lane 12, clinical isolate of *K. pneumoniae*. Lane M shows the 1-kb DNA ladder.

fragment in the DNA fingerprints of the 17 isolates. This genetic marker was absent from other glucose-nonfermenting bacteria, such as *Pseudomonas* and *Burkholderia*, as well as the glucose-fermenter *Klebsiella*, but present in one Canadian *C. meningosepticum* strain (Figure 2). Furthermore, *C. meningosepticum*, *B. cepacia*, *P. aeruginosa* strain P1, and *K. pneumoniae* differed in terms of their RAPD fingerprints.

Discussion

Our study provides evidence of the emergence of a group of atypical strains of *C. meningosepticum* causing systemic infections of patients in Taiwan. This conclusion is based on phylogenetic clustering of the four index strains according to 16S rRNA gene sequence analysis and the similarity of the biochemical characteristics, cellular fatty acid profiles, and antibiograms expressed by all 17 isolates. However, unequivocal evidence of these strains as a new species within the genus *Chryseobacterium* will require confirmation by DNA-DNA hybridization studies.

The emergence of this specific biovar of the 17 strains has important clinical implications. First, this organism is highly pathogenic for newborn infants and immunocompromised patients, usually causing nosocomial as well as community-acquired systemic infections with substantial rates of illness and death. Second, rapid, accurate identification of the strain may be challenging for clinical microbiology laboratories. An automated bacterial identification system such as the ID 32 GN (Biomerieux, St. Louis, MO), initially misidentified all 17 isolates reported in this study as *A. salmonicida*. The reason for the misidentification remains unclear, although both *A. salmonicida* and *C. meningosepticum* are relatively biochemically inert and share some common biochemical characteristics, such as being esculin hydrolysis- and gelatin hydrolysis-positive and motility negative. That *A. salmonicida* is truly indole negative may be a clue, but a positive indole reaction for these atypical strains was difficult to obtain without special conventional test media and conditions. Because this ID 32 GN system is widely used in many clinical microbiology laboratories for the identification of gram-negative rods (19), this system should be updated or modified to improve its proficiency in identifying this as well as other emerging pathogens. Third, as with typical *C. meningosepticum*, choosing optimal antibiotic regimens for treating infections caused by the atypical strain was difficult because of the multidrug-resistant nature of the organism. All 17 clinical isolates were resistant to various antimicrobial agents, especially ß-lactam antibiotics and aminoglycosides. The most effective drug we tested in this study was ciprofloxacin. The only other drug displaying better activity was piperacillin; however, its MIC values appeared higher, and 4 of the 17 isolates showed high-level resistance (ß 16 mg/mL). The role of fluoroquinolones in the treatment of *C. meningosepticum* infections may be important because of their low MICs (20,21). Successful response to treatment has also been reported with trimethoprim-sulfamethoxazole, vancomycin, rifampin, clindamycin, and erythromycin (2,4,5,9), antibiotics mainly used for treating gram-positive bacterial infections. Most of these potentially effective antibiotics will not be included in the panel of susceptibility testing for any commonly isolated gram-negative bacteria other than *Chryseobacterium*. Therefore, accurate identification of these strains by either complete biochemical investigation or updated automated identification systems is crucial in selecting appropriate antimicrobial susceptibility testing and proper antibiotic therapy.

The 17 isolates from patients with systemic infections in Taiwan generated RAPD fingerprints that differed substantially from those of *C. meningosepticum* strains from Canada. Even among the 17, 8 different RAPD types were identified. Using RAPD typing, we identified an apparently conserved 0.4-kb fragment in the DNA fingerprints of the 17 isolates. This genetic marker was absent from the types of other glucose-nonfermenting bacteria, such as *Pseudomonas* and *Burkholderia* and the glucose-fermenter *Klebsiella*. However, one Canadian *C. meningosepticum* isolate had this specific genetic marker, suggesting that the atypical *C. meningosepticum* biovar of strains reported in this study may have been present in North America. Only a specific ribotype has been reported as an epidemiologic marker of *C. meningosepticum* in human infections (11). Whether the 0.4-kb band will be useful as an epidemiologic marker or represents a unique virulence factor is the subject of further studies.

In summary, we present evidence for the emergence of a cluster of atypical *C. meningosepticum* strains in Taiwan. This organism, which is

highly pathogenic for newborns and immunocompromised patients, may be misidentified by some commercially available kit systems even at the genus level. Complete conventional biochemical testing is useful for an accurate identification. Such identification would provide clinicians with important information about the pathogenic capability of a strain and its general susceptibility profile.

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Testing Umbilical Cords for Funisitis due to *Treponema pallidum* Infection, Bolivia

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To establish the frequency of necrotizing funisitis in congenital syphilis, we conducted a prospective descriptive study of maternal syphilis in Bolivia by testing 1,559 women at delivery with rapid plasma reagin (RPR). We examined umbilical cords of 66 infants whose mothers had positive RPR and fluorescent treponemal antibody absorption tests. Histologic abnormalities were detected in 28 (42%) umbilical cords (seven [11%] had necrotizing funisitis with spirochetes; three [4%] had marked funisitis without necrosis; and 18 [27%] had mild funisitis), and 38 [58%] were normal. Of 22 umbilical cords of infants from mothers without syphilis (controls), only two (9%) showed mild funisitis; the others were normal. Testing umbilical cords by using immunohistochemistry is a research tool that can establish the frequency of funisitis due to *Treponema pallidum* infection.

Introduction

Maternal and congenital syphilis are public health problems not only in developing countries, but worldwide. Even in the United States, congenital syphilis continues to occur (1). In Bolivia, the reported rate of congenital syphilis in 1994 was 3.1 per 1,000 live births (2).

Congenital syphilis usually results from transplacental passage of *Treponema pallidum* to the fetus, causing infection of the placenta and umbilical cord. For the fetus, the likelihood of infection is greatest when the mother has primary or secondary syphilis in the last two trimesters of pregnancy (3). At birth, only half of infected newborns have clinical signs of disease (3). Placental infection is not always present, and affected placentas show varying degrees or combinations of three basic histologic findings (4-6): focal proliferative villitis, endovascular and perivascular proliferative changes in villous vessels, and relative immaturity of villi. The umbilical cord may have inflammatory infiltrates around vessels, a condition known as necrotizing funisitis (4,6). To identify which infants without clinical signs have congenital infection, laboratory tests of maternal or newborn sera, as well as of the placenta and umbilical cord, have been developed (7). Several techniques have been used to detect spirochetes in the placenta, including silver stains, immunohistochemistry (IHC), and direct fluorescence antibody testing (DFA) (6,8). The latter two are specific for syphilis because they use antibodies against *T. pallidum*. IHC also allows visualization of morphologic features of the tissue, thus permitting localization of the microorganism in tissue structures. Previous pathologic studies of placentas and umbilical cords have been retrospective and based on small population samples and have presented data from specimens analyzed because of specific perinatal problems (5,6). Thus, the frequency of histopathologic changes in the placenta and umbilical cord has not been defined.

As part of a study to document prevalence of maternal syphilis in Bolivia, we assessed the histopathologic features of umbilical cords in a cohort of live-born and stillborn infants of women with syphilis and a sample of infants born to women without syphilis. We established the frequency of histopathologic abnormalities in umbilical cords from neonates of mothers with

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syphilis, compared these results with those of the sample without syphilis, and analyzed the usefulness of IHC and DFA for detection of *T. pallidum*.

Materials and Methods

All women delivering in seven hospitals in Bolivia from June to November 1996 were eligible for the study; 1,559 women consented to participate (participation rate 63%). Women were given a risk factor questionnaire, and blood was obtained for a rapid plasma reagin (RPR) test. If a woman had a positive RPR result (1:1 dilution or above), both she and her infant were treated with penicillin according to Centers for Disease Control and Prevention (CDC) treatment guidelines (9). Maternal syphilis was defined as positive RPR and treponemal antibody absorption test results from one of two Bolivian reference laboratories chosen on the basis of their performance on a proficiency panel (CDC, Atlanta, GA). For quality control, sera from all women with syphilis and a systematic sample of 10% of the women without syphilis were tested at CDC. Umbilical cords from the subset of infants whose mothers' sera had been tested at CDC were examined; this subset included 66 umbilical cords from infants born to women with syphilis, as well as 22 control umbilical cords.

Immediately after delivery, a section of umbilical cord (approximately 3 cm to 4 cm long) was collected from the end closest to the infant, fixed in 10% formalin, and transported to CDC, where it was embedded in paraffin. We examined at least two sections from 49 cords and three to five sections from 39 cords. The tissues were studied by using hematoxylin and eosin stain, and DFA and IHC tests were performed for *T. pallidum*. The extent and location of the inflammatory process were evaluated histologically. The cords were classified into two categories according to the intensity of inflammation: mild funisitis if <10 inflammatory cells (polymorphonuclear leukocytes, lymphocytes, or macrophages) were seen at 40x magnification and marked funisitis if ≥10 inflammatory cells were seen. Necrosis, which was recorded independent of the degree of inflammation, was defined as presence of eosinophilic cellular debris surrounded by inflammation.

IHC was performed as described (10): 3-µm sections of cord were deparaffinized, rehydrated, placed in a DAKO autostainer (DAKO Corporation, Carpinteria, CA), digested in 0.1 mg/mL Proteinase K (Boehringer-Mannheim Corporation, Indianapolis, IN), and later blocked with normal swine serum. After a 1-hr incubation with polyclonal rabbit antibody against *T. pallidum* (CDC, Atlanta, GA), detection was done with a biotinylated anti-rabbit antibody, strepavidin-alkaline phosphatase complex, and naphthol/fast red substrate (DAKO Corporation). Sections were counterstained in Meyer's hematoxylin (Fisher Scientific, Pittsburgh, PA). Positive controls were tissue sections of a testis from a *T. pallidum*-infected rabbit and of liver and gastrointestinal tract from a human case of congenital syphilis. Negative controls consisted of each patient's tissue sections incubated with normal rabbit serum and an unrelated polyclonal antibody instead of the polyclonal antibody against *T. pallidum*. Positive staining by IHC was considered only if intact spirochetes were identified. In four cases, more sections from the umbilical cord were submitted for study by IHC and DFA because fine and coarse red granular staining was considered questionable for infection.

DFA was performed as described (6): 3-µm sections were deparaffinized, rehydrated through graded alcohol passages, and then digested with trypsin. Next, slides were stained with a fluorescein-labeled monoclonal conjugate to *T. pallidum* (CDC, Atlanta, GA). Slides were mounted and read with an epi-illuminated fluorescent microscope. The same positive control was used for both IHC and DFA. Positive results were reported if intact spirochetes were identified. Additional sections of five cords were submitted for DFA and IHC study because fluorescent staining was considered questionable for infection.

Frequencies of the histologic measurements were analyzed. Univariate analysis was done with the chi-square statistic or Fisher's exact test (if expected cell values were <5) by Epi-Info (CDC, Atlanta, GA) and SAS (Cary, NC) statistical packages.

Results

The histologic examination of umbilical cords from infants born to mothers with syphilis demonstrated the following: seven cords (11%) with marked funisitis, necrosis (necrotizing funisitis) and treponemes (five by IHC and DFA; two by IHC only); marked funisitis without necrosis or spirochetes in three (4%) cases; mild

funisitis in 18 (27%) cases; and no histopathologic abnormality in 38 (58%) cases. Histopathologic studies of umbilical cords from infants of mothers without syphilis identified two (9%) cases with mild funisitis; the other 20 (91%) had normal histologic features. The association of treponemes, acute and chronic inflammation, and necrosis was different in the umbilical cord structures (Table 1).

Marked funisitis was associated with both maternal syphilis and presence of treponemes in the cord. All 10 umbilical cords with marked funisitis were from infants whose mothers had syphilis; these cords were more likely to have *T. pallidum* identified by IHC or DFA than were those that did not have marked funisitis (70% vs. 4%; odds ratio [OR] 41.2; 95% confidence interval [CI] 5.5-405.7). In six of the cases of marked funisitis, acute and chronic inflammatory infiltrates were observed in one or more vessels and in the Wharton's jelly (Figure 1); all these cords were positive for *T. pallidum* by either IHC or DFA. Marked funisitis was also diagnosed in one case with acute and chronic inflammation in a vein but not in the Wharton's jelly; this cord showed spirochetes by IHC. In the seven cases described, inflammation was accompanied by necrosis. Three cords with marked funisitis had acute inflammation in a vessel but no necrosis or inflammation in the Wharton's jelly. All three were negative for *T. pallidum* by IHC and DFA.

Mild funisitis was diagnosed in 20 (30%) cords; 18 were from infants whose mothers had syphilis; none contained spirochetes. Two cords from infants born to control mothers had mild funisitis. A higher percentage of infants with mild funisitis than infants with normal umbilical cords had mothers with syphilis (90% vs. 66%; OR 4.7; p=0.04). Of cords from infants born to RPR-positive mothers, 38 (58%) had normal histologic features; one of these contained spirochetes identified by DFA. However, umbilical cords with mild funisitis did not differ substantially from normal cords with respect to presence of treponemes (5% vs. 3%).

Necrosis was identified in seven cases with marked funisitis and one case with mild funisitis. All eight cases with necrosis of the umbilical cord were from infants of mothers with syphilis; none of the controls had necrosis. Necrosis in any of the umbilical vessels or amnion was strongly associated with the presence of *T. pallidum* by IHC or DFA (seven of eight cases). When necrosis

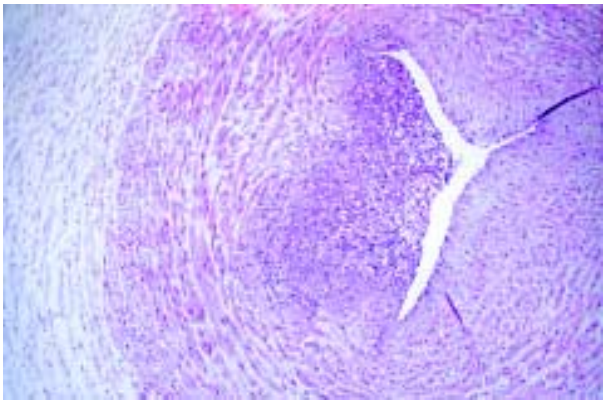

Figure 1. Umbilical artery, showing marked inflammation in the intima of the vessels.

Table 1. Inflammation, necrosis, and treponemes in umbilical cords from infants with congenital syphilis, Bolivia

Umbilical cord structure	Histologic parameter	Specimens with treponemes No. pos./no. tested (%) (N = 9)	Specimens without treponemes No. pos./no. tested (%) (N = 79)	Odds ratio (95% confidence intervals)
Umbilical artery[a]	Acute inflammation	3/8 (38)	6/78 (8)	7.2 (1.0-49)
	Chronic inflammation	3/8 (38)	4/78 (5)	11.1 (1.5-89)
	Necrosis	2/8 (25)	0/78 (0)	undefined[b]
	Normal	3/8 (38)	65/78 (83)	0.1 (0.0-0.4)
Umbilical vein	Acute inflammation	7/9 (78)	15/78 (19)	14.7 (2.4-115)
	Chronic inflammation	5/9 (56)	5/78 (6)	18.3 (3.0-125)
	Necrosis	5/9 (56)	0/78 (0)	undefined[c]
	Normal	1/9 (11)	50/78 (64)	0.1 (0.0-0.6)
Wharton's jelly	Acute inflammation	8/9 (89)	12/79 (15)	44.7 (4.8-1,043)
	Chronic inflammation	5/9 (56)	4/79 (5)	23.4 (3.6-178)
	Normal	1/9 (11)	6/79 (8)	0.03 (0.0-0.3)

[a]The umbilical artery was missing in two specimens, and the umbilical vein was missing in another.
[b]p=0.01.
[c]p<0.001.

was present in one structure, it was usually present in others; for example, necrosis of the umbilical vein was present in five (71%) of the seven cords with amnion necrosis, but in none of the 78 cords without amnion necrosis (OR undefined; $p<0.001$). Twelve umbilical cords showed neovascularization. Eight of these cords were from infants of mothers with syphilis (12%) and four were from controls (18%). Five cords had neovascularization accompanied by inflammation, and spirochetes were identified in one. Neovascularization was not associated with funisitis.

IHC demonstrated intact spirochetes and varying degrees of granular staining in seven (10.6%) umbilical cords of infants whose mothers had syphilis. In four cases, the *T. pallidum* level was high ($\geq$10 bacteria per field at 40x magnification and presence in $\geq$2 of the sections examined). In three cases, the spirochete level was lower, with <9 bacteria per field at 40x magnification and presence in only one section. Granular staining indicative of infection was present in nine cases: four by IHC and five by DFA. For all these cases, extra tissue was submitted, and in only one were spirochetes identified on reexamination.

With IHC, we were able to define the location of intact spirochetes and granular staining in the umbilical cord. In the seven positive cords, intact spirochetes were seen where there was less inflammatory infiltration (Figure 2). Granular staining was seen in the areas with marked inflammation, sometimes accompanied by intact bacteria. In the necrotic areas, no spirochetes were demonstrated, and little or no granular staining was seen (Figure 3).

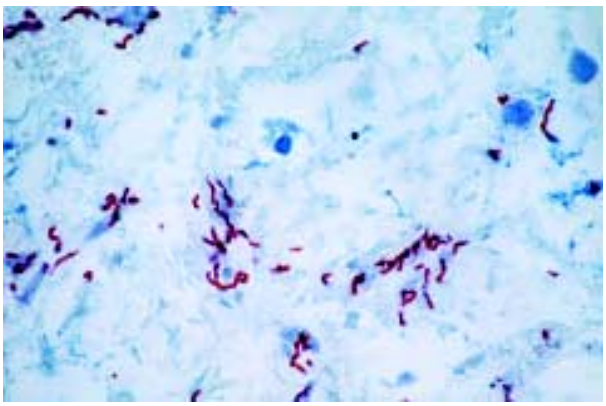

Figure 2. Immunohistochemistry for *Treponema pallidum* in an area with few inflammatory infiltrates and multiple spirochetes.

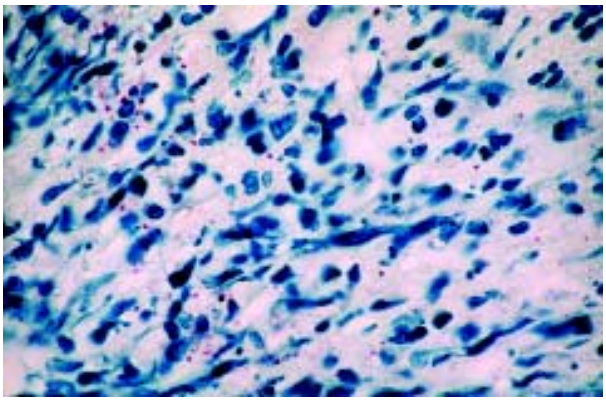

Figure 3. Immunohistochemistry for *Treponema pallidum* in an area with marked inflammatory infiltrate with granular staining; no intact spirochetes are noted.

Comparison of IHC and DFA results showed that all seven umbilical cords positive by IHC had marked funisitis and were from infants of mothers with syphilis; five of these were also positive by DFA. Two cords were positive by DFA but negative by IHC. One was from an infant whose mother had syphilis, and the other was from a control. Both these cords had normal histopathologic features.

Information about antibiotic treatment for syphilis or other conditions near term or during labor and delivery (including premature rupture of membranes) was incompletely recorded in the medical charts. Penicillin treatment for syphilis during pregnancy was documented for two women: one 6 months and the other 6 weeks before delivery. Neither umbilical cord had funisitis. Birth weight, gestational age, and physical findings for infants born to mothers with syphilis have been described (11). Of the 66 women with syphilis, 6 (9%) had stillborn infants, 1 had marked funisitis with spirochetes demonstrated by both DFA and IHC, 1 had mild funisitis with a positive DFA, and 4 had normal umbilical cord histopathologic features with no spirochetes noted on IHC or DFA.

Conclusions

We found histologic abnormalities ranging from mild to marked funisitis in 42% of umbilical cords from infants of mothers with syphilis. The presence of *T. pallidum* in umbilical cords was strongly associated with marked inflammation and necrosis (necrotizing funisitis). Newborns whose cords showed marked funisitis,

necrosis, and spirochetes are assumed to have congenital syphilis.

Necrotizing funisitis, which was described at the beginning of the 20th century, has been linked to syphilitic infection and high fetal-infant death rates (4,6). The disorder refers to a deep-seated inflammatory process in the matrix of the umbilical cord, which may be accompanied by phlebitis and thrombosis. Necrotizing funisitis is thought to result from diffusion of amniotic fluid leukotoxin, which destroys the fetal neutrophils migrating toward the amniotic cavity (12). The presence of spirochetes in umbilical cords with necrotizing funisitis has been reported in 40% (4,13) to 90% of cases (6).

We found three cords with marked funisitis but no necrosis. In these three cases, no spirochetes were present, although the cords came from infants of mothers with syphilis. We also found 18 umbilical cords with mild funisitis, all from infants born to women with syphilis. In these cords we attributed inflammation, whether mild or marked, to *T. pallidum* infection. Some authors distinguish necrotizing funisitis from acute funisitis, since the latter is characterized by acute inflammation without necrosis and can be associated with infections other than syphilis (6). We found two umbilical cords with mild funisitis from infants of women without syphilis; the inflammation in these cases could have been secondary to other pathologic conditions, such as premature rupture of membranes or bacterial infection other than syphilis (4,12).

T. pallidum infection is difficult to confirm in infants whose cords do not show spirochetes, whether they have marked funisitis without necrosis, mild funisitis, or necrosis with minimal inflammation. We noted pathologic changes in 42% of umbilical cords from infants born to women with syphilis. These infants may have been infected even though no spirochetes were identified. These data support the CDC recommendation to provide immediate penicillin treatment at delivery for all newborn infants born to women with untreated or inadequately treated syphilis during pregnancy (9).

In an ideal study of congenital syphilis, umbilical cords would be examined in conjunction with the placenta (5), and fetal outcome would be taken into account (13). However, good sensitivity for detecting *T. pallidum* from umbilical cords only has been reported (6). In epidemiologic field studies such as ours, a small portion of umbilical cord is easier to obtain and transport than the entire placenta and permits histopathologic and immunohistochemical analysis of tissues, which can be helpful in assessing populations at risk for congenital syphilis.

We compared our results of umbilical cord abnormalities from infants whose mothers had syphilis with those published by other authors (Table 2) (5,6). Qureshi et al. described placental pathology data from 25 of 162 cases of syphilis in which placentas were selectively sent to the pathology laboratory by the obstetricians (5). Schwartz et al. limited their selection to 25 women with active syphilis (6). The frequency of histologic changes in our cohort is similar to the findings of Qureshi et al. (5). Schwartz et al. (6) reported a lower percentage of normal cords and higher frequency of necrotizing funisitis and spirochetes. The other authors studied a small, selected group of umbilical cords from patients with known early syphilis and high RPR titers; in contrast, our study included all women with reactive RPR tests and with both early and late syphilis. In the latter group, transmission to the fetus is less likely (7).

Up to 75% of pregnant women with active syphilis have stillbirths (6). In the group of patients we studied, six women had documented stillbirths; of the six stillborn infants, two had histologic abnormalities of the umbilical cord and *T. pallidum* was demonstrated, so syphilis was assumed to be the cause of fetal death. In the other four cases, death cannot be attributed entirely to syphilis, especially in communities where prenatal care is poor. We found no correlation between outcome of pregnancy and umbilical cord abnormalities; however, our study may be limited by the small number of stillborn infants.

Table 2. Comparison of three studies of umbilical cord abnormalities of infants born to women with syphilis

Pathologic features	Schwartz (6)	Qureshi (5)[a]	Guarner et al.
No. of cords	25 (%)	25 (%)	66 (%)
Normal histology	12 (48)	14 (56)	38 (58)
Funisitis	13 (52)	11 (44)	28 (42)
Inflammation only	4 (16)	8 (32)	20 (30)
Necrosis	9 (36)	3 (12)	8 (12)
Spirochetes identified	22 (88)	---	7 (11)
Stillbirths	18 (72)	4 (16)	6 (9)

[a]Data extracted from text.

Most previous studies of tissues have used the silver stain (Steiner) (4,6) or DFA (6) to detect spirochetes. Problems encountered when using the silver stains include lack of specificity—most bacteria stain with Steiner—and silver precipitation frequently causing high background, which decreases sensitivity. The use of specific antibodies to *T. pallidum* increases the specificity, while sensitivity is heightened by several detection methods. DFA, which has been compared with Steiner stains for the study of syphilis in umbilical cords, has slightly better sensitivity and specificity (6). IHC had advantages for the diagnosis of congenital syphilis. We were able to define the exact location of the spirochetes in the umbilical cord; intact spirochetes were found in areas of less inflammation, but *T. pallidum* was not seen in necrotic areas. We also described granular staining, which probably corresponded to either spirochetes cut in different planes or antigenic fragments of *T. pallidum*. Other methods, such as polymerase chain reaction (PCR), can be used to enhance sensitivity (8); however, with PCR and DFA, tissue cannot be observed histologically. Therefore, we found that IHC correlated better with marked funisitis.

Although marked funisitis with necrosis (necrotizing funisitis) was identified in only 12% of umbilical cords from infants of mothers with syphilis, it is highly associated with the presence of spirochetes by either IHC or DFA. Abnormal histopathologic results are found in umbilical cords from infants of mothers with syphilis; however, normal results are found in more than half the cases. Our study demonstrates the value of histologic and IHC analysis of umbilical cords for epidemiologic research. However, routine clinical management of patients, especially in resource-poor settings, should not depend on examination of umbilical cords. Congenital syphilis can be controlled and prevented by widespread RPR testing and penicillin treatment during prenatal care.

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Microbial Genomics: From Sequence to Function

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Guest Editor, Series on Genomics

Dr. Schwartz is professor of biochemistry and molecular biology and medicine. His research focuses on emerging tick-borne infections, primarily Lyme disease and human granulocytic ehrlichiosis (HGE). Studies in his laboratory demonstrated that the infectious agent of HGE was present in ticks collected in 1984—10 years before the first description of clinical cases and human coinfection with *Borrelia burgdorferi* and the agent of HGE. More recently, his laboratory has reported on Lyme disease risk from an individual tick bite in the six lower Hudson Valley, New York, counties and the identification of a *B. burgdorferi* subtype that is more frequently associated with disseminated infection in early Lyme disease patients. Dr. Schwartz is now using genomic approaches to identify genes and proteins involved in *B. burgdorferi* pathogenesis.

The era of genomics (the study of genes and their function) began a scant dozen years ago with a suggestion by James Watson that the complete DNA sequence of the human genome be determined. Since that time, the human genome project has attracted a great deal of attention in the scientific world and the general media; the scope of the sequencing effort, and the extraordinary value that it will provide, has served to mask the enormous progress in sequencing other genomes. Microbial genome sequencing, of particular interest to the community studying emerging infectious diseases, prompted the series of articles presented in the following pages. These articles review technological and scientific advances that have occurred since publication of the *Haemophilus influenzae* genome sequence in July 1995 (1); that was the first demonstration that an entire genome sequence could be deciphered by a "shotgun" approach, i.e., the sequencing and assembly of random fragments of the genome. This is now the method of choice for sequencing of most other genomes, including human (as performed by Celera Genomics).

The articles by Fraser et al. (this issue, pp. 505-12) and by Weinstock (pp. 496-504) briefly describe some of the sequencing methods and annotation of the completed sequences. As of this writing (late June 2000), 23 bacterial genomes have been fully sequenced. More than 70 other microbial genome projects are under way; a regularly updated listing is available on the Internet (http://www.tigr.org/tdb/mdb/mdbinprogress.html). For some species, several strains have been examined, facilitating whole genome comparisons that provide insights not available by other methods (Fraser et al., this issue).

Generally, the first analysis of a completed, fully assembled genome consists of determining all the putative open reading frames (ORFs), which may constitute protein coding regions. These derived amino acid sequences are searched against sequence databases to determine the relationship to previously sequenced genes. There can be three results: a "hit" to a gene of known function, a hit to a gene of unknown function (usually referred to as a conserved hypothetical protein), or no database match. In the first instance, the newly sequenced gene is generally annotated as a homolog of the best hit. When the first bacterial genome sequences were elucidated, it was not surprising that a significant percentage (35%-45%) of identified ORFs either were of unknown function or had no database match. More surprising is that these numbers have not changed substantially as more and more sequences have been determined. Thus, close to half of all bacterial ORFs identified to date have no known function, half of which again are unique to the given

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species. This represents an enormous storehouse of unrecognized metabolic potential, and it appears obvious that many novel biochemical reactions and pathways are yet to be discovered and characterized. A recent example along these lines is the construction of 6,144 individual yeast strains, each containing an expression clone of all identified yeast ORFs. This strain collection was used for high throughput identification of three previously unrecognized enzymatic activities (2). Similar approaches should provide fertile ground for many future biochemical investigations.

Homology searching and ORF identification have been especially useful in revealing the overall metabolic capability of an organism, identifying potential targets for antimicrobial therapy, and elucidating candidate virulence genes. This information also provides a framework for comparative studies of closely related bacterial species and different strains (e.g., virulent and avirulent) of the same species. Examples of each are provided in the ensuing articles by Fraser et al. and by Weinstock. Despite the obvious value of such analyses, however, some caution must be exercised. Ultimately, the caliber of the bioinformatics tools employed for sequence homology analysis and ORF annotation determines the quality of the data. Unquestionably, errors in annotation exist, and these can result in erroneous classification of newly identified genes. Often, annotated genes are arranged into commonly identified metabolic pathways, and certain key activities are "missing." This may suggest that the organism under study may only contain a portion of the particular pathway. However, given the large reservoir of genes with unknown function, it is equally plausible that another protein has evolved to catalyze the missing reaction. This process has been referred to as non-orthologous gene displacement (3). How common such gene displacement is will only become clear through biochemical studies of the type described above.

Complete bacterial genome sequencing has revealed more extensive genetic exchange between species than suspected. Lateral or horizontal gene transfer has been inferred from differences in guanine-cytosine content or codon preference in specific regions of a genome relative to the entire genome. The best known examples of such lateral exchange are the acquisition of antibiotic resistance genes and pathogenicity islands. The extent of lateral transfer has a profound impact on the inference of phylogenetic relationships by use of specific protein sequences and is the subject of substantial debate (4-6). Fraser et al. touch on these issues and present an approach to phylogeny based on comparative genomics.

Perhaps the greatest value of complete genome sequence information is its use in generating hypotheses that can be further tested by biological ("wet") experiments. Weinstock describes how complete genome sequences may be scrutinized for clues to pathogenic mechanisms and emphasizes that this is merely a starting point for subsequent studies. In many cases, putative virulence determinants can be identified by homology to previously characterized proteins. This approach works reasonably well for pathogens closely related to those that have been extensively studied. However, for many organisms (e.g, the spirochetes *Borrelia burgdorferi* and *Treponema pallidum*), the sequence provides markedly less insight into potential pathogenic processes (7,8). Ultimately, definitive demonstration that any candidate virulence factor plays a role in pathogenesis is best accomplished by genetic manipulation. For example, disruption of a candidate gene should abolish the ability of the mutated pathogen to elicit disease in an animal model of infection, and reintroduction of the wild-type gene should reestablish virulence. For many pathogens, such genetic tools are not yet available, but one hopes that the genetics will soon catch up to the genomics.

One of the most exciting outcomes of the genomics revolution is the ability to probe an organism's global gene expression under a specified set of physiologic conditions. Variously referred to as transcription or gene expression profiling or monitoring, this technology is facilitated by highly parallel analysis of mRNA content in a cell using oligonucleotide chips or cDNA microarrays. The review by Cummings and Relman (this issue, pp. 513-25) describes some technical aspects of the technology and its specific application for the study of host-pathogen interactions. Since the technique is not truly quantitative, it is usually applied to measuring the differences in expression of "all the genes" in the organism under two different growth conditions—for example, environmental (changes in pH or temperature) or nutritional (rich vs. minimal media). The genes that are differentially expressed are assumed to be responsive to the physiologic state of the cell

under these differing conditions. For eukaryotes, this can also be used to uncover differences between normal and diseased cells or tissues. A particularly interesting extension of this approach is the study of alterations in host gene expression on exposure to, or infection with, a bacterial pathogen; this is discussed extensively by Cummings and Relman. Like all enabling technologies, DNA microarray analysis has manifold applications, and new ones will surely be developed. In the context of bacterial genomics, two additional uses are worthy of note. As already described, close to half of annotated ORFs have no known function, and some percentage of these may not be genes at all. Microarray analysis can elucidate the true nature of the "expressed genome" by confirming the expression of genes of unknown function. Of course, as with all experimental data, a positive result is meaningful, but a negative result must be interpreted with caution since a particular "gene" may be expressed only under a very selective set of conditions (perhaps one not amenable to facile experimental analysis). Microarrays can also be employed in high-throughput detection or diagnostic applications based on ribosomal RNA hybridization (9,10).

This series of review articles on genomics, like any other series covering a broad and rapidly evolving area of investigation, cannot provide comprehensive coverage of all topics. For example, information on the development of novel antimicrobial drugs and vaccines based on whole genome sequencing data (11-13) has not been included. Finally, much of the early emphasis in genomics has been on accumulating and annotating raw sequence data. While certain fundamental insights have been gained from these data (e.g., the extent of lateral gene exchange and existence of novel genes), ultimately, the most profound advances will result from using sequence information to drive the study of microbial biology, i.e., how the genome determines function. Expression profiling is the first step along this path. However, this technique measures mRNA, the "message," rather than the protein gene product. Proteomics—describing the complete protein complement of an organism—has thus developed as the adjunct to genomics. Proteomics has been made feasible by major advances in high throughput mass spectrometry, despite the fact that the core component of the technology remains two-dimensional gel electrophoresis. Descriptions of the technique and an example of its application to a microbial system can be found in recent publications (14-16).

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Genomics and Bacterial Pathogenesis

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Whole-genome sequencing is transforming the study of pathogenic bacteria. Searches for single virulence genes can now be performed on a genomewide scale by a variety of computer and genetic techniques. These techniques are discussed to provide a perspective on the developing field of genomics.

Twenty-five years ago, the development of molecular biology and recombinant DNA technology promised breakthroughs in infectious disease research. Since then, these methods have slowly teased out molecular secrets of microbial infection, gene by gene. Now, with the advent of whole-genome sequencing, a new revolution in infectious disease research has begun. Genomics is a top-down approach to the study of genes and their functions, taking advantage of DNA sequences of complete genomes. Determining the DNA sequence of a complete genome is a major activity of genomics. Although basic DNA-sequencing methods have remained the same, advances in automation and informatics enable determination of whole microbial genome sequences in <2 years. Complete knowledge of an organism's genetic makeup allows exhaustive identification of candidates for virulence genes, vaccine and antimicrobial targets, and diagnostics. The genomes of at least 13 pathogenic bacteria have been sequenced (Table 1), representing >20,000 putative genes. The genomes of at least 28 other pathogenic bacteria are being sequenced, promising >40,000 additional genes. This tally does not include an equally large number of nonpathogenic bacteria undergoing whole-genome sequence analysis. These new data dwarf previous methods of gene discovery, allowing many new genetic approaches to understanding pathogenesis.

Raw Material

Genome projects produce different types of data, depending on the stage and goals of the project (Table 2). The goal of most projects is a

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Table 1. Whole-genome sequencing of bacterial pathogens[a]

Bacterium	Status (ref.)
Actinobacillus actinomycetemcomitans	In progress
Bacillus anthracis	In progress
Bartonella henselae	In progress
Bordetella bronchiseptica	In progress
B. parapertussis	In progress
B. pertussis	In progress
Borrelia burgdorferi	Finished (1)
Campylobacter jejuni	Finished
Chlamydia pneumoniae	Finished (2)
C. trachomatis	Finished (3)
Clostridium difficile	In progress
Enterococcus faecalis	In progress
Escherichia coli K12	Finished (4)
E. coli O157:H7	In progress
Haemophilus influenzae	Finished (5)
Helicobacter pylori	Finished (6,7)
Listeria monocytogenes	In progress
Mycobacterium avium	In progress
M. leprae	In progress
M. tuberculosis	Finished (8)
Mycoplasma genitalium	Finished (9)
M. mycoides	In progress
M. pneumoniae	Finished (10)
Neisseria gonorrhoeae	In progress
N. meningitidis	In progress
Porphyromonas gingivalis	In progress
Pseudomonas aeruginosa	In progress
P. putida	In progress
Rickettsia prowazekii	Finished (11)
Salmonella serotype Typhi	In progress
S. Typhimurium	In progress
Shigella flexneri	In progress
Staphylococcus aureus	In progress
Streptococcus mutans	In progress
S. pneumoniae	In progress
S. pyogenes	In progress
Treponema denticola	In progress
T. pallidum	Finished (12)
Ureaplasma urealyticum	Finished
Vibrio cholerae	In progress
Yersinia pestis	In progress

[a]Much of these data were taken from the TIGR website (see Table 2). In-progress genome projects are those that are funded but not yet complete.

Table 2. Availability of sequence data

Internet site	Organization	Description
www.ncbi.nlm.nih.gov/Entrez/Genome/org.html	National Institute of Biotechnology Information	Many genomes represented
http://www.tigr.org/tdb/index.shtml	The Institute for Genomic Research	Genomes sequenced by TIGR
www.stdgen.lanl.gov	Los Alamos National Laboratory	Sexually transmitted disease pathogens
http://www.micro-gen.ouhsc.edu/	University of Oklahoma	Genomes sequenced at the Univ. Oklahoma
www.pasteur.fr/recherche/banques/Colibri/	Institut Pasteur	Colibri, database of the *Escherichia coli* genome
pedant.mips.biochem.mpg.de/	Pedant	Many genomes represented
http://www.ncgr.org/research/sequence/	National Center for Genome Resources	Many genomes represented
http://www.kazusa.or.jp/cyano/	Kazusa DNA Research Institute	Cyanobase, cyanobacterial genome information
www.sanger.ac.uk/Projects/Microbes/	Sanger Centre	Genomes sequenced by the Sanger Centre
www.genetics.wisc.edu/	University of Wisconsin	Genomes sequenced by the Univ. Wisconsin Genome Center
www.zmbh.uni-heidelberg.de/M_pneumoniae/genome/Results.html	University of Heidelberg	*Mycoplasma pneumoniae* genome
utmmg.med.uth.tmc.edu/treponema/tpall.html	Univ. Texas Houston Medical School	*Treponema pallidum*
http://chlamydia-www.berkeley.edu:4231/	Univ. Cal. – Berkeley	*Chlamydia* genomes
evolution.bmc.uu.se/~siv/gnomics/	Uppsala University	*Rickettsia prowazekii*
http://www.genomecorp.com/sequence_center/index.html	Genome Therapeutics Corp.	Genomes sequenced at GTC
genome.wustl.edu/gsc/Projects/bacteria.shtml	Washington University	Genomes sequenced at Washington Univ.
http://www.genoscope.cns.fr/externe/English/Projets/Resultats/rapport.html	Genoscope	Genomes sequenced at Genoscope
www.genome.washington.edu	Univ. of Washington	*Pseudomonas aeruginosa*
www.pseudomonas.com	Pathogenesis Corp.	*P. aeruginosa*
pbil.univ-lyon1.fr/emglib/emglib.html	Enhanced Microbial Genomes Library	Many genomes represented
www.pasteur.fr/recherche/banques/TubercuList/	Institut Pasteur	TubercuList, database of the *Mycobacterium tuberculosis* genome

finished contiguous DNA sequence of the bacterium's chromosome(s). The error frequency in a finished sequence has never been precisely measured but is thought to be one error (frameshift or base substitution) in 10^3 to 10^5 bases. Other types of errors, such as rearrangements, are probably even more rare. Even at the higher end of this error frequency, approximately one error per gene, the sequence is still very useful for database searches and most applications.

Finished genome sequences are annotated to varying degrees. The two most important annotations are the predicted protein coding sequences, generally called open reading frames (ORFs), and what they resemble in database searches (see below). Strictly speaking, an ORF is any stretch of codons that does not include a chain termination codon; however, only a subset of all the ORFs present in the genomic sequence actually encodes proteins and is used in genome

annotation. These ORFs are identified by predicting coding sequences. The predictions are 90% to 95% accurate. In addition, many untranslated RNAs (mainly tRNA and rRNA genes) are identified and annotated. Various other features may be part of the annotation, including elements of the predicted protein structure, such as secondary structure motifs and membrane spanning regions. Unfortunately, annotation rarely extends to noncoding regions, where promoters and regulatory signals reside. Similarly, structural features of DNA (e.g., Z-DNA) are rarely analyzed, which may bear on regulation or genome structure. At this time, the emphasis is overwhelmingly on gene products since these convert sequence data into useful products.

A near-universal trend among public (but not private) genome projects is the early release of unfinished sequence data, sometimes referred to as (rough) draft sequences. This release can occur when as little as 1x coverage (coverage being the number of bases read in DNA sequencing reactions, divided by the genome size) of the genome has been obtained by random sequencing; for an average-size 2-MB genome, this may mean 4,000 sequencing reads. Most genomes will have been sequenced at least once, although the sequence will have a high error rate and many gaps, and some regions of the genome will not be represented. These random sequence reads are assembled by a computer program that looks for overlaps between the individual sequences and generates consensus sequences, i.e, a sequence in agreement with most of the individual reads (present in stretches of contiguous nucleotides or contigs). Since there are many gaps in the sequence, hundreds to thousands of contigs are produced by this process, with a wide range of sizes (typically from 100s to 10,000s of bases)—although always much smaller than the total genome. Collections of contigs can be searched for matches to sequences of interest, allowing identification of relevant contigs and specific DNA sequences within them. This analysis prior to release of the completed sequence speeds the application of results from genome projects.

Finding Hints in Sequences

Several approaches can be used to analyze whole-genome sequences for candidate virulence factors and for vaccine and antimicrobial targets. Comparing predicted coding sequences to sequences in databases (e.g., GenBank), using the BLAST program (13,14) identifies matches to known genes. Typically, approximately 20% of the predicted ORFs in a genome do not match anything in GenBank, while another 10%–20% match genes of unknown function, often discovered in other genome projects. The fraction of genes of unknown function in a genome has been remarkably constant in microbial genome sequences, regardless of the number of genomes sequenced and available for comparison. Thus, the comparison approach is useful in recognizing good candidates among genes whose functions have been described; it is not particularly useful in discovering new virulence functions or motifs.

For microbes related to well-studied pathogens, such as gram-positive cocci or gram-negative enteric pathogens, comparing sequence data yields many database matches or "hits." For organisms more distantly related to well-studied groups, results are more modest. When this approach was used for the spirochete *Treponema pallidum*, only 70 genes out of 1,041 could be recognized as potential virulence factors (15). Since a number of these had previously been described as antigens or membrane proteins without a function implicating them in infection, only half of the 70 genes could be matched to a function associated with virulence or host interaction in another pathogen. Of these, the evidence for some of the existing database annotations was slim, at times only theoretical and not based on solid experiments. These spurious annotations can be readily perpetuated because of the volume of new genes entered without critical evaluation. Thus for *T. pallidum*, for which approximately 40% of the total ORFs did not match a gene with any annotated function (12), virulence factors are likely to be novel, and other methods for their discovery are needed.

Databases that do not search for matches to whole genes or proteins can also be searched. These include databases of protein motifs such as BLOCKS (a database of conserved regions of protein families, obtained from multiply aligned sequences [16,17]) and ProDom (18,19). Hits to these databases are based on much smaller conserved regions and do not require extensive similarity elsewhere in the sequence, as may be the case with whole-gene matches. More general characteristics of protein sequences, such as those of membrane proteins, can also be used to identify genes of interest. The rationale is that

proteins involved in host interactions (likely to be virulence factors) should be localized to the cell surface or be secreted. Transmembrane sequences can be predicted by a variety of programs such as PHD (20,21); signal sequences can be identified with programs such as SIGNALP (22,23). Transmembrane and signal sequences and other characteristics are included in annotations in databases (e.g., the one for sexually transmitted disease pathogens) (Table 2).

Other sequence-based clues have been used in this type of analysis. Tandem repeats of simple (e.g., mono-, di-, tri-, or tetranucleotide) sequences are often found in or near certain virulence genes, called contingency genes (24,25). Because changes in the number of copies of repeats alter expression or other properties of these genes, leading to antigenic or other types of variation, this feature can be analyzed to identify genes. Finally, analysis of untranslated regulatory regions, though not extensive, appears to be a fruitful area for future studies. A genetic method for identifying new virulence factors is to find genes that are coregulated with known virulence factors (26). This type of analysis could be used in silico (analysis by computer). Motifs commonly associated with binding sites for regulators, such as inverted repeats, could be identified in regulatory regions of genes involved in pathogenesis or matching known virulence factors. These motifs could then be used to search for other regulatory regions containing the motif. The associated genes would then be candidates for virulence factors.

In summary, a number of strategies have been developed to mine genomic sequences for virulence factor genes. Other approaches will likely be developed. The availability of this information on easily accessible electronic databases will make this a routine tool in future studies of pathogenic microbes. All of these factors constitute a powerful set of new tools for research planning and experimental design and interpretation.

Genetics Meets Genomics

One criticism of the sequence-gazing approach is that it is not hypothesis based. However, the theoretical analysis of genomic sequence described above requires laboratory validation of conclusions, which are the hypotheses that drive experimental design. The availability of sequence data not only generates hypotheses but also greatly speeds the task of testing them.

In systems with good genetics and suitable models to test virulence, the sequence allows design and construction of clones for making targeted knockout mutants—a type of mutation where a gene's function is knocked out by inserting DNA into or deleting the gene. These mutational methods are usually based on a polymerase chain reaction assay (PCR), since the sequence allows primers to be designed to amplify and clone the key sequences. In some organisms, wholesale construction of such mutants is under way (27). One can determine if inactivation of a gene leads to attenuation of infection in a model system. If genetic analysis is not feasible, it is still possible to test whether immunization with a gene product (either the whole protein or part of it) can lead to protection in a model. While this testing does not provide as strong a case for a role in virulence as a null mutant (a mutation that causes complete loss of function in a gene), it indicates whether the protein is a good vaccine target. In this case, the sequence allows design and construction of clones overexpressing the protein of interest in a more manipulable host (again by PCR amplification of key sequences). Often, identification and purification of proteins in the natural host are formidable tasks. However, whole-genome sequencing allows overproducers to be constructed in *Escherichia coli* or other workhorse strains.

Both of the methods described above can determine if a gene is functional when virulence is affected. However, when there is no effect, there is no indication of whether the gene is real or functional. Determining if the gene is transcribed and translated is then desirable. Reverse transcription (RT)-PCR, again basing primer design on the genome sequence, is often performed for such analysis and can be extended to determine operon structure in the genome. Genomewide transcription analysis is performed with DNA arrays. Protein prepared in a surrogate host can be used to detect antibodies in serum from infected persons, which is particularly relevant for surface protein candidates for immunodiagnostics. An immunopositive reaction indicates that a gene is transcribed and translated.

Scanning for Function

The sequence-to-mutant method described above is appropriate when genes of interest can

be identified by sequence analysis. However, there are likely to be novel genes that do not match known functions or domains and do not have characteristics used to identify surface proteins. How would one identify a secreted protein with a function not previously described and the sequence characteristics of a soluble protein? Or what about essential genes, targets for antimicrobial drugs, that may encode cytoplasmic proteins, some of which are novel and do not match known proteins? The methods described above would not be sufficient to identify these important functions.

Several methods that bridge this gap have been proposed for whole-genome function analysis. In all cases, the genome is scanned by exhaustive transposon mutagenesis, and mutants are screened en masse for functional properties. These methods can identify essential genes, virulence factors, and other types of phenotypes.

Genetic footprinting (28,29), which was developed for yeast, is also applicable to bacteria (Figure 1). This method depends on the complete genome sequence since PCR primers are made to the ends of each gene in the genome. A saturating set of transposon insertions is isolated at random in the genome, so all genes receive multiple insertions. The mutants are pooled, and the culture is split and grown under permissive and nonpermissive conditions. For essential genes, there is no permissive condition. For virulence functions, a permissive condition might be broth culture, and a nonpermissive condition might be an animal model. After growth, DNA is extracted from the cultures, and each mutant gene is assayed by PCR using one primer for the end of the gene and one primer for the end of the transposon. Each gene is assayed separately and generates a series of bands, each corresponding to a different insertion in the gene. Comparison of the permissive and nonpermissive conditions allows the identification of mutants that drop out (that is, do not grow) under nonpermissive conditions. An essential gene mutant gives no products in either permissive or nonpermissive samples. Mutants in a gene required for infection would give products with the permissive but not the nonpermissive culture. Other genes would give products under both conditions. In this way, one assays function by "knocking out" all genes.

Signature-tagged mutagenesis (30) is another dropout mutant approach, but its scheme for tracking each gene differs (Figure 2). The

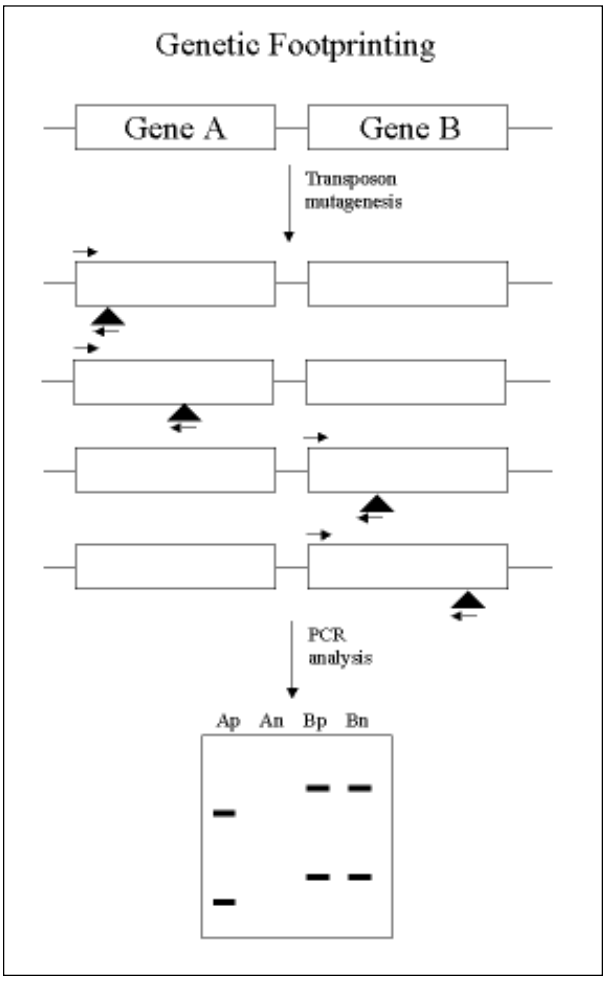

Figure 1. Genetic footprinting. Shown are two neighboring genes from the whole genome. Gene A encodes a virulence factor; gene B does not. Neither gene is essential. After transposon mutagenesis, multiple insertions (vertical triangles) are obtained in each gene (only two are shown). Polymerase chain reaction (PCR) primers (horizontal arrows) to the start of the gene and the transposon are used to amplify the sequences between insertion and start of gene. After electrophoresis, a characteristic set of bands is seen for each gene, corresponding to the location of insertions. Mutants of genes A and B grew under permissive conditions (Ap, Bp). Only mutants of gene B (Bn) grew under nonpermissive (animal model) conditions.

transposon used for random mutagenesis has been prepared to have an index region in which each transposon has a different sequence. This region can be amplified by PCR. The resulting product can be used as a hybridization probe to uniquely identify the transposon that encodes it. The initial set of random insertion mutants is arrayed on a master and then pooled and grown

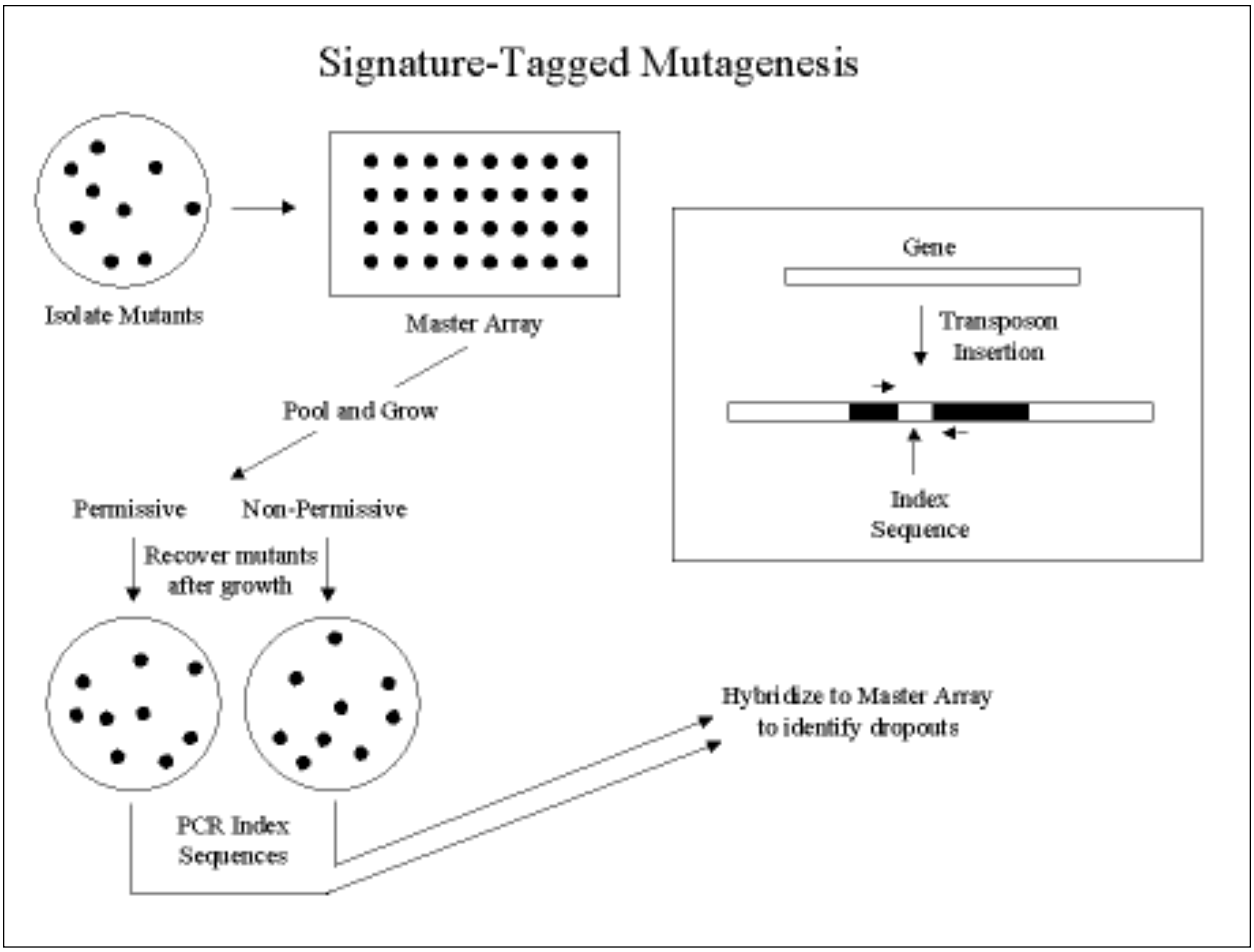

Figure 2. Signature-tagged mutagenesis. The box on the right shows a transposon insertion, indicating the index region and location of polymerase chain reaction (PCR) primers that amplify the segment unique to each transposon.

under permissive and nonpermissive conditions, as above. The mutants that emerge in each growth regimen are then collected, and their index regions are amplified and used to hybridize to the master array of original mutants. This process allows the identification of mutants that dropped out during the selection. Regions flanking the insertions in mutants of interest are then sequenced and compared to the genomic sequence to find inactivated gene(s). An important difference between signature-tagged mutogenesis and genetic footprinting is that in genetic footprinting each gene is specifically and systematically assayed, relying on the genome sequence. Thus, essential genes are readily found since they have no mutations. On the other hand, signature-tagged mutagenesis assays mutants randomly and thus could not determine that a gene could not be mutated until a large number of mutants had been tested. Nevertheless, this method has been widely used to detect virulence factor genes (31-36).

Additional methods using transposon scanning to find genes with essential or other functions will likely be developed. The methods described above often require more genetic manipulations than can be performed in some pathogenic organisms. Recent advances to overcome these limitations include using in vitro transposition to generate mutants (37) as well as new transposons with broad host ranges (38).

One Genome Is Not Enough: Comparative Genomics

Comparative genomics, which requires input of multiple genomic sequences, is relatively new, and the microbial genome era is just entering truly large-scale production. The first whole-genome

comparisons were of strains phylogenetically separated, since these were the only genomes available. Much can be learned about evolution from comparing such disparate organisms, but certain lessons can best be gleaned from comparing more closely related genomes. Recently, such comparisons have been performed with the genomes of *Mycoplasma genitalium* and *M. pneumoniae* (39,40), two strains of *Helicobacter pylori* (6), *Chlamydia trachomatis* and *C. pneumoniae* (2), and draft sequences of *Salmonella enterica* serotype Typhimurium (41) and *S.* Typhi (42) with the completed sequence of *E. coli*. These studies promise to provide pertinent, but different information about virulence functions than the analyses presented above. One type of comparison is between strains of the same genus that infect different tissues. This comparison results in lists of genes that are common or different; this outcome may ultimately be correlated with tissue-specific virulence factors. Moreover, genes that are common but not found in other genera may reflect unique morphologic characteristics as well as host interactions. A second type of comparison is between two strains of the same species. Here, one is identifying regions of variability that are to be avoided in choosing targets for vaccine or antimicrobial therapy and that may be less important in infection. This is one of the newer and very promising areas in microbial genomics. Web sites that provide genomic data will also likely provide methods of comparative analyses, similar to methods provided by the Bugspray feature on the sexually transmitted diseases database site.

Solutions without Answers

If the ultimate aim of pathogen genome sequencing is the development of vaccines, therapeutics, and diagnostics, candidate genes may be identified before the mechanism of infection is understood. The genome sequence is the "parts list," used to test each gene product for its potential usefulness by various high-throughput methods. DNA vaccines constitute one of the few documented approaches for this purpose (43-45). In this case, genes targeted for vaccine use are cloned in expression vectors, and their efficacy for vaccine use is tested without ever studying the gene product. The potential of this approach was shown with Mycoplasma. A more commonly tried method in industry, often presented at conferences although not published, is to express a subset of the total set of genes in *E. coli*, purify the products, and test them in a mouse or other small animal model. The subset of genes is usually selected by computational criteria, i.e., their similarity to known virulence genes or indications that the protein is surface localized or secreted. In addition, expression analysis, using array technology, for instance, is often used to identify genes expressed in the host. Furthermore, many organism-specific genes without database matches are included in the subset, which may comprise 500 to 1,000 genes. Expression in *E. coli* is accomplished by using standard vectors, but usually as a fusion protein to a component that can simplify purification (histidine-tag, glutathione-S-transferase, or thioredoxin, for example). Many genes may fall by the wayside because of difficulties in expression or purification, but even if only 10% make it through, at least 50 to 100 candidates are available for testing in animal models. Such a large number of candidates easily surpasses the number of proteins identified for testing by traditional means. Clearly, discovering genes to test no longer limits the identification of useful gene products; rather, the new bottleneck is finding suitable models for high-throughput testing of efficacy. In any event, it is likely that candidate genes will be identified and enter industrial development long before researchers understand their role in infection.

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Comparative Genomics and Understanding of Microbial Biology

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The sequences of close to 30 microbial genomes have been completed during the past 5 years, and the sequences of more than 100 genomes should be completed in the next 2 to 4 years. Soon, completed microbial genome sequences will represent a collection of >200,000 predicted coding sequences. While analysis of a single genome provides tremendous biological insights on any given organism, comparative analysis of multiple genomes provides substantially more information on the physiology and evolution of microbial species and expands our ability to better assign putative function to predicted coding sequences.

Perhaps no other field of research has been more energized by the application of genomic technology than the field of microbiology. Five years ago, The Institute for Genomic Research published the first complete genome sequence for a free-living organism, *Haemophilus influenzae* (1). Since then, 27 more microbial genome sequences (2-28) and 3 lower eukaryotic chromosome sequences (29-31) have been published, and at least three times that many sequencing projects are under way. Several important human pathogens are included: *Helicobacter pylori* (7,19), *Borrelia burgdorferi* (12), *Treponema pallidum* (16), *Mycobacterium tuberculosis* (15), *Rickettsia prowazekii* (18), and *Chlamydia* species (17,20); the simplest known free-living organism, *Mycoplasma genitalium* (2); the model organisms, *Escherichia coli* (8) and *Bacillus subtilis* (10); *Aquifex aeolicus* (13) and *Thermotoga maritima* (21), two thermophilic bacterial species that may represent some of the deepest branching members of the bacterial lineage; five representatives of the archaeal domain (3,9,11, 14,28); and the first eukaryote, *Saccaromyces cerevisiae* (6).

Comparative Genomics

Genomic analyses show a tremendous variability not only in prokaryotic genome size but also in guanine plus cytosine (GC) content, from a low of 29% for *B. burgdorferi* (12) to a high of 68% for *M. tuberculosis* (15). The more than twofold difference in GC content affects the codon use and amino acid composition of species. For example, glycine, alanine, proline, and arginine, represented by GC-rich codons, are found at a much higher frequency in the predicted open reading frames from GC-rich genomes (Figure 1).

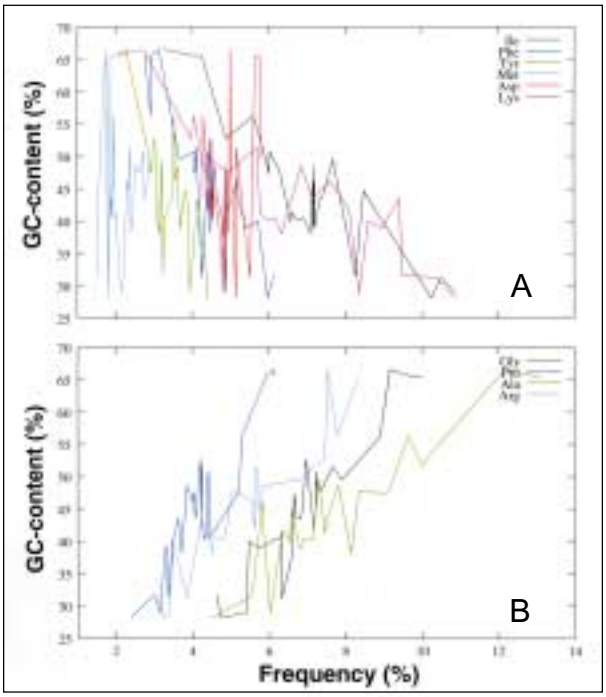

Figure 1. Comparison of amino acid frequency in microbial genomes as a function of % guanine + cytosine (G+C). A: amino acids represented by GC-rich codons; B: amino acids represented by AT-rich codons.

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Similarly, isoleucine, phenylalanine, tyrosine, methionine, and aspartic acid, represented by adenine plus thymine (A+T)-rich codons, are found at a higher frequency in the predicted open reading frames from AT-rich genomes. Genome organization also varies among microbial species, from single circular chromosomes to the most unusual situation seen with *B. burgdorferi*, whose genome is composed of an ≈1 Mbp (million base pairs) linear chromosome and 21 linear and circular extrachromosomal elements.

Results from the completed prokaryotic genome sequences show that almost half of predicted coding regions identified are of unknown biological function (Table 1). More unexpectedly, approximately one-quarter of the predicted coding sequences in each species are unique, with no appreciable sequence similarity to any other known protein sequence. These data indicate large areas of microbial biology yet to be understood and suggest that in the microbial world the idea of a model organism may not be valid.

Functions can be assigned to coding regions by making generalizations about proteins. The number of genes involved in certain functions (transcription and translation, for example) is quite similar, even when genome size differs by fivefold or more (Figure 2). This suggests that a basic complement of proteins is required for certain cellular processes. In contrast, the number of proteins in other function categories—such as biosynthesis of amino acids, energy metabolism, transporters, and regulatory functions—can vary and often increases with genome size (Figure 2). A substantial proportion of the larger microbial genomes represent paralogous genes, that is, genes related by duplication rather than by vertical descent. With few exceptions, the number of total genes that are members of paralogous gene families increases from approximately 12% to 15% in genomes of 1 Mbp up to approximately

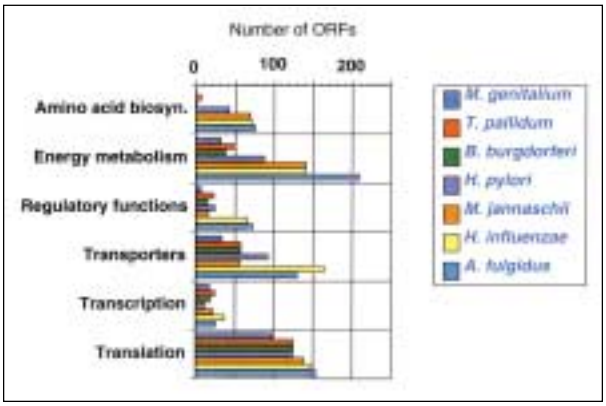

Figure 2. Comparison of the number of predicted coding sequences assigned to biological role categories in seven bacterial and archaeal species.

Table. Summary of predicted coding sequences from completed microbial genomes

Organism	Genome size (Mbp)	ORF no.	Unknown no. (%)	Unique no. (%)
Archaoglobus fulgidus	2.18	2437	1315 (54)	641 (26)
Methanobacterium thermotautotrophicum	1.75	1855	1010 (54)	496 (27)
Methanococcus jannaschii	1.66	1749	1076 (62)	525 (30)
Pyrococcus horikoshii	1.74	2061	859 (42)	453 (22)
Aquifex aeolicus	1.50	1521	663 (44)	407 (27)
Bacillus subtilis	4.20	4100	1722 (42)	1053 (26)
Borrelia burgdorferi	1.44	1751	1132 (65)	682 (39)
Chlamydia pneumoniae	1.23	1073	437 (40)	186 (17)
C. trachomatis	1.04	894	290 (32)	255 (28)
Deinococcus radiodurans	3.28	3193	1515 (47)	1001 (31)
Escherichia coli	4.60	4288	1632 (38)	1114 (26)
Haemophilus influenzae	1.83	1692	592 (35)	237 (14)
Helicobacter pylori	1.66	1657	744 (45)	539 (33)
Mycobacterium tuberculosis	4.41	3924	1521 (39)	606 (15)
Mycoplasma genitalium	0.58	470	173 (37)	7 (2)
Mycobacterium pneumoniae	0.81	677	248 (37)	67 (10)
Rickettsia prowazekii	1.11	834	311 (38)	207 (25)
Synechocystis sp.	3.57	3168	2384 (75)	1426 (45)
Thermotoga maritima	1.86	1877	863 (46)	373 (20)
Treponema pallidum	1.14	1040	461 (44)	28 (27)
Totals	41.6	40,261	18,948 (47)	10,303 (26)

50% in genomes of ≥3 Mbp. For a given organism, as genome size increases so do functional diversity and biochemical complexity. The one exception with regard to gene duplications is *B. burgdorferi*, where nearly half the genes in its 1.5-Mbp genome are paralogs. Most paralogous genes in *B. burgdorferi* are plasmid-encoded genes, many of which are putative lipoproteins. The reason for the large number of paralogous genes in this organism is not known.

The study of transport proteins in prokaryotic species elucidates the relationship between genome size and biological complexity. The ability to discriminate and transport appropriate compounds is an essential function of cell membranes and their resident proteins. The fidelity of these transport reactions is particularly critical at the cytoplasmic membrane of prokayotes since this is the primary barrier that separates the physiologic reactions of the cytosol from the external environment. Many bacterial pathogens face astounding chemical and biological challenges from their host environment (e.g., the extreme acidity of the gastrointestinal tract challenges *H. pylori*). In each host-pathogen relationship, the microbial membrane system contributes to the cell's strategy for energy production and carbon fixation while maintaining ionic homeostasis so that the enzymatic activities of the cytosol can proceed. In addition, all species encode proteins to expel toxic ions (particularly metals) and metabolites.

With complete genome sequences, evaluating the quantity and contribution of solute traffic across the membrane boundaries of pathogenic organisms is now possible. Comparisons between 11 sequenced bacterial pathogens (Table 1) indicate that approximately 6% of each genome encodes proteins (holoenzymes and subunits) involved in solute transport. This percentage is likely an underestimate since many of the gene products annotated as hypothetical proteins have hydropathy profiles reflective of known transporters. Genome size and the number of transport systems are directly related; the greatest number, 53, is annotated in the *M. tuberculosis* genome, and the smallest, 12, is found in the sequence of *M. genitalium*. Bacterial pathogens are heterotrophs; therefore, most of their import systems are used for the uptake of organic compounds (carbohydrates, organic alcohols, acids, amino acids, peptides, and amines). *M. tuberculosis* is the exception; it has 18 annotated transporters for organic substrates and 34 for the movement of ions. In this genome, there are nine copies of a P-type ATPase with a predicted substrate specificity for divalent cations. Whether this reflects a physiologic specialization allowing *M. tuberculosis* to be more resilient in its host environment is unknown.

Underlying these general trends are some unique genomic solutions to niche selection and species survival. Two pathogens, *H. influenzae* and *M. pneumonia*e, both infect the respiratory tract, yet their strategies for acquiring solutes are distinct (Figure 3). In *H. influenzae*, the genes encoding transporters show a marked diversification. For example, in systems for amino acid uptake there are transporters for 13 different

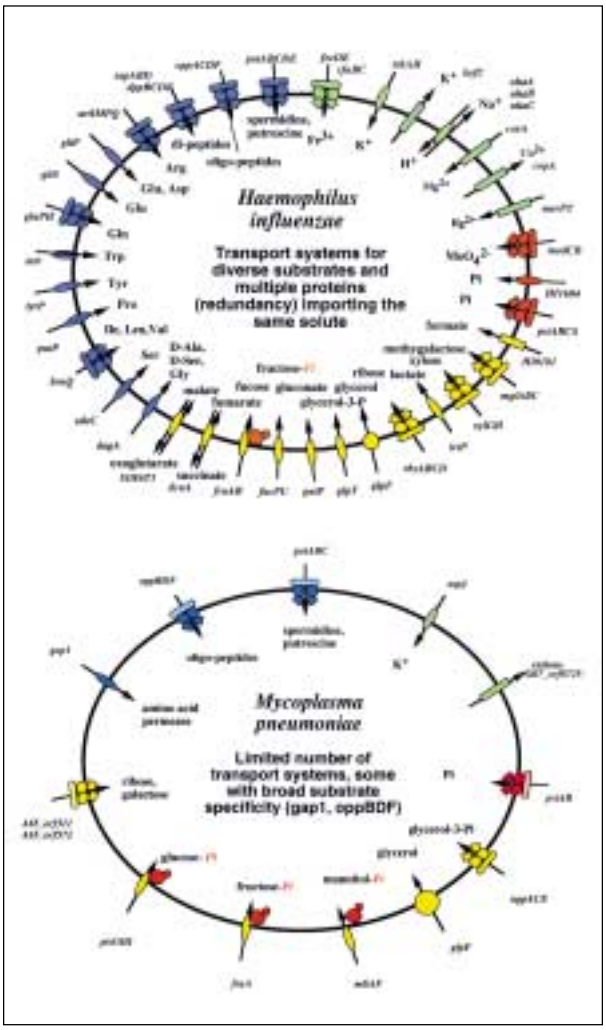

Figure 3. Comparison of the transport proteins in two human respiratory pathogens, *Haemophilus influenzae* and *Mycobacterium pneumoniae*

amino acids as well as proteins for the import of small peptides. These uptake systems work with several metabolic pathways for de novo amino acid synthesis. *H. influenzae* therefore employs a battery of redundant processes that allow it to optimize survival.

By contrast, the *M. pneumoniae* genome encodes only three transporters with substrate specificity for amino acids. This species, which may have evolved through reductive evolution from a gram-positive ancestor, has discarded all of the genes' encoding enzymes for amino acid biosynthesis (32). Instead of presenting a diverse group of porters for amino acid import, *M. pneumoniae* presents transport proteins with relatively broad substrate specificity: an oligopeptide transporter; a system for the aromatic residues tryptophan, tyrosine, and phenylalanine; and the spermidine/putrescine porter. *M. pneumoniae* uses a generalist strategy of maintaining proteins that are more versatile because of their broad substrate range. These same principles, diversification and redundancy, are repeated in the transport systems for carbohydrates (Figure 3).

In each analyzed genome, transport capacity appears to regulate the metabolic potential of that organism and dictates the range of tissues where a species can reside. Global analysis of transporters within a genome leads to several conclusions of practical consequence. First, culturing of pathogenic organisms is essential for understanding their physiology and for evaluating therapeutic agents. For species such as *T. pallidum* that have not yet successfully been grown in vitro, transporter analysis provides a clear starting point for the development of a defined culture medium based on information about the range of substrates a given cell can import and metabolize (16). Second, knowledge of transport processes and metabolic pathways they sustain provides novel solutions to the development of antimicrobial agents. An integrated view of cellular biochemistry enables selection of the pathway(s) essential for cell viability. Third, comparisons between genomes elucidate the diverse survival strategies found in pathogens with distinct evolutionary histories.

In addition to transport proteins, other membrane proteins in human pathogens play important roles in cell adhesion and as potential antigenic targets. Perhaps not surprisingly, in most human pathogens whose genome sequencing has been completed, mechanisms for generating antigenic variation on the cell surface have been proposed as a result of genome analysis. The following mechanisms for generating antigenic variation have been described: slipped strand mispairing within DNA sequence repeats found in 5'-intergenic regions and coding sequences as described for *H. influenzae* (1), *H. pylori* (7), and *M. tuberculosis* (15); recombination between homologous genes encoding OSPs, as described for *M. genitalium* (2), *M. pneumoniae* (5), and *T. pallidum* (16); and clonal variability in surface-expressed proteins, as described for *Plasmodium falciparum* (29) and possibly *B. burgdorferi* (12). Studies of clinical isolates of some species have demonstrated phenotypic variation in the relevant cell surface proteins (33), suggesting that (at least for human pathogens) evolution of antigenic proteins occurs in real time, as cell populations divide.

The Institute for Genomic Research has recently launched the Comprehensive Microbial Resource (CMR), a database designed to facilitate comparative genomic studies on organisms whose genome sequencing has been completed. CMR (http://www.tigr.org) includes the sequence and annotation of each of the completed genomes and associated information (such as taxon and Gram stain pattern) about the organisms, the structure and composition of their DNA molecules (such as plasmid vs. chromosome and GC content), and many attributes of the protein sequences predicted from the DNA sequence (such as pI and molecular weight). With CMR, a user can query all the genomes at once or any subset of them, as well as make complex queries based on any properties of the organism or genome. CMR can be used to mine the completed genomes in ways not possible with single genome databases, furthering the progress of comparative genomics.

Evolutionary Studies of Complete Genomes

Studies of complete genomes have provided an unprecedented window into the evolution of life on this planet. For example, analysis of bacterial, archaeal, and eukaryotic genomes has confirmed the uniqueness of the archaeal lineage. Comparative studies of genome sequences have also revealed that lateral gene transfer has been very common over evolutionary time, occurring between both close and distant

relatives (21). While the value of genome sequences in studies of evolution has been widely applauded, evolutionary analysis, which can provide great insight into genome sequences, is less well appreciated.

In any comparative biological study, an evolutionary perspective allows one to focus not only on characterizing the similarities and differences between species but also on explaining how and why those similarities and differences may have arisen (34). One area in genome analysis where an evolutionary perspective is useful is in distinguishing similarities due to homology (i.e., common ancestry) from those due to convergence (i.e., a separate origin). An example of the uses of distinguishing convergence from homology is the study of ribosomal RNA (rRNA) genes, which have been cloned from thousands of species; comparisons of these gene sequences are used extensively in evolutionary studies of these species. In early studies of rRNA sequences, most thermophiles were noted to have rRNA genes with high GC content relative to mesophiles. Since the rRNA genes in these thermophiles were similar in sequence and not just GC content, many of the thermophiles (e. g., the bacterial genera *Aquifex* and *Thermotoga*) were considered closely related. However, recent studies show that these genera are not closely related and the similarities in their rRNA genes are due to convergence (35). The most likely theory is that, to be stable at high temperatures, rRNAs need high GC contents, and therefore, even unrelated thermophiles will have similar sequences because many positions in the rRNA gene will converge to G or C (36). Finding this convergence explains the selective constraints on rRNA genes and shows that these genes may not be the best markers for evolutionary studies of species.

A highly practical use of evolutionary analysis in genome studies is predicting the function of genes (37). Predictions of gene function, a key step in the annotation of genomes, help researchers decide what types of experiments might be useful for a particular species or even a particular gene. Predictions are frequently made by assigning the uncharacterized gene the annotated function of the gene it is most similar to (similarity is measured by a database searching program such as BLAST). However, such predictions are frequently inaccurate because the annotated function may not be the best match (which would lead to error propagation if only the best match were used) and sequence similarity is not the best predictor of function. Several studies have shown that information about the evolutionary relationships of the uncharacterized gene can greatly improve predictions of function. For example, many gene families have undergone gene duplication. Since gene duplication is frequently accompanied by divergence of function, identifying the duplicate lineage (or orthology group) of a particular gene can greatly improve predictions of the gene's function. One orthology identification method is a clustering system developed by Tatusov et al. (38). This method (COG, for clusters of orthologous groups) classifies groups of genes by levels of sequence similarity. Although rapid and accurate in many cases, a clustering method such as COG does not always accurately infer the evolutionary history of genes. For this reason, and because orthologs do not always have the same function, we have developed a phylogenetic-tree–based function prediction method. This method involves inferring the evolutionary relationships of genes and then overlaying onto this history any experimentally determined functions of the genes. For uncharacterized genes, predictions are made according to their position in the tree relative to genes with known functions and according to evolutionary events (such as gene duplications) that may identify groups of genes with similar functions (39). Whatever method is used, information about the evolution of a gene can greatly improve function predictions.

Characterizing the evolutionary history of a particular gene is useful for other reasons. Identifying gene duplication events can provide insight into the mechanisms of gene duplication between genomes (e.g., proximity, age). Comparisons of the evolutionary history of different gene families can be used to infer recombination patterns within species as well as lateral gene transfers between species. While the likelihood of extensive gene transfers between species has thrown our concepts of the evolutionary history of species into disarray (21), identifying particular gene transfer events can be of great practical use. For example, there is a good correlation between regions of genomes responsible for pathogenicity and regions that have undergone lateral gene transfer (40). In analysis of eukaryotic genomes, identifying genes in the nucleus that have been

transferred from the organellar genomes can best be done by phylogenetic analysis. Genes derived from the mitochondrial genome should branch most closely with genes from alpha-Proteobacteria, and genes derived from the chloroplast genome should branch most closely with cyanobacterial genes. In most cases, nuclear genes derived from these organelles still encode proteins that function in the organelles.

Evolutionary analysis is also very important for inferring gene loss. For example, we have used phylogenomic analysis to show that the mismatch repair genes *MutS* and *MutL* have been lost separately in multiple pathogenic species (e.g., *H. pylori*, *M. tuberculosis*, *M. genitalium*, and *M. pneumoniae*) (37). Several studies have shown that defects in mismatch repair increase pathogenicity, probably because these defects increase the mutation rate, which allows faster evolutionary response to immune systems and other host defenses. With more and more completed genome sequences, finding any other genes that may have been consistently lost in pathogenic species or strains will be possible. Identifying gene loss can also be useful in making function predictions for genes or species. For example, genes with a conserved association with each other might be lost as a unit—if one is lost, there is probably not much reason for the others to persist. The correlated presence and absence of genes constitute the basis of the phylogenetic profiles method of Pellegrini et al. (41), a very important tool in predicting functions.

The study of the evolutionary relationship of the *M. tuberculosis* complex has been greatly enhanced by the availability of two complete sequences from different strains (15 and www.tigr.org) and most sequences from the *M. bovis* genome (www.sanger.ac.uk). The H37Rv laboratory strain of *M. tuberculosis* was first isolated in 1905 and has been passed for many decades; substantial differences have been demonstrated between recent clinical isolates and genomes of laboratory strains with long histories of passage. A highly infectious clinical isolate of *M. tuberculosis*, CDC1551, was involved in a recent cluster of tuberculosis cases in the United States (42). Whole genome analysis of single nucleotide polymorphisms, insertions and deletions, and gene duplications provides comparisons that were previously unobtainable. Studies examining a limited set of *M. tuberculosis* genes from various strains suggest a limited sequence diversity between strains and in the complex, with a nucleotide polymorphism rate of approximately 1 in 10,000 bp (43). Detailed comparison of strains H37Rv and CDC1551 indicates a higher frequency of polymorphism, approximately 1 in 3,000 bp, with approximately half the polymorphism occurring in the intergenic regions. In other words, 50% of the polymorphisms are in 10% of the genome. While this rate is higher than that suggested (43), it still represents a lower nucleotide diversity than found in limited comparisons from other pathogens.

Examination of insertion and deletion events and gene duplication between species and strains allows insight into the evolutionary relationship of the *M. tuberculosis* complex. For example, a phospholipase C region, present in CDC1551 and absent in H37Rv, is also present in *M. bovis*. The simplest explanation for this is that the common ancestor of *M. tuberculosis* and *M. bovis* contained this region, and the region was subsequently deleted in the H37Rv lineage.

Membrane lipid proteins are identified by a unique signature sequence that is the target for a specific lipoprotein signal peptidase and that allows the cleaved protein product to attach by cysteinyl linkage to a glyceride–fatty acid lipid. Among the genes encoding membrane lipid proteins in strain H37Rv are two in tandem (*Rv2543* and *Rv2544*). Nucleotide identity of >85% suggests that these two genes arose through duplication. The homologous genome region in strain CDC1551 contains the orthologs MT2618 and MT2620, respectively, as well as a third gene, *MT2619*, which by sequence similarity appears to represent an additional duplication (Figure 4). The increased induction of cytokines by CDC1551 is associated with the membrane lipid component (42). Modification of the lipid

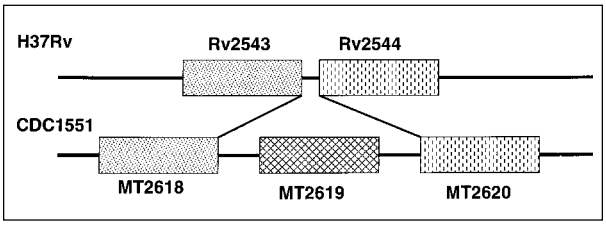

Figure 4. Polymorphic insertions in *Mycobacterium tuberculosis*. A genomic region containing membrane lipid protein genes likely to have arisen through gene duplication. H37Rv contains two genes (*Rv2543* and *Rv2544*), while the homologous region in CDC1551 appears to have undergone additional gene duplication (*MT2618*, *MT2619*, and *MT2620*).

component by various protein components may contribute to differences in the immune response to *M. tuberculosis* infection in the host.

These examples illustrate how evolutionary information can benefit genome analysis. Complete genome sequences are also very useful. Gene loss, for example, cannot be readily identified without knowing the complete genome sequence of an organism. Since there are feedback loops between evolutionary and genome analyses, combining them into a single composite *phylogenomic* analysis may be advantageous (37,44). As more and more genomes are completed, the benefits of combined evolutionary and genome analysis should become even more apparent.

Dr. Fraser is president and director of the Institute for Genomic Research. She is involved in genome analysis of microbial species and the use of comparative genome analysis in elucidating species diversity.

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Using DNA Microarrays to Study Host-Microbe Interactions

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Complete genomic sequences of microbial pathogens and hosts offer sophisticated new strategies for studying host-pathogen interactions. DNA microarrays exploit primary sequence data to measure transcript levels and detect sequence polymorphisms, for every gene, simultaneously. The design and construction of a DNA microarray for any given microbial genome are straightforward. By monitoring microbial gene expression, one can predict the functions of uncharacterized genes, probe the physiologic adaptations made under various environmental conditions, identify virulence-associated genes, and test the effects of drugs. Similarly, by using host gene microarrays, one can explore host response at the level of gene expression and provide a molecular description of the events that follow infection. Host profiling might also identify gene expression signatures unique for each pathogen, thus providing a novel tool for diagnosis, prognosis, and clinical management of infectious disease.

The complex interaction between a microbial pathogen and a host is the underlying basis of infectious disease. By understanding the molecular details of this interaction, we can identify virulence-associated microbial genes and host-defense strategies and characterize the cues to which they respond and mechanisms by which they are regulated. This information will guide the design of a new generation of medical tools.

Genomic sequencing will provide the data needed to unravel the complexities of the host-pathogen interaction. As of August 10, 2000, draft sequence was available for 87% of the human genome (http://www.ncbi.nlm.nih.gov/genome/seq/), and at least 39 prokaryotic genomes, including those of more than a dozen human pathogens, had been completely sequenced (http://www.tigr.org/tdb/mdb/mdbcomplete.html). The pace of gene discovery rapidly accelerates, but its potential for explaining life at the molecular level remains largely unrealized because our understanding of gene function lags increasingly far behind. For example, even in the heavily studied *Escherichia coli*, no function has been assigned to more than one third of its genes (1). High-throughput methods for assessment of function are clearly required if this wealth of primary sequence information is to be used.

Global profiling of gene expression is one attractive approach to assessing function. Because a gene is usually transcribed only when and where its function is required, determining the locations and conditions under which a gene is expressed allows inferences about its function. Several independent high-throughput methods for differential gene expression (including SAGE and differential display) may enable function annotation of sequenced genomes (2). DNA microarray hybridization analysis stands out for its simplicity, comprehensiveness, data consistency, and high throughput.

Transcription control plays a key role in host-pathogen interaction (3,4); thus, genomewide transcription profiling seems particularly appropriate for the study of this process. This review focuses on microarray-based approaches for studying transcription response because they hold exceptional promise for the study of infectious disease. Microarray-based genotyping applications, although expected to make substantial contributions in this field, are covered only briefly here.

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High-Density DNA Microarrays: Basic Tools

First described in 1995 (5), high-density DNA microarray methods have already made a marked impact on many fields, including cellular physiology (6-11), cancer biology (12-17), and pharmacology (18,19). The first results of gene expression profiling of the host-pathogen interaction have just begun to emerge. Before exploring these results, we briefly review the methods.

Technology

The key unifying principle of all microarray experiments is that labeled nucleic acid molecules in solution hybridize, with high sensitivity and specificity, to complementary sequences immobilized on a solid substrate, thus facilitating parallel quantitative measurement of many different sequences in a complex mixture (20,21). Although several methods for building microarrays have been developed (22,23), two have prevailed. In one, DNA microarrays are constructed by physically attaching DNA fragments such as library clones or polymerase chain reaction (PCR) products to a solid substrate (5) (Figure 1). By using a robotic arrayer and capillary printing tips, we can print at least 23,000 elements on a microscope slide (P. Brown, pers. comm.; Figure 2). In the other method, arrays are constructed by synthesizing single-stranded oligonucleotides in situ by use of photolithographic techniques (24,25). Advantages of the former method include relatively low cost and substantial flexibility (which explain its wide implementation in the academic setting); in addition, primary sequence information is not needed to print a DNA element. Advantages of the latter method include higher density (>280,000 features on a 1.28X1.28-cm array) and elimination of the need to collect and store cloned DNA or PCR products. Continued commercial interest in microarray technology promises increasing array element density, better detection sensitivity, and cheaper, faster methods. Technical descriptions of microarray construction methods and hybridization protocols are available (26-28; and http://cmgm.stanford.edu/pbrown/mguide/index.html).

Messenger RNA from eukaryotic cells is usually specifically labeled by affinity purification of mRNA with an oligo-dT resin, followed by

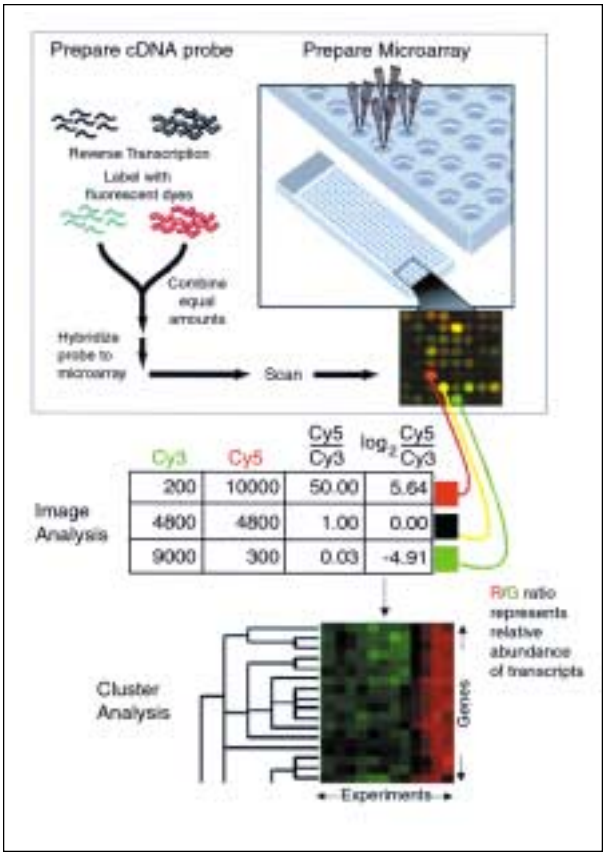

Figure 1. Measuring relative gene expression by using DNA microarrays. Capillary printing is used to array DNA fragments onto a glass slide (upper right). RNA is prepared from the two samples to be compared, and labeled cDNA is prepared by reverse transcription, incorporating either Cy3 (green) or Cy5 (red) (upper left). The two labeled cDNA mixtures are mixed and hybridized to the microarray, and the slide is scanned. In the resulting pseudocolor image, the green Cy3 and red Cy5 signals are overlaid—yellow spots indicate equal intensity for the dyes. With the use of image analysis software, signal intensities are determined for each dye at each element of the array, and the logarithm of the ratio of Cy5 intensity to Cy3 intensity is calculated (center). Positive log(Cy5/Cy3) ratios indicate relative excess of the transcript in the Cy5-labeled sample, and negative log(Cy5/Cy3) ratios indicate relative excess of the transcript in the Cy3-labeled sample. Values near zero indicate equal abundance in the two samples. After several such experiments have been performed, the dataset can be analyzed by cluster analysis (bottom). In this display, red boxes indicate positive log(Cy5/Cy3) values, and green boxes indicate negative log(Cy5/Cy3) values, with intensity representing magnitude of the value. Black boxes indicate log(Cy5/Cy3) values near zero. Hierarchical clustering of genes (vertical axis) and experiments (horizontal axis) has identified a group of coregulated genes (some shown here) and has divided the experiments into distinct classes.
(Illustration by J. Boldrick, Stanford University.)

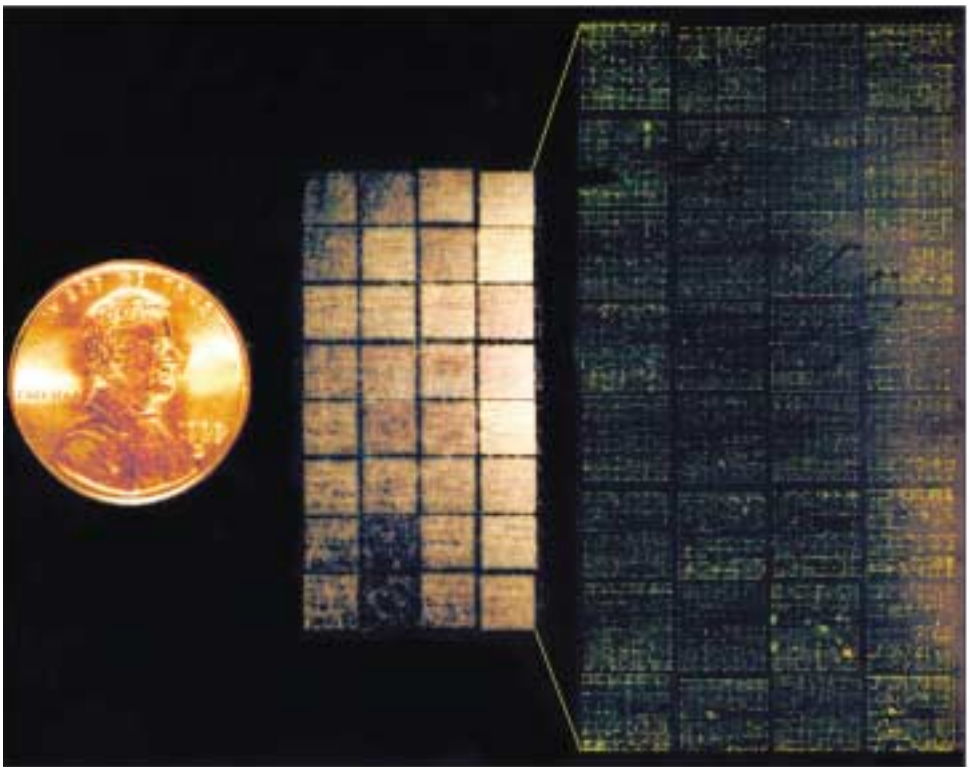

Figure 2. DNA microarray—Lymphochip. (Center) Lymphochip version 8.0, printed on a coated glass microscope slide using a 32-tip printing head, contains 17,856 cDNA clones (overhead illumination) (14). (Left) U.S. penny, for scale. (Right) Scanned image demonstrating differential hybridization of Cy3- and Cy5-labeled cDNA to this microarray. (Illustration by A. Alizadeh, M. Eisen, and P. Brown, Stanford University; and L. Staudt, National Cancer Institute.)

incorporation of dye-labeled nucleotides into cDNA molecules by reverse transcriptase (RT) with random or oligo-dT oligonucleotide primers (Figure 1). In prokaryotes, the absence of polyadenylation on transcripts makes labeling of mRNA more difficult. One method is labeling of total RNA either by covalent linkage (29) or by incorporating dye-labeled nucleotides into complementary DNA through RT and random oligonucleotide primers (30). In spite of the high copy number of labeled ribosomal and tRNA molecules in the hybridization reaction, specific hybridization of mRNA to the array can be achieved under appropriate stringency. An alternative method is to prime reverse transcription with a mixture of reverse-strand oligonucleotides specific for open reading frames (ORFs), either those used to construct the microarray (M. Laub and L. Shapiro, pers. comm.) or a minimally complex mixture of octamers sufficient to hybridize to the 3' end of every ORF (31). This method results in higher signal-to-noise ratios by preferentially synthesizing cDNA from coding regions.

For printed DNA microarrays, relative transcript abundance is measured by labeling two samples with different fluorescent dyes (e.g., Cy3 and Cy5), hybridizing them simultaneously, and determining the fluorescence ratio for each spot on the array (Figure 1). On oligonucleotide arrays, multiple probes from the same gene, each with a corresponding mismatch probe that serves as internal control, as well as labeled transcript of known amounts for standard genes makes quantitative measurement of transcript abundance possible after hybridizing a single labeled sample (25). For both techniques, use of fluorescent labeling enhances sensitivity and the dynamic range of measurement.

Gene expression array experiments can also be performed by hybridizing a single labeled mRNA sample to "macroarrays" of DNA elements on positively charged filters (10,11,32-34). Because this format does not require any special arraying or scanning equipment, specialty arrays can be made and analyzed relatively cheaply. Human, mouse, and microbial macroarrays are also commercially available (SigmaGenosys, The Woodlands, TX; Research Genetics, Huntsville, AL; Clontech Laboratories, Palo Alto, CA; Genome Systems, St. Louis, MO). The major disadvantages of this format are reduced sensitivity (32), limited elements, and the need for higher concentrations of labeled cDNA.

Microarray Data Analysis

Microarrays are likely to become a standard tool of the microbiology laboratory. However, because genomewide datasets are large and comprehensive, analysis of an experiment can become daunting. Careful experimental design can simplify analysis and interpretation of the dataset by minimizing the number of variables that affect gene expression. For example, strain differences can be minimized by using isogenic mutants, tissue complexity can be reduced by studying clonal cell lines, and complex regulatory pathways can be tamed by experimental modulation of transgene expression (6).

Because microarray experiments result in such large amounts of data, false-positive results are likely. Analyzing multiple independent experiments may eliminate spurious results (32). Also important is validation of differentially expressed genes by independent methods. When checked by a number of methods including quantitative RT-PCR (6, 35), Northern blotting (33, 34, 36), and protein expression (33, 34), most differentially expressed genes have been confirmed. For example, 72 of 72 mRNAs found to be regulated in response to cytomegalovirus (CMV) infection were confirmed by either prior reports or Northern blotting (37). Future challenges for microarray researchers will include developing databases and algorithms to manage and analyze vast genomic-scale datasets.

Image Analysis Software

The first step after hybridization is capturing an image of the array and from it, extracting numerical data for each element (Figure 1). Several software applications, including those packaged with most commercial scanners, can perform this task. However, not all programs use the same algorithms to calculate signal intensity, and each of the programs exports a different constellation of signal quality measurements, complicating comparisons between data acquired with different applications (38). If gene expression datasets are to be compared, these measurements must be standardized. Furthermore, standard, robust statistical methods must be developed for assigning significance values to gene expression measurements.

Databases

Although many laboratories are now capable of collecting microarray data, few have access to a database that can effectively meet their data requirements. With considerable investment of resources, a few full-featured, relational gene expression databases have been developed, but these are not available for public deposition of data (e.g., http://genome-www4.stanford.edu/MicroArray/MDEV/index.html; http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/dbase.html). Recently released, the freely available AMAD software package (http://www.microarrays.org/software.html) provides basic microarray data storage and retrieval capabilities to the average laboratory.

A grander goal for the community is establishing a consolidated resource for public distribution of microarray data (39-41). Again, the lack of a standard format for microarray data interferes with creating such a resource (38,39). The European Bioinformatics Institute, recognizing this obstacle, has proposed defining a standard based upon XML, a computer markup language that combines data and formatting in a single file for distribution over the World-Wide Web (40; http://www.ebi.ac.uk/arrayexpress/).

Algorithms

Inferring biologically meaningful information from microarray data requires sophisticated data exploration. Most global gene expression analyses have used some form of unsupervised clustering algorithm (16,42-44) to find genes coregulated across the dataset (Figure 1). A primary justification for this approach is that shared expression often implies shared function (38,43). In datasets containing many experiments, clustering can also group experiments on the basis of gene expression profiles, an approach that has been successful in classifying tumor-derived cell lines (19, 45) and tumor subtypes (12-17).

When a coregulated class of genes is known, supervised clustering algorithms, which are trained to recognize known members of the class, can assign uncharacterized genes to that class. For example, a machine-learning method known as a support vector machine has been used to classify yeast genes by function on the basis of shared regulation (46). Robust determination of coregulated gene clusters may be achieved by using a tiered approach: unsupervised clustering to identify coregulated genes followed by testing and refinement with supervised algorithms (47).

Although clustering algorithms will continue to be a mainstay in the analysis of gene expression datasets, a wealth of other data-mining techniques have yet to be applied (38,48). Preliminary reports indicate that many algorithms and visualization methods are being developed, but their ability to extract biologic insight has yet to be established (49-51).

The study of microbial pathogens, and prokaryotes in general, will require the development of some specialized analysis tools. First, the compact and modular structure of prokaryotic genomes—and in particular, the presence of operons and pathogenicity islands—suggests that important insights may be gained by mapping gene expression information onto genomic structure. In addition, because gene expression will be measured in many different pathogens, often under the same environmental conditions, tools for cross-species comparison of gene expression data will permit the detection of conserved transcription responses.

Examining a Microorganism: Application of DNA Microarrays

Microarray technology promises to speed the study of uncharacterized or poorly characterized microbes by contributing to annotation of the microbial genome, enabling exploration of microbial physiology, and identifying candidate virulence factors.

Designing a Microbial Genome Microarray

Designing a whole-genome DNA microarray for a fully sequenced microbe is conceptually straightforward. Several sensitive microbial gene-finding programs can quickly and accurately predict most ORFs (52-57). DNA fragments representing each of the ORFs can be obtained by PCR amplification that uses ORF-specific oligonucleotides, the design of which can be automated with primer design software such as Primer3 (58). Homology-searching algorithms should be used to choose regions of genes that will not cross-hybridize with other regions of the genome. After a simple purification step, PCR fragments can be arrayed by a robotic arrayer (5). This basic approach has been used to construct a 4,290-ORF *E. coli* microarray (10, 11) and a 3,834-ORF *Mycobacterium tuberculosis* microarray (30) as well as full-genome arrays for *Helicobacter pylori* (S. Falkow, pers. comm.) and *Caulobacter crescentus* (L. Shapiro, pers. comm.). Microarray fabrication based on photolithographic synthesis of oligonucleotides in situ is also a viable approach and has been successfully used for the production of an *E. coli* complete ORF chip (*E. coli* Genome Array, Affymetrix, Santa Clara, CA).

The utility of microarrays is not restricted to fully sequenced organisms. A powerful screening tool can be obtained by arraying DNA libraries, as has been done for the eukaryotic pathogen, *Plasmodium falciparum* (59). A DNA microarray of 3,648 random genomic clones was used to identify >50 genes for which expression differed significantly between the trophozoite and gametocyte stages. The major limitation of this approach is that the identity of any element of interest must be determined after the experiment.

Annotating the Function of a Microbial Genome

For many pathogens, the number of genes for which function information is available is usually low. Moreover, the relative insufficiency of genetic tools can make obtaining such information difficult. However, because >70% of bacterial proteins have orthologs in other organisms (60,61), one can leverage extensive knowledge of function from the model organisms to infer function for a pathogen's genome. Similarity searches alone will predict functions of many genes.

We expect the study of genomewide expression patterns to contribute even further to annotation of function. The rationale for this belief follows from the observation that shared expression often implies shared function (38). As suggested by Brown and Botstein (21), the inclusion of a gene with a characterized ortholog in a coregulated gene cluster can predict the function of the remaining genes in that cluster, thus bootstrapping the function annotation of the pathogen's genome. This assertion is borne out in a study of global gene expression in *Saccharomyces cerevisiae*. Clustering of 2,467 gene expression profiles across a series of 78 experiments representing eight cellular processes demonstrated coregulation of genes that participated in shared cellular function (43). Therefore, the acquisition of a pathogen's gene expression data from even a modest number of experimental conditions may lead to testable hypotheses about function for a substantial number of genes, even those lacking sequence similarity to genes whose function has been characterized.

Probing a Microbe's Physiologic State

The assumption that genes are preferentially expressed when their function is required allows inference of gene function directly from physiologic gene response. For example, genes preferentially transcribed during the diauxic shift in yeast are predicted to contribute in the metabolic transition to respiration (9). Thus, gene expression studies will contribute to function annotation by identifying the specific environmental and physiologic conditions in which each gene is expressed. Furthermore, as annotation improves, the direction of this inference may be reversed, i.e., if information on function is known for many genes, genomic expression profiling may reveal the physiologic state of the organism.

Two studies have used whole-genome DNA arrays to explore gene expression response to environmental stimuli in *E. coli*. First, treatment with isopropyl-ß-D-thiogalactopyranoside (IPTG) was shown to induce only the *lac* operon, and to a lesser extent, the melibiose operon (11). In a second study, comparison of strains grown in minimal versus rich media revealed 344 genes that were differentially expressed between the two conditions: preferential expression of the translation apparatus in rich media and the amino acid biosynthetic pathways in minimal media were entirely consistent with prior data (10). Finally, examination of gene expression during heat shock revealed 119 genes with altered expression levels, all but 35 of which were previously recognized as heat shock genes (11). These studies confirm that the physiologic state of bacteria can be inferred from gene expression data.

In the first report of global gene expression monitoring in a bacterial pathogen, oligonucleotide microarrays were used to measure the relative transcript levels of 100 *Streptococcus pneumoniae* genes during the development of natural competence and during stationary phase (29). The results confirmed induction of the *cin* operon and identified 11 genes differentially regulated in stationary versus exponential phase. Of course, gene expression monitoring is not restricted to the study of bacterial pathogens. Transcription of the CMV genome was measured during infection by using an array of 75-mer oligonucleotides representing each of the 226 predicted CMV ORFs (62). By blocking translation or DNA replication, the researchers revealed a detailed classification of CMV genes into four kinetic classes, in agreement with previous reports, and assigned many ORFs, for which expression data were not previously available, into these groups.

Identifying Candidate Virulence Factors

Because expression of virulence-associated genes is tightly regulated (4), measuring a pathogen's gene expression in microenvironments specific to the pathogen and germane to the disease process is critical. Exploration of pathogen gene expression in the host environment may be technically challenging because of the relatively small number of pathogens present in an infected animal (29). Until more sensitive detection protocols are developed, examining global gene expression will be more practical in environmental conditions that mimic aspects of the host environment, such as elevated temperature, iron limitation, and changes in pH (4, 63) and in cell culture models. In fact, a microarray has been used to monitor gene expression in *M. tuberculosis* while it infects cultured monocytes (64). Even after measurement of bacterial gene expression from infected hosts becomes feasible, the ex vivo datasets will facilitate deconstruction of the in vivo gene expression response into component responses, leading to detailed understanding of the pathways of virulence factor regulation.

Identifying candidate virulence factors through a global gene expression method relies on two assumptions. First, because virulence-associated genes are often coordinately regulated (4), new virulence factors are likely to be coregulated with known ones. By clustering gene expression profiles across a large number of conditions, we can precisely monitor coregulation, thus revealing subtleties of regulation and leading to the identification of bona fide regulons. Second, because virulence-associated genes are tightly regulated (4), genes that are specifically expressed during infection or under conditions mimicking infection are candidate virulence factors. This assumption has been justified by numerous studies using in vivo expression technology (IVET) and differential fluorescence induction (DFI), in which genes induced during infection are often required for virulence (4, 65). When RNA from in vivo microbial samples can be efficiently isolated and labeled, microarrays will provide substantial advantages over IVET and

DFI technologies for identifying putative virulence factors, including immediate identification of differentially expressed genes and detection of temporal profiles of transcription induction and repression. As is demanded for candidate genes identified by any expression screening approach, a role in pathogenesis must be confirmed by mutation and subsequent assays of virulence.

By identifying factors expressed in the host, microarray methods may also identify potential vaccine targets. Furthermore, one could identify candidate epitopes for vaccine development for intracellular pathogens by predicting whether genes that are preferentially expressed inside host leukocytes will encode promiscuous human leukocyte antigen class II ligands (66).

Gene expression studies may also reveal key regulatory differences that lead to differing virulence between closely related pathogen strains. For example, variations in virulence of *Listeria monocytogenes* serotypes have been correlated with differential transcription of PrfA-regulated virulence genes (67, 68). However, because microarrays cannot measure expression of genes that are absent from the reference strain, genotypic differences such as horizontal transfer of virulence factors will not be detectable by this method.

Pharmacogenomics

Yet another application for microarrays is the study of drug effects on microbial cellular physiology, as revealed by global gene expression patterns (69). This approach has been used to identify drug-specific gene expression signatures in yeast and human cells (18,19,70). Correlation of gene expression with drug activity may suggest molecular details of drug action, and correlation of transcription profiles in untreated cells with drug response may reveal mechanisms for sensitivity and resistance (19).

This approach has recently been used to characterize gene expression response in *M. tuberculosis* exposed to known inhibitors of the mycolic acid biosynthesis pathway, isoniazid and ethionamide (30). Both of these compounds elicited a similar gene expression response profile, characterized by pronounced transcription induction of five adjacent genes encoding fatty acid biosynthesis enzymes. Because a proven isoniazid target, KasA, was among these genes, the authors proposed that the adjacent, coregulated loci might be targets for new anti-tuberculosis drugs. Finally, these results suggested that the mode of action of a novel compound may be inferred from gene expression response to that compound.

Using microarrays to detect microbial polymorphisms linked to known drug-resistance phenotypes will also influence diagnosis and subsequent drug treatment. For example, an oligonucleotide array was used to detect mutant alleles of the *M. tuberculosis rpoB* gene, which are known to confer resistance to rifampicin (71).

Microbial Genotyping

One microarray application that interrogates DNA rather than RNA is the identification of genomic deletions in mutant strains and environmental isolates by measuring the number of DNA copies at each locus, a technique termed array-based comparative genome hybridization (72). This technique was used to identify several large deletions in a number of BCG vaccine strains and reconstruct their phylogeny (73).

Oligonucleotide arrays have also been used for fine-scale genotyping of polymorphisms in related pathogens. Accurate identification of *Mycobacterium* species using a GeneChip containing a set of 82 polymorphic oligonucleotides from the 16S ribosomal RNA gene demonstrated the potential power of this approach for molecular diagnostics (71). As additional microbial genome ORF microarrays become available, molecular surveys of the genomic structure of multiple strains will become far more precise and feasible. Two caveats should be mentioned: the ability to characterize genome insertions relative to the reference sequence is lacking, and the degree to which sequence variability can be characterized on the basis of microarray hybridization is unknown.

Examining a Host: Application of DNA Microarrays

Designing Microarrays for Host Organisms

The currently described human DNA microarrays are largely composed of expressed sequence tags (ESTs). Culling ESTs from many different tissue sources and limiting representation of any single Unigene cluster (see http://www.ncbi.nlm.nih.gov/UniGene/Hs.stats.shtml) have resulted in better than 50% representation of the predicted 80,000-100,000 human coding regions (28). A variety of human DNA and

oligonucleotide microarrays are available commercially (e.g., Incyte, Palo Alto, CA; Affymetrix; NEN Life Science Products, Boston, MA).

For in vivo studies of host response, infection of animal models will often be necessary. If the animal is a primate, human DNA microarrays might be used to monitor host gene expression because of the high level of primary sequence similarity between species. Sequence similarity is too low to permit reliable cross-hybridization with nonprimate vertebrates, but microarrays composed of mouse and rat sequences have been described (74) and are available (e.g., Incyte, Affymetrix).

Understanding Pathogenesis

Microarrays promise to accelerate our understanding of the host side of the host-pathogen interaction. A large fraction of the genome can be simultaneously interrogated, and clustering of the data may identify groups of genes that implicate activation or repression of key regulatory pathways. Microarrays also allow the temporal sequence of transcription induction and repression to be followed, a prerequisite for determining the order of events following an encounter. Finally, ascertainment of the host cell's physiologic state, particularly apoptosis and necrosis, by genomewide profiling will facilitate separation of primary and secondary effects.

One important caveat of studying transcription in any system is that post-transcription regulatory events cannot be detected. This is particularly important in the case of host response because many important host cell events, such as cytoskeletal rearrangements, occur after transcription (75). Therefore, some key aspects of the molecular program may not be easily characterized by gene expression profiling. Eventually, it may be possible to monitor simultaneously the levels, activities, and interactions of all proteins in the cell (76).

Although analyzing gene expression of infected tissues is feasible, cellular heterogeneity may make analysis of host response complicated. Examining the response in infected cultured cells by using cell types most likely to encounter the pathogen may reduce the complexity of the system being examined. Results obtained in cell culture systems will be instrumental in interpreting gene expression profiles of specific cell types from whole tissue datasets.

The first application of global gene expression methods to pathogenesis used oligonucleotide arrays to monitor gene expression in primary human fibroblasts infected by human CMV (37). The transcript abundance of 258 out of 6,600 human genes changed by more than fourfold compared to uninfected cells at either 8 or 24 hours after infection. Some of these changes, such as induction of cytokines, stress-inducible proteins, and many interferon-inducible genes, were consistent with induction of cellular immune responses.

A similar experimental design has been used to examine the global effects of HIV-1 infection on cultured CD4-positive T cells. One study concluded that HIV-1 infection resulted in differential expression of 20 of the 1,506 human genes monitored and that most of these changes occurred only after 3 days in culture (36). In contrast, the preliminary results of an independent study using a similar design indicated that substantial HIV-induced transcription changes began very early after inoculation (77). The latter study confirmed activation of nuclear factor-κB (NF-κB), p68 kinase, and RNase L.

DNA expression arrays have recently been used to examine the response of host cells to infection by bacterial pathogens. Transcription profiling of macrophages and epithelial cells infected by *Salmonella* confirmed increased expression of many proinflammatory cytokines and chemokines, signaling molecules, and transcription activators and identified several genes previously unrecognized to be regulated by infection (33,34). The macrophage study demonstrated that exposure to purified *Salmonella* lipopolysaccharide resulted in a very similar response profile to whole cells and that activation of macrophages with gamma interferon before infection modified the response (34). In epithelial cells, overexpression of κB (an inhibitor of NF-κB) blocked induction of gene expression for a number of regulated genes, underscoring the importance of NF-κB in the proinflammatory response (33).

Similarly, the transcription response of human promyelocytic cells to *L. monocytogenes* infection has been determined by both oligonucleotide arrays and filter-based arrays (32). Comparison of these data with the *Salmonella* infection data suggests that the proinflammatory response is grossly conserved: in both cases

many key components including interleukin-1, intercellular adhesion molecule-1, and macrophage inflammatory protein 1-β are induced. Although differences were observed between the two experiments, including induction of apoptosis-promoting genes by *Salmonella* versus induction of anti-apoptotic genes by *L. monocytogenes*, the disparities between cell lines, methods, and genes assayed in these reports make direct comparison difficult. However, we speculate that differences in pathogen virulence strategies may account for some of these differences in host response at the molecular level.

The initial reports demonstrate the potential power of using microarrays to characterize host response but also suggest that interpretation of host gene expression profiles will be challenging. For example, modulation of mRNAs encoding components of the prostaglandin E2 biosynthetic pathway suggested that CMV induced synthesis of this proinflammatory second messenger (37). The authors of this study proposed three potential explanations for this observation: this pathway could be induced by a cellular response intended to limit spread of the infection by promoting the killing of infected cells; viral regulators could induce prostaglandin E2 production to lure monocytes, which could subsequently be infected, leading to viral dissemination within the host; and these genes could be induced secondarily through induction of interleukin-1β since a similar pattern of regulation was observed in cells treated with that cytokine. Microarrays can identify interesting cellular events, but because expression patterns cannot distinguish between these mechanisms, the need for further investigation is obvious.

The experiments described above are strictly exploratory and attempt to catalog the transcription events that occur after an infection. However, expression profiling also lends itself to a more hypothesis-driven experimental design. For example, comparison of host responses to related strains of the same pathogen could explain differences in pathogenesis. In fact, comparison of gene expression in human monocytes infected by two distinct strains of Ebola virus, one infectious for humans and one not, revealed divergent transcription responses (78). Similarly, by examining responses to isogenic mutant pathogen strains lacking single virulence genes, or virulence factor-associated biologic activities, one might attribute components of the response to specific virulence attributes, which in turn might yield mechanistic insight into those virulence factors. Finally, comparing transcription responses to families of structurally related virulence factors, e.g., bacterial pore-forming toxins, may explain how pathogens expressing similar virulence factors can cause different pathologic responses.

Diagnostic Gene Expression Profiles

Most microarray-based gene expression studies in humans have searched for genes that are differentially expressed in various pathologic states. For example, clustering gene expression profiles can classify tumors into separate molecular subtypes (12-17). In the case of diffuse large B-cell lymphoma, two distinct molecular classes exhibit substantially different survival rates, suggesting that future clinical intervention, at least in the case of cancer, could be guided by diagnostic gene expression profiling (14). Microarrays have also been used to measure the response of cultured cells to distinct external stimuli, including drugs (19) and environmental toxins (79).

How can this paradigm be applied to the diagnosis of infectious disease? In collaboration with Pat Brown (Stanford) and Lou Staudt (National Cancer Institute), we hypothesize that the unique constellation of virulence factors expressed by a specific pathogen will elicit a unique transcription response in the host (80). By extension, the cascade of events leading to inflammation and acquired immunity, including secretion of mediators and subsequent cell-cell interactions, might leave a unique trail of transcription signatures in the leukocytes participating in that response. Despite conserved overall virulence strategies, microbial pathogens exhibit specialization and unique attributes for any given strategy at the molecular level (81). Thus, by measuring the aggregate gene expression pattern in peripheral blood mononuclear leukocytes, for example, we may find signatures diagnostic of infection by specific pathogens or categories of pathogens.

The potential advantages of using host gene expression signatures as diagnostic markers of infection are profound. First, this technique might permit early detection of exposure to pathogens, even uncultivatable or uncharacterized

pathogens. Second, variations in host signatures could be used to infer time since exposure. Third, because host response may continue in the absence of the pathogen, this method might detect exposure to pathogens that only transiently colonize the host, are sequestered in poorly accessed anatomic sites, or do not colonize the host at all (e.g., *Clostridium botulinum* and *C. perfringens*, in some cases). Finally, a single, easily collected sample could be used for diagnosing exposure to a wide array of agents.

Before the proposed method becomes an accepted diagnostic tool, one must determine whether exposure to a pathogen leads to a robust, persistent, and specific gene expression signature in peripheral blood mononuclear leukocytes and whether this signature is universal in patients of different genetic backgrounds. Experiments are under way in our laboratory to assess the feasibility of this approach. Thus far, identification of gene expression profiles common to many different pathogens is leading to a more detailed understanding of early events in the development of immune response, and inflammation in particular, but the goal of these experiments (to define unique signatures for each pathogen) has not yet been realized.

Conclusion: The Two-Way Conversation

The few published studies reviewed here represent what is certain to be the beginning of a deluge of genome-scale pathogen data. At Stanford University alone, microarray-based studies of *Bordetella pertussis*, *Salmonella*, *H. pylori*, *Campylobacter jejuni*, *V. cholerae*, *M. tuberculosis*, and *E. coli*, as well as the nonpathogenic microbes *Streptomyces coelicolor* and *C. crescentus*, are under way (S. Falkow, G. Schoolnik, S. Cohen, and L. Shapiro, pers. comm.).

The longer term goals of functional genomics and microarray technology in infectious diseases include describing the host-pathogen interaction in molecular detail and identifying critical target molecules and pathways for diagnosis and intervention. Realizing these goals will require additional technology, extensive data collection, sophisticated computational tools, and efforts to discern cause and effect. We are on the verge of being able to listen to the two-way conversation between pathogen and host through devices of immense power.

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Pertussis Infection in Fully Vaccinated Children in Day-Care Centers, Israel

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We tested 46 fully vaccinated children in two day-care centers in Israel who were exposed to a fatal case of pertussis infection. Only two of five children who tested positive for *Bordetella pertussis* met the World Health Organization's case definition for pertussis. Vaccinated children may be asymptomatic reservoirs for infection.

Pertussis, an acute disease of the upper respiratory tract caused by the gram-negative bacillus *Bordetella pertussis*, lasts 6 to 8 weeks and has three clinical stages. The initial (catarrhal) stage resembles a common cold with a mild cough. The second (paroxysmal) stage is characterized by episodes of repetitive coughing during a single expiration, followed by a sudden inspiration that generates the typical "whoop." The final (convalescent) stage, which lasts 1 to 2 weeks, marks a decrease in the severity and frequency of the cough (1).

Since the introduction of routine childhood vaccine, pertussis has been considered preventable, and pertussis-associated illness and deaths are uncommon (2). However, vaccine-induced immunity wanes after 5 to 10 years, making the vaccinated host vulnerable to infection (3). This susceptibility has been described in outbreaks of pertussis infection in highly vaccinated populations (3-6).

A recent study by Yaari et al. showed that infection in a vaccinated person causes milder, nonspecific disease, without the three classical clinical stages (7). Whooping cough is seen in only 6% of such cases; instead, the illness is characterized by a nonspecific, prolonged cough, lasting several weeks to months. Because of these atypical symptoms, pertussis infection is underdiagnosed in adults and adolescents, who may be reservoirs for infection of unvaccinated infants (8-10). In a study in France, up to 80% of infections in unvaccinated children were acquired from siblings and parents, suggesting that adults and even young siblings play a fundamental role in the transmission of pertussis (11).

We demonstrated *B. pertussis* infection in fully vaccinated children ages 2-3 years and 5-6 years who had contact with an infected child. We investigated whether younger or recently vaccinated children may be protected from classical clinical illness but remain susceptible to infection and become asymptomatic carriers.

The Study

We examined the family of a 4-month-old infant who died of pertussis in Israel, as well as children at two day-care centers that two siblings had attended during the infant's illness. The two siblings, ages 2 and 5 years, attended different day-care centers, for ages 2-3 years and 5-6 years, respectively. Both siblings continued to attend the centers despite paroxysmal cough for 4 to 5 weeks. Thirty other children attended the day-care center for the 2- to 3-year-old group. Sixteen other children attended the center for the 5- to 6-year-old group.

In the infant's family, a third sibling, age 11 years, also had a paroxysmal cough of 4 to 5 weeks' duration. The 35-year-old mother had a 3-month history of persistent cough. An 18-year-old aunt, who took care of the infant and lived in

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the same house, reported a mild respiratory illness without paroxysmal cough. None of the family members had a whooping episode, cyanosis, or pneumonia (Figure).

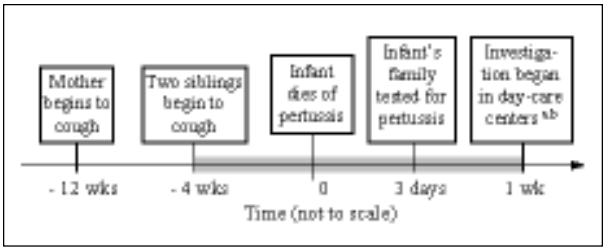

Figure. Timeline of pertussis infection in children in two day-care centers, Israel.

All the children in the day-care centers had been immunized in infancy with all four doses of Pasteur diphtheria-tetanus toxoid pertussis (DTP) vaccine, which includes a booster dose at 12 months of age. The Pasteur vaccine contains 1 immunization dose (ID) of purified diphtheria toxoid, 1 ID of purified tetanus toxoid, and >4 IU of *B. pertussis*. All family members of the infant were also fully vaccinated with four doses of DTP. The infant had received only the first dose of vaccine at 2 months of age.

The five family members of the infant and the 46 children in the two day-care centers were tested for *B. pertussis*. Two nasopharyngeal specimens were taken with Dacron swabs (Medical Wire, MEDECO, Corsham, UK); one specimen was used for culture and the other for polymerase chain reaction (PCR) testing. The culture specimen was immediately spread on charcoal agar plates (Hy Labs, Rehovot, Israel), which were incubated at 37°C for 14 days. Serum samples were also taken from every study participant for specific testing for immunoglobulin (Ig) M, IgA, and IgG antibodies to *B. pertussis* by an enzyme immunoassay (EIA) with whole-cell antigens (Panbio, East Brisbane, Australia) (12). Primers for the repeated insertion sequences were used in a semi-nested PCR assay (13-14). The upstream primer sequence gATTCAATA ggTTgTATgCATggTT and downstream primer AATTgCTggACCAT TCgAgTCgACG were used in the first PCR, which included 5 µL sample DNA, reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 1 µM of each primer, 200 µM deoxynucleotide triphosphate, and 1 U Taq polymerase (Boehringer Mannheim, Germany) in a 25-µL volume (14). Statistical analysis was performed by the two-tailed Fisher's exact test.

A person with positive PCR results was considered to have *B. pertussis* colonization of the nasopharynx. A person with positive IgM serum antibodies was considered to have had a recent infection. There were no culture-positive results, and nasopharyngeal aspirates were not available from the infant. Positivity by PCR or IgM did not indicate presence of symptoms.

Information on clinical symptoms was obtained from each person by a detailed questionnaire. The children in the day-care centers were followed clinically for 8 weeks after laboratory testing. All family members had been treated with erythromycin before testing, but no antibiotics were administered to the children in the day-care centers.

Eleven percent of the children in the two day-care centers were PCR positive, indicating nasopharyngeal colonization: 4 (25%) of the 16 5- to 6-year-old and 1 (3%) of the 30 2- to 3-year-old children ($p < .05$). Nine (55%) 5- to 6-year-old children were positive for serum IgM antibodies, and 4 (25%) were IgA positive. Three (10%) of the 2- to 3-year-old children were IgM positive, and 1 (3%) had IgA antibodies. Nasopharyngeal colonization was found more frequently in the 5- to 6-year-old than in the 2- to 3-year-old children (4/16 vs. 1/30, $p < .05$). This trend was also constant with IgM and IgA serum antibodies (9/16 vs. 3/30, $p < .001$ and 4/16 vs. 0/30, $p < .01$, respectively). In the index family, four of five members were positive by PCR, including all three siblings of the infant and the 18-year-old aunt. The 35-year-old mother, who was treated with erythromycin before testing, was negative by PCR. All five family members, including the mother, had high levels of IgM antibodies, indicating recent infection. The 4-month-old infant was seronegative for all subclasses of Ig antibodies to *B. pertussis*. No cultures were grown from the three groups.

According to a modified World Health Organization (WHO) case definition, two of the five children colonized with *B. pertussis* in the two day-care centers had the typical course of pertussis infection, with 3 weeks of paroxysmal cough (Table) (1). The other three children who were positive by PCR had only a mild, nonspecific cough during follow-up.

Table. Clinical and laboratory profiles of children positive for *Bordetella pertussis* by polymerase chain reaction (PCR) in Israel

Day-Care Center	PCR+	IgM[a]+	IgA+	Culture+	Clinical Pertussis[b]
Ages 2-3					
Child 1	Yes	Yes	No	No	Yes
Ages 5-6					
Child 2	Yes	Yes	Yes	No	Yes
Child 3	Yes	Yes	Yes	No	No[c]
Child 4	Yes	Yes	Yes	No	No[c]
Child 5	Yes	Yes	Yes	No	No[c]

[a]Ig = Immunoglobulin.
[b]Paroxysmal cough >3 weeks; modified World Health Organization case definition (1).
[c]Nonspecific cough during 4 weeks of follow-up.

Conclusions

The effects of whole-cell pertussis vaccine wane after 5 to 10 years, and infection in a vaccinated person causes nonspecific symptoms (3-7). Vaccinated adolescents and adults may serve as reservoirs for silent infection and become potential transmitters to unprotected infants (3-11). The whole-cell vaccine for pertussis is protective only against clinical disease, not against infection (15-17). Therefore, even young, recently vaccinated children may serve as reservoirs and potential transmitters of infection.

We used PCR, EIA, and culture to confirm *B. pertussis* infection in two highly vaccinated groups of children in two day-care centers. Three (10%) of 30 2- to 3-year-old children were seropositive for recent infection; one had nasopharyngeal colonization and a clinical illness that met the modified WHO case definition. In the day-care center for the 5- to 6-year-old group, 9 (55%) of 16 children were IgM positive, 4 (25%) of whom had nasopharyngeal colonization. Of these four children, three had nonspecific cough, and only one met the modified WHO definition for pertussis. None of the children in our study, including those who met the WHO definition, had been examined by a physician before our investigation.

Children who were seropositive and remained both asymptomatic and PCR negative probably had sufficient immunity from vaccines or natural boosters to protect them against persistent colonization and clinical disease. Their seropositivity could not be due to vaccine because the children were tested more than a year after having been vaccinated. Yet not all the children were protected from infection and from colonization with the bacteria. Whether a child who is serologically or PCR positive for pertussis and is clinically asymptomatic is a potential transmitter of infection has not been established. What is certain, however, is that vaccine-induced immunity against infection does not persist throughout adulthood. In France, booster vaccinations have been recommended for adolescents and teenagers (18). We found that immunity does not even persist into early childhood in some cases. We also observed that DPT vaccine does not fully protect children against the level of clinical disease defined by WHO. Our results indicate that children ages 5-6 years and possibly younger, ages 2-3 years, play a role as silent reservoirs in the transmission of pertussis in the community. More studies are needed to find the immunologic basis of protection against infection and colonization and thus an effective way to eradicate pertussis.

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Prevalence of Non-O157:H7 Shiga Toxin-Producing *Escherichia coli* in Diarrheal Stool Samples from Nebraska

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We determined the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in diarrheal stool samples from Nebraska by three methods: cefixime-tellurite sorbitol MacConkey (CT-SMAC) culture, enterohemorrhagic *E. coli* (EHEC) enzyme immunoassay, and $stx_{1,2}$ polymerase chain reaction (PCR). Fourteen (4.2%) of 335 specimens were positive by at least one method (CT-SMAC culture [6 of 14], EHEC enzyme immunoassay [13 of 14], $stx_{1,2}$ PCR [14 of 14]). Six contained serogroup 0157, while non-0157 were as prevalent as 0157 serogroups.

Disease caused by Shiga toxin-producing *Escherichia coli* (STEC) ranges from self-limiting diarrhea to hemorrhagic colitis and hemolytic uremic syndrome (HUS). Serotype O157:H7, the most frequently implicated STEC causing hemorrhagic colitis and HUS, has been isolated from large foodborne outbreaks, as well as sporadic cases, in North America and abroad. However, 60 STEC serotypes have been implicated in diarrheal disease, and several non-O157:H7 serotypes have been implicated as the cause of foodborne outbreaks and HUS in the United States, Europe, and Australia. Studies from Canada, Europe, Argentina, and Australia suggest that non-O157:H7 STEC infections are as prevalent, or more so, than O157:H7 infections.

E. coli O157:H7 is easily differentiated from other *E. coli* by its inability to rapidly ferment sorbitol; however, non-O157:H7 STEC are phenotypically similar to commensal nonpathogenic *E. coli* and are not detected with sorbitol MacConkey agar. To detect non-O157:H7 STEC, nonculture methods are used (enzyme immunoassay [EIA] or polymerase chain reaction [PCR]), which are typically only performed in reference laboratories. The purpose of this study was to determine the prevalence of non-O157:H7 STEC in persons with diarrhea in Nebraska.

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The Study

Nine regional clinical microbiology laboratories in Nebraska sent stool samples from March 1, 1998, to October 31, 1998, to the Nebraska Public Health Laboratory, University of Nebraska Medical Center. All stool samples that were sent to a participating laboratory with a physician's order to screen for enteric pathogens were included. Thus, all samples were from patients with a differential diagnosis of bacterial gastroenteritis. Patients who had been in the hospital for >2 days before diarrhea developed were excluded. The samples were added to a Para-Pak C&S stool transport container (Meridian Diagnostics, Cincinnati, OH) and sent by courier to the Nebraska Public Health Laboratory.

Samples were plated to cefixime-tellurite sorbitol MacConkey (CT-SMAC) agar plates and screened for typical sorbitol-negative colonies (1). Presumptive colonies were identified as *E. coli* by API strips (Biomerieux Vitek, Hazelwood, MO) and serotyped with RIM *E. coli* O157:H7 (Remel, Lenexa, KS). Samples were injected into 10 mL MacConkey broth (Difco, Detroit, MI) and incubated overnight at 37°C. The Premier enterohemorrhagic *E. coli* (EHEC) assay was performed by using 50 µL of overnight growth. The reaction mixtures were read spectrophotometrically at 450 nm and scored as positive or negative. PCR was performed by first extracting DNA from the overnight culture of MacConkey broth using a QIAamp Tissue kit (Qiagen, Santa

Clarita, CA). The following set of primers, which detects both stx_1 and stx_2, was used: 5'TTTACG ATAGACTTCTCGAC3' and 5'CACATATAA TTA TTTCGCTC3' (2). *E. coli* O157:H7 strain G5244 was used as positive control (Centers for Disease Control and Prevention [CDC] strain collection). Samples Shiga-toxin positive by either EHEC enzyme EIA, PCR, or both were plated onto sheep-blood agar (Remel) and streaked for isolation. After overnight growth, multiple *E. coli*-like colonies were selected for retesting by using the Premier EHEC assay. Positive colonies were identified to species level by using API strips and serotyped by CDC.

Multiplex PCR (3) was performed on isolated Shiga toxin-positive colonies to detect specific genes encoding Shiga toxins 1 and 2 (stx_1 and stx_2), intimin (*eaeA*), and enterohemolysin A (*ehxA*). Genomic DNA suitable for pulsed-field gel electrophoresis (PFGE) was prepared (4) and digested with *Xba*I (Roche, Indianapolis, IN). *E. coli* O157:H7 G5244 was used as a standard. PFGE patterns were captured by a Bio-Rad Gel-Doc system and were analyzed by Molecular Analyst software (Bio-Rad, Hercules, CA).

Of the nine clinical laboratories that submitted 335 samples during the study period, five submitted samples positive by CT-SMAC culture, EIA, or *stx* PCR (Table 1). Fourteen 4.2%) samples were positive by at least one of the methods; 13 of these were obtained either through direct culture by using CT-SMAC or through Shiga toxin screening and subsequent colony isolation. Six of the thirteen were serotype O157:H7 or O157:NM; seven were non-O157 serotypes. All seven of the non-O157 isolates were the predominant species found in the culture when the sample was plated on sheep-blood agar. All six *E. coli* O157:H7 or O157:NM isolates were detected by using CT-SMAC culture and *stx* PCR; five of six were detected by EIA. All of seven non-O157 isolates were detected by EIA or *stx* PCR. One sample (isolate B3) that was negative by EIA but positive by PCR and CT-SMAC culture was subsequently found to be positive by EIA when tested individually. One sample from laboratory C was positive for EIA and *stx* PCR (both tests were weak positives), but no Shiga toxin-positive colony was obtained upon repeat subculture. The low prevalence of this organism in the stool sample may reflect STEC carriage in this patient. By the combined results of both culture and EIA as the reference standard (14 samples positive, 321 samples negative), the sensitivity and specificity of the *stx* PCR (14 samples positive, 321 samples negative) were each 100%.

PCR was performed on the 13 isolated STEC to detect stx_1, stx_2, *eae*, and *ehxA* (Table 2). All isolates, regardless of serotype, encoded *eae* and *ehxA*; three of five O157 isolates encoded both stx_1 and stx_2; two of seven non-O157:H7 isolates encoded stx_2 (both O111:NM). PFGE showed that all but two STEC isolates were unrelated; isolates D1 and D2 (both O111:NM), which were isolated from samples sent from the same laboratory, were indistinguishable (data not shown).

Table 1. Results of CT-SMAC culture, enzyme immunoassay, and *stx* polymerase chain reaction

Laboratory	Isolate	Serotype	CT-SMAC[a]	EIA	PCR
A	A1	O157:H7	+	+	+
	A2	O157:H7	+	+	+
	A3	O26:H11	-	+	+
	A4	O157:NM	+	+	+
B	B1	O145:NM	-	+	+
	B2	O103:H2	-	+	+
	B3	O157:NM	+	-	+
C	NI[b]	NI	-	+	+
D	D1	O111:NM	-	+	+
	D2	O111:NM	-	+	+
	D3	O157:H7	+	+	+
	D4	O157:H7	+	+	+
E	E1	Orough:H2	-	+	+
	E2	O26:H11	-	+	+

[a](+) Denotes a positive CT-SMAC culture, EIA, or *stx* PCR; (-) denotes a negative CT-SMAC culture, EIA, or *stx* PCR; CT-SMAC = cefixime-tellurite sorbitol MacConkey agar; EIA = enzyme immunoassay; PCR = polymerase chain reaction.
[b]NI = Not isolated.

Table 2. Results from multiplex polymerase chain reaction (PCR) amplification

Isolate	Serotype	*stx1*[a]	stx2	*eae*	*ehxA*
A1	O157:H7	+	+	+	+
A2	O157:H7	-	+	+	+
A3	O26:H11	+	-	+	+
A4	O157:NM	+	+	+	+
B1	O145:NM	+	-	+	+
B2	O103:H2	+	-	+	+
D1	O111:NM	+	+	+	+
D2	O111:NM	+	+	+	+
D3	O157:H7	-	+	+	+
D4	O157:H7	+	+	+	+
E1	Orough:H2	+	-	+	+
E2	O26:H11	+	-	+	+

[a](+) Denotes presence of gene as assessed by PCR; (-) denotes absence of gene as assessed by PCR.

Conclusions

In a 1997 study of 30,000 diarrheal stool samples, *E. coli* O157:H7 was the fourth most prevalent bacterial enteric pathogen (5). However, the incidence of non-O157 STEC in the United States is not well established. Studies from Europe have shown that the prevalence of STEC in diarrheal samples is 0.3% to 9.3%; serogroup O157 prevalence is 0% to 2.7%. In Australia, serotype O111:NM is the most frequent cause of serious human disease and has been associated with outbreaks. In a recent study of 3,289 diarrheal samples from clinical laboratories in the United States, non-O157 STEC were more prevalent than O157 serotypes (6). STEC was found to be as prevalent (1.2%) as *Shigella* sp. (1.4%), and almost as prevalent as *Salmonella* sp. (2.4%) and *Campylobacter* sp. (2.0%). Testing for *E. coli* O157:H7 alone would have missed up to 50% of STEC.

Our study is the first to address the prevalence of non-O157 STEC in diarrheal samples from the Great Plains region of the northern United States, where cattle and other animal reservoirs of STEC are abundant. In our study, 4.2% of the samples were positive for STEC by CT-SMAC culture, PCR, or Meridian EHEC EIA. Though this prevalence is higher than previously reported in the United States, other studies have shown that northern states have a higher prevalence of *E. coli* O157:H7 (7). In addition, Nebraska has a large rural population, whose members may have contact with animal reservoirs that carry STEC. Five different non-O157 STEC serotypes were isolated: O111:NM, O26:H11, O145:NM, O103:H2, and Orough:H2. Four of these are associated with HUS (O111:NM [8–10], O26:H11[11], O145:NM [12], and O103:H2 [13]). In addition, serotypes O111:NM and O26:H11 have been associated with diarrhea in weaned calves (14). Although most STEC cases are linked to eating undercooked hamburger (15), contact with food animals has also been implicated as a source of infection (16).

Shiga toxins 1 and 2 are the main virulence factors associated with hemorrhagic colitis and HUS, presumably because they interact with endothelial cells at the site of infection and in the glomeruli and arterioles of the kidney (17). stx_1 and stx_2 are highly related yet immunologically distinct. STEC produce other accessory virulence factors, including intimin (*eae*) and enterohemolysin A (*ehxA*). The former is responsible for the characteristic histopathologic feature known as the attaching and effacing (A/E) lesion (18); *ehxA* is a hemolysin encoded by the 90-kb virulence-associated plasmid found in most STEC infecting humans (19). A study of 237 isolates from 118 serotypes found a significant association between stx_2 and *eae* in isolates that caused hemorrhagic colitis and HUS in humans (20) and between *ehxA* and severe disease. STEC isolates from asymptomatic human carriers usually do not encode *eae* and therefore may not have a mechanism to adhere to intestinal epithelial cells (21). However, STEC isolates lacking the *eae* gene have been associated with hemorrhagic colitis and HUS (22). All STEC isolates obtained in this study encoded both *eae* and *ehxA*. The six O157 isolates also encoded stx_2, as did both O111:NM isolates, which indicates that at least these eight isolates could produce serious disease. Five of seven non-O157 isolates encoded stx_1 only; however, STEC isolates associated with hemorrhagic colitis and HUS that encoded only stx_1 have been reported (23).

All O157:H7, O157:NM, and O26:H11 isolates in our study were distinct by PFGE RFLP patterns, which suggests that these cases were sporadic. However, two O111:NM isolates (D1 and D2) from the same community had indistinguishable PFGE RFLP patterns. The isolates were from different patients and were cultured within 1 day of each other. This suggests that an outbreak of O111:NM may have occurred that was not detected by standard laboratory techniques. However, without epidemiologic information, these results are difficult to interpret.

This study demonstrated that non-O157 STEC serotypes are at least as prevalent as serogroup O157 in diarrheal samples from Nebraska. Non-O157 STEC isolates presumably were the cause of diarrhea in 7 of 14 positive samples. These non-O157 isolates carried known STEC virulence genes and were the predominant organism found in culture. Other bacterial pathogens such as *Salmonella, Shigella,* and *Campylobacter* were not isolated from these seven samples. Our results suggest that *stx* PCR is as sensitive and specific as CT-SMAC culture and EIA combined, and therefore may be used as an alternate method to diagnose diarrheal infections caused by STEC. Clinical laboratories may need to implement serotype-independent methods to avoid underdiagnosis of STEC-mediated bacterial gastroenteritis.

Dr. Fey is an assistant professor in infectious disease and pathology and microbiology and the associate director of the Nebraska Public Health Laboratory at the University of Nebraska Medical Center. His interests include the epidemiology and antibiotic resistance of diarrheal pathogens as well as the genetics and pathogenesis of staphylococci.

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Fecal Colonization with Vancomycin-Resistant Enterococci in Australia

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To assess the rate of fecal vancomycin-resistant enterococci (VRE) colonization in Australia, we examined specimens from 1,085 healthy volunteers. VRE was cultured from 2 (0.2%) of 1,085 specimens; both were *vanB Enterococcus faecium*, identical by pulsed-field gel electrophoresis, but with a pattern rare in Melbourne hospitals.

Colonization and infection with vancomycin-resistant enterococci (VRE) are emerging worldwide. In Europe, agricultural use of the glycopeptide growth promoter avoparcin has been implicated in the emergence of *vanA* VRE in the food chain (1), and fecal colonization rates of 2%-28% have been reported in some communities (2,3). In the United States, where avoparcin is not used, both *vanA* and *vanB* VRE strains appear to be largely nosocomially spread, with fecal VRE colonization rare in nonhospitalized patients (4,5). By contrast, in Australia, where avoparcin has been used widely (10,000 kg/year) in agriculture for many years (6) and the per capita consumption of antibiotics is one of the highest in the world (7), VRE (mostly *vanB* [8]) has only recently emerged as an important nosocomial pathogen. We assessed the rate of fecal VRE colonization in a population of healthy Australians.

The Study

In mid-1997, fecal specimens from 1,085 healthy volunteers were collected and frozen at -70°C as baseline specimens for a water quality study in Melbourne, Australia. These previously described (9) volunteers were from 294 families with young children who lived in a lower- to middle-class suburb. They were specifically chosen because they were representative of young Australian families in eating habits and medical care. Thus, the elderly, the unmarried, and the very poor were not represented. Study participants had no history of diarrhea or antibiotic use at the time of specimen collection.

For VRE screening, frozen samples were thawed and spread onto enterococcosel agar (BBL Microbiology Systems, Cockeysville, MD) containing 6 µg/mL vancomycin and incubating at 35°C for 48-72 hours. Each sample was also incubated in enterococcosel broth (BBL) without vancomycin for 48 hours, before being spread onto enterococcosel agar containing 6 µg/mL vancomycin. From esculin-positive colonies, three of each morphologic appearance were selected from the direct agar and the subcultured enterococcosel broth cultures of each specimen and provisionally identified as either *Enterococcus faecium* or *E. faecalis* by routine criteria (gram-positive cocci, L-pyrrolidonyl-ß-naphthylamide hydrolase–positive, nonmotile, catalase-negative, and pigment-negative) (10,11). All isolates fulfilling these criteria were assessed for susceptibility to vancomycin and teicoplanin by the E-test method (AB Biodisk, Dalvagen, Sweden). Isolates with an MIC to vancomycin ≥2 µg/mL were analyzed for the presence of *vanA, B, C1,* or *C2/3* genes by polymerase chain reaction (8); if *vanA*- or *vanB*-positive, they were then confirmed as either *E. faecium* or *E. faecalis* by automated biochemical analysis (BioMerieux Vitek Inc., MO) and by polymerase chain

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reaction, and were assessed by pulsed-field gel electrophoresis (PFGE) (8,12,13).

To be certain that this analysis of frozen specimens was a valid assessment of the rate of fecal VRE carriage in this population, two additional studies were conducted. First, to verify that -70°C storage did not affect the overall recovery of enterococci, a randomly selected subset of 112 of the 1,085 specimens were cultured on enterococcosel agar (BBL) without antibiotic and assessed for enterococci by routine identification techniques (10,11). In addition, to assess whether -70°C storage could selectively impair recovery of VRE, fresh fecal specimens were spiked with concentrations (10^4-10^8/mL) of isolates of *E. faecalis* (*vanA*, n=1, *vanB*, n=2) or *E. faecium* (*vanA*, n=2; *vanB*, n=2), then stored at -70°C for 2 weeks before being thawed and cultured.

Results

Enterococci were identified in 106 of the subset of 112 specimens, a rate consistent with previous reports (14). VRE were also consistently recovered from our frozen spiked specimens. These findings suggest that -70°C storage of specimens does not affect the recovery of enterococci in general, or VRE in particular.

In the community study, VRE was isolated from 2 (0.2%) of the 1,085 (95% confidence intervals 0%-0.4%) specimens. Both isolates were *vanB E. faecium* and were detected in both agar and broth cultures. E-test MIC results at 24 hours for both isolates were 12 µg/mL for vancomycin and 0.125 µg/mL for teicoplanin. Although they were identical by PFGE, they displayed a PFGE pattern that is uncommon among VRE isolates from Melbourne hospitals. The two participants who were *vanB E. faecium* positive were not related. One was a 34-year-old man and the other a 30-year-old woman; both were married, and each had two children. None of the other family members had detectable fecal VRE colonization. The man had a history of stable ulcerative colitis and was receiving sulfasalazine.

Conclusions

This study suggests that fecal colonization with VRE is present but uncommon in Australia. Despite widespread agricultural use of avoparcin and high community rates of antibiotic use, *vanA* *E. faecium* was not identified. *vanB E. faecium*, which is the most common clinical VRE strain in Australia (8) and whose nosocomial transmission remains a concern (15), was identified in healthy Australians.

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Falciparum Malaria in European Tourists to the Dominican Republic

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Thirteen cases of falciparum malaria acquired by Europeans in the Dominican Republic occurred from June 1999 to February 2000. The cases were identified by the European Network on Imported Infectious Disease Surveillance (TropNetEurop).

Malaria, falciparum malaria in particular, represents a serious health hazard to travelers to disease-endemic areas. As international air travel to tropical destinations has become more popular, imported cases have also increased in countries where malaria is not endemic (1-3). The importance of appropriate drug prophylaxis has been stressed repeatedly (4).

Like most countries in the Caribbean, large parts of the Dominican Republic are considered low risk for falciparum malaria (5). In general, only border regions to Haiti and provinces in the northwest have been associated with endemicity. This pattern has been reversed recently: starting with an index patient in June 1999, 12 additional European patients acquired falciparum malaria in the Dominican Republic from November 1999 through February 2000. The cases were identified and reported within TropNetEurop, a sentinel surveillance network of clinical sites throughout Europe whose goal is to monitor imported infectious diseases. The network has a reporting system at sentinel clinics throughout Europe, known for its speed of reporting (usually within few days of diagnosis) and for members' sites that serve as regional referral centers.

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All but three patients (two Spanish, one Austrian) were Germans (Table). All had traveled to Punta Cana, a town in the eastern tip of the Dominican Republic, or to nearby beach resorts. Excursions were made only to the nearest town, Higüey, which was not considered malarious. The patients were not required to and did not receive malaria chemoprophylaxis for this journey and did not practice exposure precautions. Within 1-2 weeks after their return, patients visited general practitioners or emergency rooms, reported fever, and were hospitalized after diagnoses of falciparum malaria were established by blood films. For all patients, the clinical course was uneventful, and drug treatment was successful (Table).

The clustering of cases during a comparatively short time suggests a change in the epidemiologic situation in the Dominican Republic and may herald future outbreaks among tourists. According to information from the Dominican Republic, malaria cases increased in 1999 after Hurricane George: as of November 20, 1999, 3,003 cases had been reported, compared to 2,000 cases for all of 1998. In the eastern part of the country, an outbreak of falciparum malaria among the local population was noted and traced back to Haitians working in construction. With anopheline vectors and abundant breeding sites in that area, transmission of falciparum malaria is easy (6). Current recommendations for visitors

Table. Patients with falciparum malaria from the Dominican Republic

No.	Sex[a]	Age	Nationality	Month/year presentation	Journey	Therapy
1	F	26	German	06/99		
2	M	28	German	11/99	14 days in Punta Cana (honeymoon with #3)	Mefloquine
3	F	28	German	11/99	14 days in Punta Cana (honeymoon with #2)	Mefloquine
4	F	34	German	11/99	7 days in Punta Cana	Mefloquine
5	F	28	Spanish	11/99	7 days in Punta Cana	Chloroquine
6	F	45	German	11/99	14 days in Punta Cana	Atovaquone/ proguanil
7	M	27	German	11/99	Flight assistant, overnight stays in Puerto Plata (October) and Punta Cana (November)	Quinine
8	F	30	German	11/99	10 days in Punta Cana	Mefloquine
9	F	47	Austrian	12/99	14 days in Punta Cana	Quinine
10	F	28	Spanish	12/99	6 days in Punta Cana	Chloroquine
11	F	30	German	12/99	10 days in Punta Cana	Mefloquine
12	F	31	German	12/99	16 days in Punta Cana	Chloroquine
13	M	24	German	02/00	10 days in Punta Cana	Quinine

[a]F = female; M = male.

to the Dominican Republic should include an antimalaria strategy and strict adherence to personal protection measures against mosquito bites.

For 1998, official statistics from the World Tourism Organization put the number of visitors from Germany to the Dominican Republic at 366,599 (7). Corresponding numbers from Austria and Spain are 30,017 and 110,782, respectively. If these numbers were used as the basis for a crude denominator, the annual incidence of falciparum malaria in tourists to the east coast of the Dominican Republic would be 2.73/100,000 for German tourists and 3.3/ and 1.8/100,000 for Austrian and Spanish tourists, respectively. No reports were received of infections among tourists from other nations, including the United States, Canada, and the United Kingdom. This may reflect use of different malaria chemoprophylaxis or exposure prophylaxis for travel to the Dominican Republic.

This report demonstrates the effectiveness and importance of sentinel surveillance methods for monitoring imported infectious diseases in Europe. Discussion of the index case among the member sites of TropNetEurop increased awareness within the network and led to the other reports within days of initial diagnosis. The malaria patients might otherwise have gone unnoticed since they were seen at different hospitals throughout Europe.

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Two Cases of *Mycobacterium microti*–Derived Tuberculosis in HIV-Negative Immunocompetent Patients

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We describe two cases of *Mycobacterium microti* infection causing pulmonary tuberculosis (TB) in HIV-seronegative immunocompetent patients in Germany. The isolates were identified as *M. microti* of the llama and vole types, according to spoligotype patterns. Our data demonstrate that *M. microti* can cause severe pulmonary TB in immunocompetent patients.

Mycobacterium microti, which causes tuberculosis (TB) mainly in small rodents such as voles, has been considered nonpathogenic for humans (1-3). Considering the DNA sequences of the 16S rRNA gene and the 16S-to-23S internal transcribed spacer region, *M. microti* proved to be a member of the *Mycobacterium tuberculosis* complex. Primary isolation and differentiation by classical biochemical tests are complicated by the very slow growth of this species (1,2). The new molecular spoligotyping method has recently permitted the simultaneous detection and typing of *M. microti* (1). This technique has been applied in two retrospective studies of *M. microti* infections in the Netherlands and England, enabling identification of cases in llamas, cats, and ferrets, as well as in five humans, only one of whom was described as immunocompetent (1,4). A case in an HIV-positive patient has been described in detail by Foudraine and co-workers (5). We report two cases of *M. microti* infection causing pulmonary TB in Germany, representing the second and third reported cases in HIV-seronegative immunocompetent patients.

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Case 1

In January 1999, an HIV-seronegative 53-year-old man was hospitalized with a solid lesion in the upper lobe of the right lung, first identified in 1989. Computer tomography (CT) of the chest showed two dense infiltrates, 3x2 cm and 6x5 cm, without cavitation. The patient reported nonproductive cough and weight loss of 3 kg in 2 years. He was a cigarette smoker (approximately 1 pack/day for 30 years). Chronic abuse of alcohol began in 1992, after the initial diagnosis. An elevated erythrocyte sedimentation rate was the only abnormal laboratory finding. A tuberculin skin test (5 units of PPD) had a 7-mm induration. Sputum and bronchoalveolar lavage fluid were smear negative for acid-fast bacilli (AFB). Tuberculous granuloma were found in bronchial and peribronchial lung tissue obtained by bronchoscopy; however, no AFB or signs of malignancy were detected. Anti-TB therapy was started with isoniazid, rifampin, and pyrazinamide. The patient's clinical condition improved, and by November 1999 the pulmonary infiltrate had almost completely resolved. In interviews, the patient said that he had no extended exposure to household or farm animals. He lives in his own apartment in a town (population 14,000) in central Germany. No tuberculin skin test-positive contact persons have been identified.

One mycobacterial culture was grown from bronchoalveolar lavage fluid in liquid media (12B vial, BACTEC460TB, Becton Dickinson Microbiology Systems, Cockeysville, MD) after 5 weeks of incubation. Limited growth was observed on Stonebrink medium (small colonies visible after >6 weeks of incubation), and no growth was detected on Loewenstein-Jensen medium. The isolate was identified as *M. tuberculosis* complex by gene probes (ACCUProbe, GenProbe, San Diego,CA) and as llama-type *M. microti* by the characteristic spoligotype pattern (Figure 1) (1). After reculturing, the strain showed normal growth in BACTEC 460TB (Becton Dickinson), allowing drug-susceptibility testing that indicated susceptibility to isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin. Because of the limited growth on solid media, classical biochemical tests could not be performed. The bacteria showed normal cell morphologic features in several Ziehl-Neelsen-stained microscopic preparations from cultures.

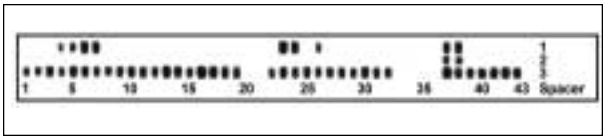

Figure 1. Spoligotype patterns of the isolates obtained from patient 1 (lane 1) and patient 2 (lane 2) and control strain H37Rv (lane 3)

Case 2

A severely ill 58-year-old man sought medical attention in April 1999. The patient was in poor general condition, with weight loss (> 8 kg in the last year), night sweats, occasional fever, fatigue, and productive cough. He had a history of smoking (10 cigarettes/day for at least 20 years) and diabetes mellitus (diagnosed in 1980). A chest X-ray in December 1983 showed no abnormalities of the heart and lung. The current illness had onset in early 1998, after which the patient was examined intensively by a number of physicians. Several X-rays of the chest indicated pulmonary disease with progression to bilateral lesions, predominantly in the right lung (Figure 2a); however, diagnosis was inconclusive. In September 1998, the general condition of the patient had so deteriorated that he was unable to work. Cytopathologic examination of a hemorrhagic pleural effusion in October 1998 showed lymphocytic-granulocytic pleuritis. X-ray examination (Figure 2b) and CT scan (data not shown) in April 1999 showed bilateral fibrosis of the lung parenchyma with a partial honeycomb pattern. Comparison with the X-ray in early 1998 documented a clear progression of the changes in the right lung.

Laboratory values were normal except for a slightly elevated leukocyte count. A tuberculin skin test (Mendel-Mantoux) and tests for HIV-1 and HIV-2 were negative. Fiberoptic bronchoscopy showed chronic atrophic bronchitis without signs of malignancy. For the first time, a bronchial secretion specimen was smear positive for AFB, and severe pulmonary TB was diagnosed. After treatment was begun with isoniazid, ethambutol, rifampin, streptomycin, and pyrazinamide, the patient's clinical condition improved, he recovered his normal weight, and the clinical symptoms resolved. Despite restrictive alterations in ventilatory function that correlated with radiologic and histomorphologic findings, the patient's condition improved and he has returned to work. An X-ray in January 2000 showed a consistent picture of the fibrosing lung disease.

One liquid culture (MGIT, Becton Dickinson) from a gastric aspiration obtained in April 1999 was positive for Mycobacteria in mid-June after 9 weeks of incubation. *M. tuberculosis* complex-specific gene probes (ACCUProbe) were positive. By the spoligotyping method, the isolate was unequivocally identified as vole-type *M. microti* (Figure 1). In contrast to the llama-type isolate, even after reculturing, the vole isolate showed very slow growth in liquid media and no growth on solid media, making drug-susceptibility testing and biochemical characterization impossible. In the Ziehl-Neelsen-stained microscopic preparation of both the bronchial secretion and several cultures, the bacterial cells showed normal morphologic features. In subsequent interviews, the patient said that he had not been exposed to household or farm animals. He lives in a village in a rural area in southern Germany. No tuberculin skin test-positive contact persons have been identified.

Conclusions

We describe the first two cases of pulmonary TB due to *M. microti* of the llama and vole types, respectively, in German patients. *M. microti* caused severe pulmonary TB with extensive histopathologic changes in

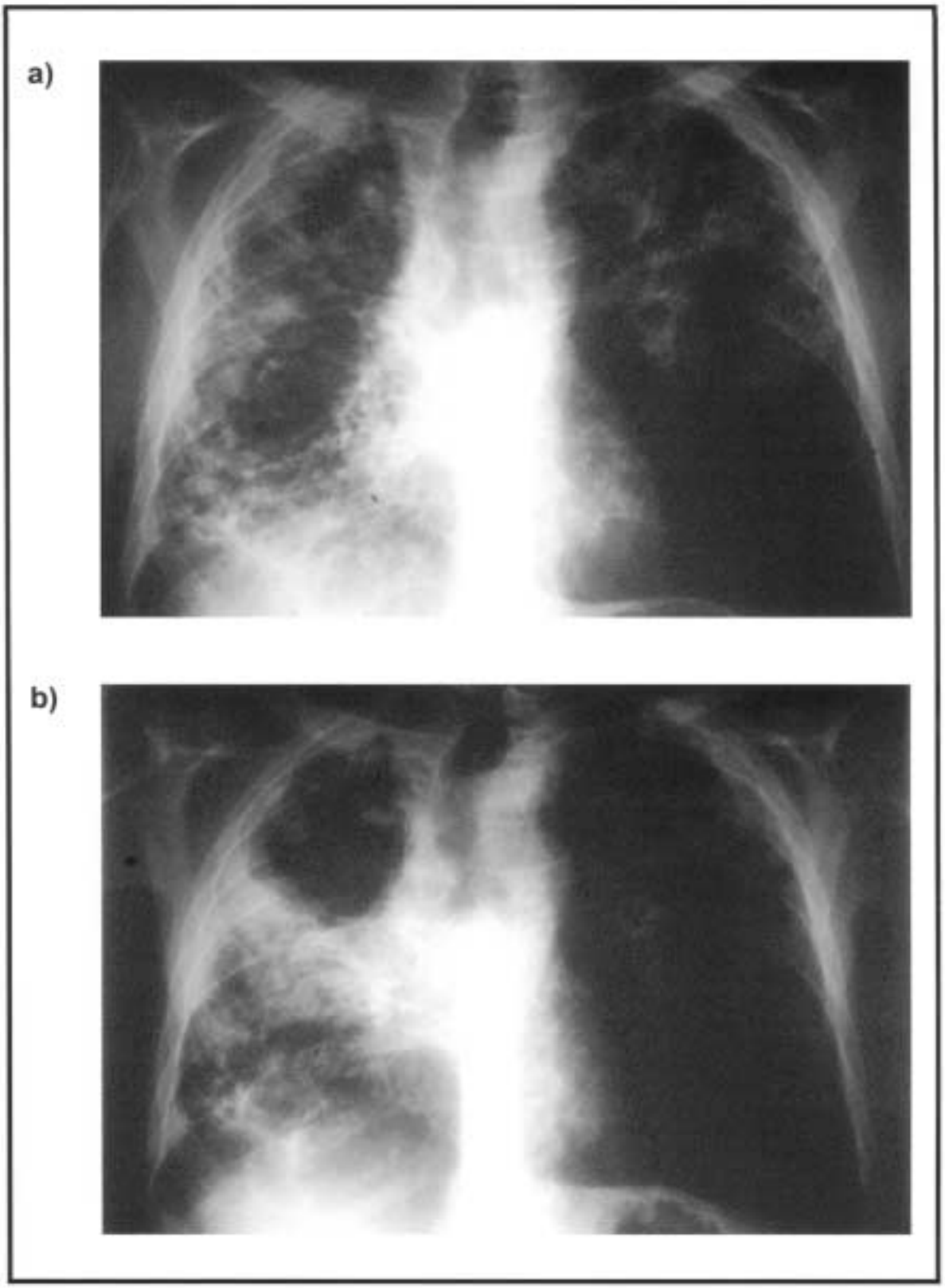

Figure 2. X-rays obtained in February 1998 (a) and April 1999 (b), of the chest of a 58-year-old patient.

the lung of an HIV-seronegative immunocompetent patient. As in the case reported by van Soolingen et al. (1), our data confirm the potential of *M. microti* to cause clinical illness in immunocompetent patients.

In contrast to the case reported by Foudraine and co-workers (5), both patients responded well to treatment with isoniazid, rifampin, and pyrazinamide or isoniazid, ethambutol, rifampin, streptomycin, and pyrazinamide, which indicates that regular anti-TB therapy may be appropriate for patients with *M. microti* infection.

As reported previously (1), primary isolation and biochemical characterization of *M. microti* are complicated by the slow growth of the bacteria. The resultant diagnostic delay, especially if atypical pulmonary lesions are present, may lead to false diagnosis and inadequate treatment. Thus, infection with *M. microti* should be considered in the differential diagnosis of patients with typical signs of TB but no bacteriologic proof. In these cases, spoligotyping is a fast, reliable technique for identification and differentiation of *M. microti* and other members of the *M. tuberculosis* complex (1,6,7).

The vole-type strain showed only limited growth during primary isolation and reculturing, impeding further characterization. In contrast, our results indicate that the llama-type *M. microti* may show normal growth in BACTEC 460TB. Moreover, we demonstrated that drug-susceptibility testing of clinical isolates of this subtype may be performed in liquid media.

Transmission from rodents to humans has been discussed as a possible pathway for the acquisition of *M. microti* infection (1). Although patients were interviewed, no source of infection could be identified.

Because of the difficulty with primary isolation and differentiation, the prevalence and clinical importance of *M. microti* may have been underestimated. Further studies based on molecular methods are needed to characterize the epidemiology of this emerging human pathogen.

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Trichinella pseudospiralis Outbreak in France

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Four persons became ill with trichinellosis after eating meat from a wild boar hunted in Camargue, France. Nonencapsulated larvae of *Trichinella pseudospiralis* were detected in meat and muscle biopsy specimens. The diagnoses were confirmed by molecular typing. Surveillance for the emerging *T. pseudospiralis* should be expanded.

Until 1995, *Trichinella pseudospiralis*, a nonencapsulating species of the genus *Trichinella* and the only species that infects both mammals and birds, was not considered a potential pathogen of humans and domestic animals, since it had been detected only in sylvatic animals (raccoon dog, corsac fox, tiger cat, tawny eagle, and rook) in remote regions (Caucasus, Kazakhstan, Tasmania) (1). However, this pathogen has since been detected in wildlife in the United States (2), in domestic and synanthropic animals and humans in Russia (3), and in humans in Thailand (4). In October 1999, a human outbreak of trichinellosis in France was traced to infected wild boar meat. We describe the epidemiologic, clinical, and laboratory investigations that confirmed *T. pseudospiralis* as the etiologic agent.

The Study

In October 1999, four adults living in Miramas, a small town in southeastern France, sought medical attention for asthenia, fever, nausea, and watery diarrhea. Three of the patients were members of the same household (father, mother, and son); the fourth was a friend. Their family physicians prescribed symptomatic therapy for gastroenteritis, but their conditions worsened and they were referred to the Infectious and Tropical Diseases Unit of a teaching hospital in Marseille. In initial interviews, all four patients said that they had eaten undercooked barbecued wild boar *(Sus scrofa*) meat on October 7. The father and his friend hunted the boar in Camargue, a swampy region in the Rhone River Delta.

Frozen wild boar meat was thawed, artificially digested, and washed (5) to obtain *Trichinella* larvae from muscle tissue for examination. Individual larvae were suspended in 5 µl water and stored at -30°C. Larvae were identified by polymerase chain reaction (PCR) analysis in which 10 µl 0.1 M Tris-HCL, pH 7.6, was added to the larvae, overlaid with mineral oil, and heated at 90°C for 10 minutes. PCR was done with *Taq* DNA polymerase, 10X PCR buffer, and deoxynucleoside triphosphates (dNTPs) (Takara, Otsu, Shiga, Japan). The 30-µl PCR cocktail contained 10X PCR buffer at a final concentration of 1.5 mM $MgCL_2$, 200 mM dNTPs, 50 pmoles of each primer, and 0.5 unit *Taq* DNA polymerase. For amplification, 2 µl of a single larva preparation was used. Amplifications consisted of 35 cycles as follows: denaturation at 94°C for 20 seconds, annealing at 58°C for 1 minute, and elongation at 72°C for 1 minute. The primer set oTsr1 (5'-CGA AAA CAT ACG ACA ACT GC-3') and oTsr4 (5'-GTT CCA TGT GAA CAG CAG T-3') amplifies a region in the LSU-RNA known as the expansion segment V (ESV) (6). Larvae from reference strains of *T. spiralis* (code ISS3), *T. pseudospiralis* (code ISS13), and *T. britovi* (code ISS2) were used as controls.

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Crude and excretory-secretory *Trichinella* antigens were prepared from larvae for enzyme-linked immunosorbent assay (ELISA) and immunoblotting (7). An indirect ELISA was used to detect parasite-specific immunoglobulin (Ig) G in human serum samples (8). Briefly, antigens were used at a concentration of 5 µg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.6. Serum samples were studied at several dilutions (range 1:200 to 1:6,400), and the conjugate (Bio-Rad, Hercules, CA) was at 1:10,000 dilution. Electrophoresis of crude and excretory-secretory antigens from *T. spiralis*, *T. pseudospiralis*, and *T. britovi* was done with 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels under reducing conditions. Proteins were transferred to nitrocellulose and incubated with human sera diluted 1:100. Serum samples from five persons with confirmed trichinellosis and five known to be *Trichinella* free were used as positive and negative controls.

The incubation period for the hunters (patients 2 and 4), who both ate >300 g of boar meat, was half as long, and their clinical symptoms (fever and myalgias) lasted twice as long as those of the other two patients, who ate <300 g. Other clinical and laboratory abnormalities were not correlated with the estimated size of the inoculum. Diarrhea was the initial symptom for all patients (Table). When the patients were hospitalized on October 31, they had fever, asthenia, and myalgias (torn muscle pain worsened by exertion); none had vomiting or rash. The patients were treated with albendazole (800 mg/day) for 10 days combined with prednisone (30 mg/kg/day) for the first 3 days.

Table. Epidemiologic, clinical, laboratory, and outcome characteristics of four patients infected with *Trichinella pseudospiralis*, southeastern France

	Patients			
Characteristics	1	2	3	4
Age (years)	28	62	60	47
Sex	M	M	F	M
Amount of boar meat eaten (g)	<300	>300	<300	>300
Incubation time (days)	17	7	14	7
Duration of fever (days)	13	29	14	20
Duration of myalgias (days)	17	30	17	34
Measured peak temperature (°C)	39.8	39.8	38.6	39.2
Arthralgias	No	No	No	Yes
Diarrhea	Yes	Yes	Yes	Yes
Vomiting	No	No	No	No
Periorbital edema	Yes	Yes	Yes	No
Conjunctivitis	No	Yes	No	No
Rash	No	No	No	No
Insomnia	Yes	No	No	No
Asthenia	Yes	Yes	Yes	Yes
Dizziness	Yes	No	No	No
leukocytes (N[b] = 4.0-10x10^9/L)[a]	10.0	11.8	8.3	16.7
Eosinophilia [peak] (N = 0-4.0x10^9/L)	1.9 [2.3]	3.1 [3.7]	1.6 [3.1]	5.3 [5.3]
Hemoglobin (N = 14-17 g/dL [men], 12-15 g/dL [women])	14.2	14.0	10.3	14.0
Fibrinogen (N = 2.5-4.5 g/L)	5.0	4.3	3.8	4.1
Erythrocyte sedimentation rate (N = <10 mm/h)	25	19	3	ND[c]
Creatine kinase [peak] (N = <200 UI/L)	5,874 [8,398]	787 [2,670]	4,277 [4,277]	286 [286]
Lactate dehydrogenase (N = 100–620 UI/L)	1,968	1,079	1,921	716
Aspartate transaminase (N = <50 UI/L)	22	61	186	27
Alanine transaminase (N = <60 UI/L)	90	61	212	47
Triglycerides (N = 0.4-1.8 g/L)	1.96	2.44	3.08	1.70
Plasma total protein (N = 62–80 g/L)	64	56	52	50
Plasma albumin (N = 39–50 g/L)	35	30	27	27

[a]Unless otherwise noted, laboratory findings were recorded at the time of admission to the hospital.
[b]N: Normal range.
[c]ND: Not determined.

The asthenia and myalgias initially worsened but then gradually improved, and all four patients recovered completely within 4 months.

All patients had elevated peripheral blood eosinophil counts (1.6 to 5.3 x 10^9/L) and decreased plasma albumin levels. Serum creatine phosphokinase concentrations were elevated, with peak levels of 286 to 8,389 U/L (Table).

A frozen meat sample from the boar was highly infected (187 larvae/g), but all larvae were dead. Examination of muscle samples by the compression method showed that all larvae were nonencapsulated. Histologic examination of deltoid muscle tissue from biopsies performed on all four patients on November 3 showed active myositis with numerous necrotic fibers, inflammatory infiltrates of mononuclear cells, and *Trichinella* larvae (Figure 1). All sera analyzed by ELISA were positive at dilutions up to 1:6,400 with both crude and excretory-secretory antigens. The protein electrophoresis patterns observed with these larval antigens differed; however, serum samples from the four patients and control samples from persons infected with other *Trichinella* species (*T. spiralis* or *T. britovi*) recognized both antigens (prominent bands in the approximate range of 40 to 75 kD). The expansion segment V of larvae from the wild boar (310 bp) was identical by PCR to that of the *T. pseudospiralis* reference isolate (Figure 2).

Figure 1. Sections of a muscle biopsy specimen from a patient infected with *Trichinella pseudospiralis* on day 32 after infection. The identified larva is nonencapsulated. Inflammatory cells are noted in the interstitium. Upper panel: hematoxylin and eosin stain at 50X magnification. Bottom panel: Masson trichrome stain at 100X magnification.

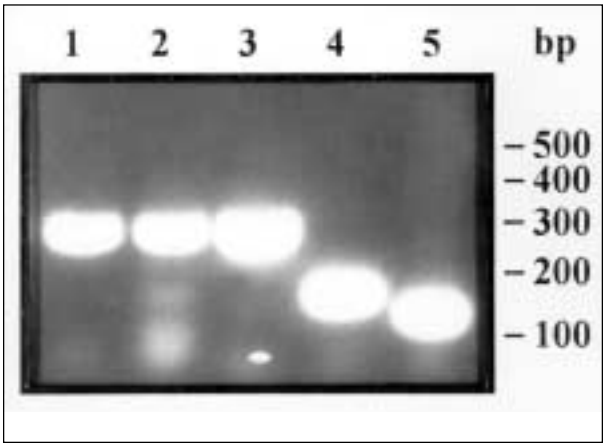

Figure 2. Gel agarose electrophoresis of the polymerase chain reaction amplification products of *Trichinella* sp. larvae. Lines 1 and 2, larvae in wild boar meat from Camargue, France; line 3, larva from the reference strain for *T. pseudospiralis*; line 4, larva from the reference strain for *T. spiralis*; and line 5, larva from the reference strain for *T. britovi*. Molecular weight markers: 50 base pairs (bp) DNA ladder (Pharmacia).

Conclusions

This is the first report of human *T. pseudospiralis* infection in Europe. The first reported human case was detected in New Zealand, but the infection was probably acquired in Tasmania (9). This is only the third reported human *T. pseudospiralis* outbreak in the world. The first, in Thailand, affected 59 persons; one died (4). The second, in Kamchatka, Russia, affected approximately 30 persons (3). The clinical findings in our patients are consistent with previous reports of uncomplicated *T. pseudospiralis* (4) and *T. spiralis* infections (10). None of our patients had the life-threatening cardiopulmonary, renal, and central nervous system complications typical of trichinellosis infection. However, our patients had two unusual clinical features: fever persisted 13 to 29 days, considerably longer than previously reported for patients infected with *T. pseudospiralis* (4) or *T. spiralis* (10); and all four patients recovered completely within 4 months, in contrast with previous reports of severe asthenia and myalgias persisting for longer periods (4,11). Our patients received early treatment with effective anthelminthics and responded rapidly, which may explain the

shorter duration of clinical symptoms. The laboratory findings in our four patients are consistent with those reported in trichinellosis; however, low plasma albumin and elevated triglyceride levels have not been reported in earlier outbreaks. The results of the Western blot analysis of sera from our patients demonstrated that immunoblotting cannot be used to identify the etiologic agent on the basis of recognized antigens. These findings contrast with those of a previous report (11) of an unusual Western blot pattern in serum samples from a patient thought to be infected only with *T. pseudospiralis*. However, subsequent investigation showed that this patient was also infected with another tissue nematode (*Haycocknema perplexum*)(12,13).

In Europe, *T. pseudospiralis* has been detected in a raccoon dog in the Krasnodar region of Caucasus (14), in two night birds of prey in central Italy (15), and recently in four raccoon dogs, one wild boar, and one brown rat in Finland (1). Although *T. pseudospiralis* can be considered a sylvatic genotype, the recent finding of this parasite in domestic pigs and brown rats on a farm in Kamchatka (3) suggests that, in certain epidemiologic situations, this parasite is transmitted to the human environment and should be considered a new potential parasite for domestic pigs. Pigs raised on ecologic (organic) farms are more likely to feed on infected wild animal carcasses than those raised on industrialized farms (16). These epidemiologic data suggest that either the prevalence of *T. pseudospiralis* infection is increasing in sylvatic and domestic animals of Europe and other continents or that techniques for diagnosing human and animal infections have improved, allowing identification of this nonencapsulated *Trichinella* species. Trichinelloscopy (visualizing *Trichinella* larvae by transillumination of small pieces of muscle from the diaphragm pillars between two thick slides) is used for trichinellosis screening in slaughterhouses. Because the collagen capsule is lacking, *T. pseudospiralis* larvae can easily be mistaken for muscle fiber. Therefore, trichinelloscopy is ineffective for screening in slaughterhouses or for diagnosis of human biopsy specimens. The finding that all larvae detected after artificial digestion of frozen meat were dead suggests that freezing may make game meat safe from *T. pseudospiralis* infection.

Experimental infection of domestic pigs and wild boars with *T. pseudospiralis* (17,18) showed that these hosts can harbor a substantial number of *T. pseudospiralis* larvae up to 20 weeks, but most muscle larvae had disappeared by 40 weeks after infection. However, several factors can contribute to the infectivity and persistence of this parasite species in domestic and sylvatic swine: the genetic variability of *T. pseudospiralis* isolates (6); the genetic variability of pigs and wild boars; and the occurrence in nature of stress, starvation, or concomitant infections that can induce immunosuppression in wild boars.

Twelve cases of trichinellosis associated with eating wild boar meat were reported from June 1994 to December 1995, in southeastern France, clustered around seven geographic foci (19). Some of these cases may have been caused by *T. pseudospiralis*, since neither histology of muscle biopsy nor recently developed molecular typing methods were used to verify the specific diagnosis. Molecular typing has opened new avenues for scientific investigations of trichinellosis and promises better understanding of the emerging pathogen that causes it. The broad spectrum of *T. pseudospiralis* hosts (both mammals and birds), the difficulty in detecting this parasite by trichinelloscopy, the potentially severe clinical picture in humans, and the increasing occurrence of this parasite indicate that expanded surveillance is needed to monitor the introduction and spread of trichinellosis.

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Double Infection with a Resistant and a Multidrug-Resistant Strain of *Mycobacterium tuberculosis*

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An immunocompetent patient was dually infected with a resistant and a multidrug-resistant strain of *Mycobacterium tuberculosis* (TB). The multidrug-resistant strain, which belongs to the W-strain/Beijing family, was first isolated after 3 months of therapy. Inappropriate treatment led to further drug resistance and unsuccessful therapy. Thus, additional infections with resistant *M. tuberculosis* strains should be considered when tuberculosis therapy fails.

During the last decade, drug-resistant *Mycobacterium tuberculosis* (TB) strains have emerged, posing a major threat to global TB control efforts. The incidence of drug-resistant TB has increased in many parts of the world, not only in developing countries but also in industrialized countries, where the prevalence of drug-resistant TB had been low (1). The emergence of drug resistance during antituberculosis therapy results mainly from inadequate therapy, i.e., improper prescription of treatment regimens, addition of single drugs to failing treatment regimens, and patient noncompliance. However, inconsistent drug-susceptibility patterns or delayed responses to TB therapy may also indicate exogenous reinfection with a strain resistant to multiple drugs or mixed infection with a sensitive and a multidrug-resistant TB strain. Such infections occur in immunocompromised and immunocompetent persons (2-7) and may be more common in areas with high prevalence of resistant TB.

We report the case of an immunocompetent patient initially infected with an isoniazid- and streptomycin-resistant TB strain, who after the first 3 months of TB therapy was found to be infected with a second multidrug-resistant TB strain, resulting in treatment failure. In all, the patient's cultures were resistant to nine anti-TB drugs.

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The Study

A 24-year-old man from Kazakhstan was admitted to hospital A in February 1996, 2 weeks after his arrival in Germany for further diagnosis of a cavernous lesion in the upper lobe of his left lung. The patient reported coughing, but no weight loss, night sweats, or hemoptysis. An elevated erythrocyte sedimentation rate was the only abnormal laboratory finding. He was seronegative for HIV. A tuberculin skin test was positive (12 mm), acid-fast bacilli were detected in gastric aspirates, and two sputum cultures were positive for TB. Antituberculosis therapy was started with isoniazid, rifampin, ethambutol, and pyrazinamide. The patient was then transferred to hospital B, where therapy was continued with three drugs only (ethambutol was discontinued). After 2 months of therapy, the patient showed clinical improvement, resulting in a sputum smear- and culture-negative phase of approximately 4 weeks (Table). When susceptibility testing of the first culture obtained 2 months previously revealed resistance to isoniazid and streptomycin, ethambutol was readded to the treatment regimen. Isolates were identified as *M. tuberculosis* complex by using gene probes (ACCUProbe, GenProbe, San Diego, California, USA). Drug susceptibility was determined by the proportion method on Löwenstein-Jensen medium, the modified proportion method in BACTEC 460TB, or both.

Suspicion of nonadherence to therapy during the first 3 months led to transfer of the patient to hospital C and 3 weeks later to hospital D, where

Table. Treatment regimens and characteristics of *Mycobacterium tuberculosis* isolates[a]

Therapy (months)	Hospital	Treatment regimen	Culture	Susceptibility testing: Culture obtained	Susceptibility testing: Current resistance pattern	Susceptibility testing: Time delay (months)[b]	Spoligotype
0	A/B	H, R, E,[c] Z	pos.	02/28/96	H, S	2	type I
1	B	H, R, Z	pos.	03/17/96	H, S	2	type I
2	B	H, R, E, Z	neg.	-	-	-	-
3	B	H, R, E, Z	neg./ pos.	-	n.d.	-	n.d.
4	C	H, E, Pt	pos.	06/06/96	H, S, R, (E), (Rb)	2	type II
5	D	R, E, Z	pos.	07/12/96	H, S, R, (E), (Rb)	2	type II
6	D	E, Z, Rb, Of	pos.	-	n.d.	-	n.d.
7-12	D	E, Z, Rb, Pt	pos.	-	n.d.	-	n.d.
13	D	E, Z, Rb, Pt	pos.	03/15/97	H, S, R, E, Rb, Pt	1	type II
14-18	D	Z, Pt, Ci	pos.	04/25/97	H, S, R, E, Rb, Pt, Z	5	type II
19	D	Z, Pt, Rb, Ci	pos.	-	n.d.	-	n.d.
20-21	D	Rb, Ci, Am, Pa	pos.	-	n.d.	-	n.d.
22-24	D	Rb, Ci, Am, Pa, Cl	pos.	-	n.d.	-	n.d.
25-28	D	Rb, Ci, Am, Pa, Cl	pos./ neg.	02/18/98	H, S, R, E, Rb, Pt Z, Pa	4	type II
29-32	D	Rb, Ci, Am, Pa, Cl	pos.	06/23/98	H, S, R, E, Rb, Pt, Z, Pa, Am	4	type II
33	D	Rb, Ci, Cl, Ca	pos.	-	n.d.	-	n.d.

[a]Abbreviations and symbols: H, isoniazid; S, streptomycin; R, rifampin; E, ethambutol; Rb, rifabutin; Pt, protionamide; Z, pyrazinamide; Pa, *p*=aminosalicylic acid; Am, amikacin; Of, ofloxacin, Ci, ciprofloxacin; Cl, clofazimine; Ca, capreomycin; borderline results are shown in parentheses; pos., positive culture result; neg., negative culture result; n.d., not determined.
[b]Time between the date on which the specimen was obtained and the date on which the drug-susceptibility pattern was available for the clinician (drug-susceptibility tests were not always performed directly after the cultures were grown but were done retrospectively).
[c]E was given in the first 4 days of therapy only.

treatment was administered on a closed ward (Table). At that time, cultures tested positive again, and the chest X-ray showed slight deterioration. On August l, 1996, the latest susceptibility tests showed resistance to isoniazid, rifampin, and streptomycin, and an intermediate result for rifabutin and ethambutol. Therapy was switched to ethambutol, pyrazinamide, rifabutin, and ofloxacin. Subsequently, treatment regimens were changed several times, but cultures continued to be positive for mycobacteria. In early 1997, atelectasis of the left lower lobe and thickening of the wall of the cavernous lesion in the left upper lobe became apparent. Lung resection was suggested, but thoracic surgeons declined to operate because of the extensive lung involvement. By year-end, susceptibility tests showed resistance to seven drugs: isoniazid, rifampin, ethambutol, pyrazinamide, protionamide, rifabutin, and streptomycin. A regimen with rifabutin, ciprofloxacin, amikacin, para-aminosalicylic acid, and clofazimine led to a short phase of negative sputum cultures, but a chest X-ray showed no improvement. Later, additional resistance to para-aminosalicylic acid and amikacin was documented.

To elucidate the reasons for therapy failure, we compared the current susceptibility patterns with the treatment regimens applied earlier. We found that during several phases of treatment, this patient was treated with only one effective drug. During extended periods, he was culture positive, but no susceptibility tests were performed, even after relapse. Additionally, treatment regimens were changed, and single drugs were added several times without determination of the actual resistance pattern. Treatment regimens, drug-susceptibility patterns, and detailed information on the history of the case are summarized in the Table.

All isolates obtained were submitted to spoligotyping and IS*6110* fingerprinting (5,8). Spoligotypes (Figure), as well as the IS*6110* restriction fragment length polymorphism (RFLP) patterns (data not shown) of the first two isoniazid- and streptomycin-resistant cultures, were identical (type I) but differed clearly (IS*6110* identity of less than 30%) from those of

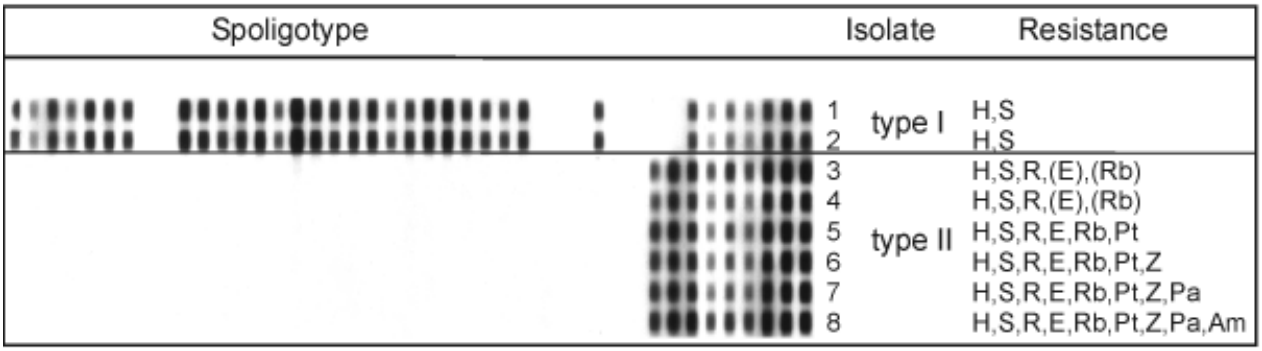

Figure. Spoligotypes and drug-resistance patterns of isolates of patient's first (1 and 2, type I) and second culture-positive phase (3-8, type II). The spoligotypes of the first two cultures differ clearly from those of the latter ones. The eight isolates were obtained on the following dates: 1, February 28, 1996; 2, March 17, 1996; 3, June 6, 1996; 4, July 12, 1996; 5, March 15, 1997; 6, April 25, 1997; 7, February 18, 1998; and 8, June 23, 1998. H, isoniazid; S, streptomycin; R, rifampin; E, ethambutol; Rb, rifabutin; Pt, protionamide; Z, pyrazinamide; Pa, *p*=aminosalicylic acid; Am, amikacin. Borderline results are displayed in parentheses.

later multidrug-resistant isolates (second phase of sputum culture positivity, type II; see Table). These results indicate that the patient was infected with a second TB strain, which showed initial resistance to isoniazid, streptomycin, and rifampin, and borderline resistance to ethambutol and rifabutin. IS*6110* RFLP patterns of multidrug-resistant isolates from the patient were compared with those from other patients who had been treated in hospitals B and C during the same period and with IS*6110* RFLP patterns from resistant TB strains isolated from unrelated patients living in other areas of Germany. Gelcompar software was used for this analysis (Windows 95, version 4.0; Applied Maths, Kortrijk, Belgium) (5). No isolate showing an identical IS*6110* RFLP pattern was identified (data not shown). Spoligotype and the IS*6110* RFLP patterns of the patient's multidrug-resistant strain were similar to those of the W-strain or Beijing family, which have been found in New York, USA, and Beijing, China (9,10).

Conclusions

We report an immunocompetent patient with pulmonary TB who had double infection with a resistant and a multidrug-resistant TB strain, leading to therapy failure. After 2 years of treatment, resistance to eight antituberculosis drugs—including the most potent first- and second-line treatments—occurred, despite clinically supervised hospital therapy. Four months later, resistance to a ninth drug occurred. Progressive disease caused by a second multidrug-resistant TB strain, as demonstrated by molecular strain typing methods, was the initial cause for this occurrence. A possible variation of the initial strain has been excluded since the spoligotype patterns of the multidrug-resistant isolates completely differed from the two isolates of the first TB period (spoligotype patterns have been shown to be highly stable among serial patient isolates [11]).

In this case, TB therapy was often based on drug-resistance data not representing the current drug-resistance pattern, resulting in improper treatment and many periods in which the patient received only one effective drug; the second TB strain could have acquired further resistance during these many periods of monotherapy. Earlier identification of the second infection might have led to treatment with a more appropriate drug regimen, resulting in a more successful outcome. The second multidrug-resistant TB strain could have been acquired by mixed-strain infection or exogenous reinfection (2-7). However, our investigation did not identify a possible index patient. Moreover, the fact that the patient was an immigrant from Kazakhstan, a country with high rates of resistant TB (1), suggests that he may have been infected with the second multidrug-resistant strain in his homeland.

This patient was seronegative for HIV and had no clinical measurements indicative of immunosuppression, suggesting that additional

infection with multidrug-resistant TB during treatment can be largely independent of a host's immune status. These mixed-strain infections with at least one resistant strain may lead to unsuccessful TB therapy. Although few have been reported (6, 7), such cases may become more frequent in areas with high rates of drug-resistant TB. Standard TB treatment apparently is not sufficient to protect patients from infection with a second multidrug-resistant TB strain.

Clinicians should consider the possibility of additional infection with multidrug-resistant TB in cases when TB therapy fails. In such cases, inappropriate treatment regimens and delayed follow-up of susceptibility tests can permit additional resistance to develop, which can dramatically complicate TB therapy. However, regardless of the cause, when a clinical course is abnormal, adding single drugs to failing treatment regimens should be avoided, and retreatment programs should not be initiated before culture sensitivity results are available.

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Antimicrobial-Drug Use and Changes in Resistance in *Streptococcus pneumoniae*

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Resistance of *Streptococcus pneumoniae* to antimicrobial drugs is increasing. To investigate the relationship between antimicrobial use and susceptibility of *S. pneumoniae* isolates at 24 U.S. medical centers, we obtained data on outpatient antimicrobial-drug use for the regions surrounding 23 of these centers. We found an association between decreased penicillin susceptibility and use of beta-lactam antimicrobial drugs.

Resistance of *Streptococcus pneumoniae* to penicillin and other beta-lactams is increasing worldwide (1-4). The major mechanism of resistance involves the introduction of mutations in genes encoding penicillin-binding proteins (5). Selective pressure is thought to play an important role, and use of beta-lactam antibiotics has been implicated as a risk factor for infection and colonization (6-14). Wide geographic spread of resistant clones has been described (3,15). However, the effect of geographic patterns of antimicrobial-drug use on the emergence and spread of resistance is not known.

We performed two previous surveillance studies of *S. pneumoniae* isolated at medical centers in the United States, one in 1994-95 (16), the other in 1997-98 (17). We report the relationship between antimicrobial-drug use in the geographic areas surrounding these medical centers and the change in penicillin resistance of *S. pneumoniae* over a 3-year period.

The Study

Multicenter national surveillance of *S. pneumoniae* was performed from November 1994 to April 1995 (16) and again from November 1997 to April 1998 (17). All isolates during these two surveillance studies were recovered from consecutive nonhospitalized patients from either the lower respiratory tract or a sterile site (blood or cerebrospinal fluid). Briefly, isolates were transported from study centers to a central laboratory, where they were confirmed as *S. pneumoniae* by conventional identification methods (16). Susceptibility testing was performed by the reference broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (18). Susceptibility was determined by using the established NCCLS breakpoints (19). For penicillin, breakpoints of 0.1 to 1.0 µg/mL for intermediate and ≥2 µg/mL for resistant were used; for this analysis, both intermediate and resistant categories were considered resistant.

Twenty-four medical centers were surveyed during both study periods. For 23 of these centers, data for outpatient antimicrobial-drug use were obtained for the surrounding metropolitan statistical area. These data were expressed in terms of number of prescriptions written per 100,000 population per month during the 48-month period that included the two surveillance studies (20). This period (May 1994 through April 1998) included four consecutive respiratory virus seasons. We divided the 23 medical centers into high-, intermediate-, and low-use centers for each antimicrobial-drug class. With the change in penicillin resistance as the dependent variable of interest, we used one-way ANOVA to compare mean change in resistance to penicillin between high-, intermediate-, and low-use centers. We then analyzed covariance models to evaluate the relationship between antimicrobial-drug use categories and changes in penicillin resistance. Alpha was set at 0.05, and all p-values were two-tailed.

We compiled the penicillin and erythromycin susceptibility test results for *S. pneumoniae*

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isolates collected in 1994-95 and 1997-98 from all 23 centers (Table 1). Overall, penicillin nonsusceptibility (MIC ≥ 0.1 µg/mL) increased by 8.9%. In 1994-95, 269 (22.2%) of the 1,211 *S. pneumoniae* isolates were intermediate or fully resistant to penicillin, while in 1997-98, 337 (31.1%) of the 1,083 isolates were in these categories. When the change in percent penicillin susceptibility at each

Table 1. Change in resistance[a] among *Streptococcus pneumoniae* isolates at 23 U.S. medical centers, 1994-95 and 1997-98

Medical center	No. of isolates	Study period	Erythromycin	Change (%)	Penicillin I + R[b]	Change (%)
Seattle, WA	37	1994-95	5.4		35.1	
	50	1997-98	30.0	24.6	38.0	2.9
Denver, CO	62	1994-95	3.2		14.5	
	26	1997-98	7.7	4.5	15.4	0.9
Phoenix, AZ	57	1994-95	12.3		40.4	
	54	1997-98	35.2	22.9	40.7	0.3
Houston, TX	63	1994-95	22.2		25.4	
	48	1997-98	43.8	21.6	64.6	39.2
Dallas, TX	58	1994-95	6.9		22.4	
	36	1997-98	27.8	20.9	30.5	8.1
Rochester, MN	35	1994-95	8.6		14.2	
	48	1997-98	20.8	12.2	22.9	8.7
Milwaukee, WI	65	1994-95	18.5		33.8	
	55	1997-98	10.9	-7.6	20.0	-13.8
Evanston, IL	49	1994-95	8.2		14.3	
	35	1997-98	14.3	6.1	14.3	0.0
Chicago, IL	41	1994-95	17.1		34.1	
	41	1997-98	19.5	2.4	19.5	-14.6
Indianapolis, IN	63	1994-95	7.9		20.7	
	55	1997-98	18.2	10.3	25.5	4.8
St. Louis, MO	57	1994-95	8.9		24.6	
	55	1997-98	12.7	3.8	29.1	4.5
Detroit, MI	63	1994-95	6.3		19.0	
	60	1997-98	10.0	3.7	30.0	11.9
Cleveland, OH	42	1994-95	11.9		19.0	
	60	1997-98	20.0	8.1	23.2	4.2
Philadelphia, PA	47	1994-95	2.1		2.1	
	42	1997-98	11.9	9.8	21.4	19.3
Syracuse, NY	23	1994-95	8.7		8.7	
	50	1997-98	8.0	-0.7	20.0	11.3
Rochester, NY	58	1994-95	6.9		10.4	
	50	1997-98	12.0	5.1	20.0	9.6
New York, NY	64	1994-95	4.7		12.6	
	53	1997-98	3.8	-0.9	20.8	8.2
Hartford, CT	61	1994-95	3.3		8.2	
	51	1997-98	7.8	4.5	27.4	19.2
Washington, DC	60	1994-95	13.3		23.3	
	28	1997-98	28.6	15.3	35.7	12.4
Chapel Hill, NC	60	1994-95	10.0		31.7	
	49	1997-98	38.8	28.8	57.1	25.4
Decatur, GA	61	1994-95	23.0		36.1	
	52	1997-98	26.9	3.9	44.2	8.1
Mobile, AL	68	1994-95	16.2		20.6	
	58	1997-98	37.9	21.7	41.3	20.7
Miami, FL	17	1994-95	5.9		52.9	
	27	1997-98	29.6	23.7	51.8	-1.1
TOTAL	1,211	1994-95	10.2		22.2	
	1,083	1997-98	20.6	10.4	31.1	8.9

[a]Includes both intermediate- and high-level resistance to penicillin and erythromycin.
[b]I + R = both intermediate and fully resistant.

center was considered, the overall mean increase in penicillin resistance was 8.3% (-14.6% to 39.2%) among the 23 centers that participated in both surveys (Table 1).

Antimicrobial-drug use data for beta-lactams, tetracyclines, quinolones, and macrolides were calculated for the high-, intermediate-, and low-use tertiles in our analysis (Table 2). The mean increase in penicillin resistance was compared among high-, intermediate-, and low-use centers for the major antibiotic classes (Table 3). The beta-lactams were most strongly associated with an increase in penicillin resistance (2.8%, 8.8%, and 13.3% increases in low-, intermediate-, and high-use tertiles, respectively, p=0.20).

Univariate analysis of covariance was performed, with change in penicillin resistance as the dependent variable and the antimicrobial-drug use category for each antimicrobial-drug class as independent variables. When all classes for which data were available (beta-lactams, tetracyclines, macrolides, and quinolones) were entered into a model, only the macrolides and beta-lactams were statistically significant ($p < 0.1$) as explanatory variables and were therefore included in the final model (Table 4). Higher beta-lactam use was strongly associated with increased resistance to penicillin ($F=8.7$, $p=0.008$). Conversely, higher macrolide use was associated with decreased resistance to penicillin ($F=5.4$, $p=0.031$). The overall model explained a significant amount of the variance in penicillin resistance at these 23 centers ($F=4.8$, $p=0.02$).

Table 2. Prescriptions for antibiotics at medical centers with high, intermediate, and low antimicrobial-drug use[a]

Class/tertile	Mean	Median	Range	SD
Beta-lactams				
High	1,640	1,620	1,186-2,557	411
Intermediate	1,027	1,040	948-1,136	69
Low	859	870	777-917	51
Macrolides				
High	929	865	800-1,286	166
Intermediate	738	722	687-787	35
Low	609	623	528-673	52
Quinolones				
High	282	258	222-424	63
Intermediate	197	200	177-216	16
Low	143	146	91-170	27
Tetracyclines				
High	77	75	61-100	15
Intermediate	56	58	50-59	3
Low	33	34	25-45	7

[a]All values are expressed in units of mean number of prescriptions per 100,000 population per month during the period between the two surveillance studies (May 1994-April 1998).

Table 3. Mean increase in percent penicillin resistance[a] of *Streptococcus pneumoniae* by category[b] of antimicrobial-drug use

Class	High	Inter-mediate	Low	p-value[c]
Beta-lactams	13.3	8.8	2.8	0.20
Quinolones	13.0	6.3	5.3	0.39
Macrolides	4.0	12.4	8.9	0.39
Tetracyclines	5.3	7.7	11.8	0.56
All classes	13.3	3.3	7.6	0.27

[a]Includes both intermediate- (MIC 0.12-1 µg/mL) and high-level (MIC ≥2 µg/mL) resistance to penicillin.
[b]Each center was categorized by total number of outpatient prescriptions for the antimicrobial class per 100,000 population per month in the surrounding metropolitan statistical area.
[c]One-way ANOVA p-value, two-tailed.

Table 4. Analysis of covariance model, with change in penicillin resistance at each of the 23 medical centers as the dependent variable

Source	Type III sums of squares	Parameter estimate (*B*)	F	p-value
Overall model	990[a]		4.8	0.02
Intercept	56	4.5	0.5	0.47
β-lactam use	893	8.6	8.7	0.008
Macrolide use	553	-6.7	5.4	0.031
Error	2,054			
Total	4,616			
Corrected total	3,045			

[a]$R^2 = 0.325$.

A separate analysis showed no significant association between beta-lactam, macrolide, quinolone, or tetracycline use and change in the percentage of erythromycin resistance. However, an overall increase in erythromycin resistance was observed (Table 1).

Conclusions

Numerous studies have associated antimicrobial-drug use patterns in hospitals with the emergence of resistance among nosocomial pathogens (21-25). However, *S. pneumoniae* is usually acquired outside the hospital environment; therefore, establishing a relationship between antimicrobial-drug use and resistance requires outpatient data, as well as susceptibility test results. A large-scale study of this type is costly and difficult to perform in the United States, given the problems inherent in collecting accurate data from multiple outpatient settings. To generate hypotheses and support the

planning of such a study, we used data collected for other purposes to explore the relationship between outpatient antimicrobial-drug use and resistance among *S. pneumoniae* isolates.

We found an association between the outpatient use of beta-lactam antimicrobial drugs in metropolitan areas and changes in the penicillin susceptibility of *S. pneumoniae* isolates sampled from tertiary care centers in those metropolitan areas. Determining whether this association is spurious or causal requires further investigation, given the limitations of our study design. Since information about each patient's previous antimicrobial-drug use was not available, we were unable to make a direct connection between patient use and risk for resistance. Furthermore, since antimicrobial-drug use data are presented as the total number of prescriptions per month in the population, the data may not accurately reflect use. Patient compliance, dosage prescribed, and duration of antibiotic use may differ from region to region. In addition, the data are for large populations, and the *S. pneumoniae* isolates represent a small sample from one study center in each metropolitan statistical area. These samples may not accurately reflect the true prevalence of resistance in the study population. For this reason, we grouped the study centers into tertiles on the basis of use, to decrease the impact of a small number of resistant isolates at a single study center. Finally, this analysis was retrospective. These surveillance surveys were not designed to evaluate the association between antimicrobial-drug use and changing resistance patterns among *S. pneumoniae*.

However, the use of antimicrobial agents in a population would be expected to contribute to the emergence and spread of resistance within that population, and our data support this hypothesis for beta-lactam use and penicillin resistance. The fact that beta-lactam use was associated with increased penicillin, but not erythromycin, resistance among pneumococcal isolates in our study suggests a specific association. Furthermore, this positive association with penicillin resistance was not seen for antimicrobial-drug classes other than the beta-lactams; neither was resistance associated with the total number of antimicrobial-drug prescriptions.

Erythromycin resistance also increased during our study. The lack of a strong association between use and erythromycin resistance may reflect the fact that beta-lactams were the most commonly prescribed in the metropolitan statistical areas we studied, and the impact of these drugs was therefore greater and easier to detect. In addition, a relationship between resistance to penicillin and resistance to virtually all other oral antimicrobial-drug classes has been described (2,16-17), making colinearity a potential problem in evaluating the impact of specific classes on resistance to a single antimicrobial agent or class. If the relationship between penicillin resistance and resistance to other antimicrobial-drug classes is due to the clonal spread of already multidrug-resistant strains (rather than emergence of resistance under antimicrobial pressure), the impact of a specific class of agent on the spread of a specific resistance in *S. pneumoniae* might vary by region, depending on the coresistance pattern of the predominant PRSP clones in that area.

Other investigators have reported an association between prescriptions for outpatients and rates of resistance in Western Europe (26), Hungary (27), and Iceland (28). In these studies, lower use of antimicrobial drugs in general and beta-lactams in particular is associated with lower rates of isolation of resistant strains. Our study supports this association and underscores the importance of implementing measures to decrease the inappropriate use of antibiotics in the outpatient setting (29).

Despite several limitations, our data support the hypothesis generated in previous studies that outpatient antimicrobial-drug use plays an important role in the development and spread of resistance. In future epidemiologic studies, antimicrobial-drug use should be carefully matched with resistance in well-defined populations and should include prospective evaluation of interventions to reduce the use of certain classes of antimicrobial agents for outpatients.

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Digging for Pathogens: Ancient Emerging Diseases—Their Evolutionary, Anthropological and Archaeological Context.
Charles L. Greenblatt, editor. Balaban Publishers, Rehovot, Israel, 1998.

Digging for Pathogens presents the proceedings of the first conference held by the Center for the Study of Emerging Diseases, convened in Jerusalem in May 1997. Specific areas of interest of the Center include parasite adaptability and evolution, human genetics and behavior, vaccine and antibiotic development. The subject of this monograph is "paleo- and prospective parasitism," or ways in which knowing the causes of ancient diseases contributes to our understanding of emerging ones.

The book is divided into three sections. The first two—The Evolutionary Context and The Anthropological and Archaeological Context—provide a strong background for understanding the respective roles of parasites, arthropod vectors, vertebrate hosts, and humans in affecting disease outcomes. This book is the first to focus on the isolation, characterization, and interpretation of ancient DNA (aDNA) in relation to infectious disease and pathology (1). The titular third section, Digging for Pathogens, expertly summarizes the technical and practical aspects of paleopathology, including the fate of biologic markers (Eglinton, chapter 15), aDNA (Herrmann and Hummel, chapter 16), and arthropods as reservoirs and vectors of pathogens, both ancient and modern (Spigelman and Greenblatt, chapter 17).

Each chapter presents enough original ideas to fuel a new generation of graduate students. Martin and Rothschild (chapter 3) authoritatively cover the history of infection, bone diseases, endocrine disorders, and tumors in vertebrates. Cano (chapter 5) discusses how aDNA sequences provide insight into molecular evolution and the evolution of virulence, exemplified by bees and *Bacillus* symbionts fossilized in amber. Schlein and Jacobson (chapter 7) and Halevy (chapter 8) discuss the evolution of the Trypanosomatidae, based on common cellulase and ergosterol profiles. Ewald (chapter 4) suggests that cholera was responsible for the decentralization and migration of the Harappan civilization. A more compelling argument for the migration of the Harappan civilization, however, is drought. Satellite images of northern India and Pakistan show the ancient course of the Sarasvati River, which dried up during the global climatic change climaxing in the great drought of 2200 to 2000 BCE (2,3). Severe aridity and wind turbulence led to the abandonment of great civilizations from the Aegean Sea, Egypt, Mesopotamia, and northern India (3). The Harappan civilization, originally clustered along the length and tributaries of the Sarasvati, migrated east toward the Ganga, with "asynchronous but sustained abandonment of different cities" (Ewald, chapter 4).

The book would have been improved by providing a straightforward subject index and pairing terms in the Keywords section with page numbers. As expected of a book with multiple authors, the articles range unevenly from research papers to excellent comprehensive reviews. Martin and Rothschild's Earth History and the Evolution of Sickness (chapter 1) and Ubelaker's Ancient Disease in Anthropological Context (chapter 10) are examples of the latter. As editor, Greenblatt has successfully smoothed most transitions.

Digging for Pathogens deserves a wider audience than infectious disease specialists, evolutionary biologists, and parasitologists. For university students of microbe-host interactions, who are increasingly trained in molecular biology but may have scant grasp of evolution or organismic biology, this book should be required reading.

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Will Avilamycin Convert Ziracine into Zerocine?

To the Editor: Avilamycin and evernimicin (Ziracine), which belong to the everninomicin class of drugs, are oligosaccharide antibiotics active against numerous gram-positive bacteria, including emerging pathogens such as glycopeptide-resistant enterococci, methicillin-resistant staphylococci, and penicillin-resistant pneumococci (1,2). The two drugs share the same mode of action—inhibition of translation—by binding to the same target in the large 50S ribosomal subunit (3). As a result, they also display cross-resistance: bacteria resistant to avilamycin are resistant to evernimicin (and vice versa), but not to other classes of drugs (4). Two mechanisms of resistance to this class of drugs are amino-acid substitution in ribosomal protein L16 (5) and mutations in the peptidyltransferase domain of 23S ribosomal RNA (6).

Avilamycin has been used in Europe for several years as a growth promoter for food animals, particularly pigs. Enterococci resistant to everninomycins have been isolated from animals receiving avilamycin as a food additive (4). Evernimicin is being evaluated as a human therapeutic agent. Indirect evidence indicates that the use as growth promoters of antibiotics that display cross-resistance with those used in human therapy contributes to antibiotic resistance in bacteria responsible for human infections (7). As early as 1969, a recommendation was published in the United Kingdom that antibiotics used to treat infections in humans not be used as animal food additives (8). In 1999, four antibiotics were banned as food additives in the European community.

Antibiotic resistance in human pathogens has become a major health issue, complicated by the fact that no new class of drugs has been introduced for human therapy in the last 25 years. Since the use of avilamycin in animals has favored selection of enterococcal strains that are cross-resistant to evernimicin (4), these bacteria can colonize the human gut or form a pool of resistance genes that can spread to human commensals or pathogens (9). The continued use of avilamycin as a growth promoter is likely to diminish the effectiveness of evernimicin if Ziracine or any member of the class is used for human therapy. Since everninomicins will be prescribed mainly for infections due to multiresistant gram-positive cocci, avilamycin should be prospectively banned as an animal growth promoter.

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Medical Examiners, Coroners, and Bioterrorism

To the Editor: Federal, state, and local agencies are developing plans to detect and respond to bioterrorism. Medical examiners and coroners should be included in these plans. A multifaceted response team for bioterrorist events includes health-care providers and law enforcement, public health, and public safety officials. Since medical examiners and coroners generally work independently from other members of this team, special efforts may be necessary to ensure their inclusion in the planning process.

Medical examiners and coroners have state statutory authority to investigate violent, suspicious, sudden, or unexplained deaths (1), including those due to homicide, trauma, and inapparent or poorly explained causes, such as drugs, toxins, and infectious agents. The role of these medical professionals in bioterrorism response can be twofold: response to a known terrorist attack and surveillance for unusual deaths or clusters of deaths that may represent an undetected attack. Deaths from terrorism are homicides and therefore under the jurisdiction of medical examiners and coroners. These investigators are skilled in preserving medicolegal evidence that may be important for subsequent criminal proceedings and in handling situations that involve mass deaths, as shown by their participation in the investigations of the Oklahoma City bombing, aviation accidents, and heat-related deaths (2-4).

Medical examiners and coroners may also play an important role in the detection of bioterrorism since they may recognize unusual deaths before health-care providers become involved. Patients who die of infectious diseases or poisoning often die at home (5,6). Even patients who come to a health-care facility for treatment may die precipitously and unexpectedly, without a clear diagnosis, and may come under the jurisdiction of medical examiners and coroners. For example, in the 1993 outbreak of hantavirus pulmonary syndrome in the southwestern United States, medical examiners played an important role in recognizing the novel, rapidly fatal infectious syndrome (7). Autopsies are an effective way of obtaining an accurate diagnosis for deaths from infectious diseases and toxic exposures. In 1979, autopsy pathologists played a critical role in recognizing inhalational anthrax cases caused by an accidental discharge from a former Soviet bioweapons laboratory (8). In 1985, Illinois medical examiners identified the cause of death of persons who ingested acetaminophen that had been intentionally contaminated with cyanide (9).

To be fully integrated into the medical, law enforcement, and public health plans for detecting and responding to bioterrorism, medical examiners and coroners will need information about the biological and chemical agents likely to be used, access to laboratories capable of identifying these agents, adequate data management systems for mortality surveillance, and improved autopsy facilities and procedures to ensure that prosectors are protected from infectious and chemical agents. As a beginning, the Centers for Disease Control and Prevention recently funded a model medical examiner surveillance program for bioterrorism mortality in New Mexico. Further collaboration among federal, state, and local agencies and medicolegal death investigators will be required for the components of such a program to be effective on a national scale.

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Seroprevalence of Human Hantavirus Infection in the Ribeirão Preto Region of São Paulo State, Brazil

To the Editor: Hantavirus pulmonary syndrome (HPS) has been identified in the region of Ribeirão Preto, São Paulo State, Brazil, since 1993 (1-4). As of September 1999, 38 HPS cases had been reported in Brazil, 16 in the state of São Paulo (2). Between May 1998 and August 1999, the Adolfo Lutz Institute (ALI) in São Paulo city serologically confirmed five cases—three fatal—in the Ribeirão Preto region: two from Guariba, one from Jardinópolis, one from Cajuru, and one from Cassia dos Coqueiros (Luiza Teresinha Madia de Souza, ALI, pers. comm.).

Despite these reports and suspicions of additional cases, the prevalence of hantavirus infection and HPS in the region is not known. Laboratory confirmation has not been available locally, and sending serum samples to ALI for laboratory evaluation is not feasible in most cases. Thus, only presumptive diagnoses could be made until the Sin Nombre virus (SNV) enzyme-linked immunosorbent assay (ELISA) was developed.

To estimate the occurrence and distribution of human hantavirus infection, we used SNV ELISA to conduct a serologic survey of a sample of hospital patients requiring venipuncture for routine procedures. The patients came from three regional cities: Ribeirão Preto, Guariba, and Jardinópolis. Between February and June 1999, a total of 567 samples were collected: 257 from the public hospital of Guariba, 110 from the public hospital in Jardinópolis, and 200 from the General Hospital of the School of Medicine of Ribeirão Preto. When we compared our sample with the general population, the patients in the study sample were slightly older but similar in sex distribution.

Sixteen additional samples were evaluated to confirm the effectiveness of SNV ELISA in diagnosing hantavirus infection: 12 from patients in whom HPS was clinically suspected and 4 previously confirmed by ALI in the city of São Paulo between May 1998 and August 1999. Known HPS convalescent-phase plasma provided by the Centers for Disease Control and Prevention (CDC) was used as positive control. Negative controls were selected by simple random sampling from all previously negative samples.

Positive and negative recombinant SNV antigens provided by CDC were coated on microtiter plates at 1:2,000 dilution in phosphate-buffered saline overnight at 4°C and used in a standard immunoglobulin G testing format. Reverse transcriptase-polymerase chain reaction analysis of serum from two fatal cases of HPS occurring in the cities of Franca and Araraquara suggested the presence of two genetically distinct hantaviruses in the area surrounding Ribeirão Preto. Antigen prepared from local virus is not considered to be necessary for immunoassays because the local virus is not sufficiently different from other isolates to require special antigen preparation (5).

All samples were screened in duplicate on both positive and negative antigens in the assay. A sample was considered positive if absorbance on the positive antigen was greater than absorbance on both the negative control antigen and the negative control of the plate. To confirm the diagnosis, samples satisfying these criteria were tested in duplicate along with 14 negative samples. Samples were considered positive when their subtractive absorbance was greater than the calculated mean subtractive absorbance of the 14 negative samples and three standard deviations.

From our serologic survey, the seroprevalence of human hantavirus infection was determined to be 1.23% (7/567) overall, 0.5% (1/200) in Ribeirão Preto, 0.4% (1/257) in Guariba, and 4.5% (5/110) in Jardinópolis. If one assumes the inhabitants sampled were representative, the seroprevalence provides an estimate of surviving past or recent hantavirus infections in the area. As the overall antibody prevalence of 1.23% is more than twice that observed in the U.S. populations at risk for hantavirus infection, such infections are not rare in the Ribeirão Preto region (6).

Three of the four HPS samples previously confirmed by the ALI in São Paulo tested positive by our ELISA. Of the remaining 12 suspected HPS cases assayed, three were positive. Two of these three were later confirmed as positive by the ALI (Luiza Teresinha Madia de Souza, ALI, pers. comm.) Thus, we report three previously unconfirmed HPS cases, one fatal, in the Ribeirão Preto area between May 1998 and August 1999.

Since the rodent reservoir is not known and the virus has not been isolated, rodent capture is currently being conducted in areas where human infections have been found. In addition, positive cases are being retrospectively investigated.

Thanks to Thomas Ksiazek for providing recombinant SNV antigen and positive serum and to Robert Shope for his assistance.

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Molecular Characterization of *Mycobacterium abscessus* Strains Isolated from a Hospital Outbreak

To the Editor: In recent years there have been several reports of sporadic and epidemic hospital-acquired infections caused by rapidly growing mycobacteria, namely, *Mycobacterium abscessus*, *M. chelonae,* and *M. fortuitum*. *M. abscessus* has been well documented as a cause of cutaneous and soft tissue infections and has been implicated in chronic ear infections, bacteremia associated with hemodialysis equipment, and peritoneal dialysis–related infections (1).

Differentiating mycobacteria to the species level is difficult because of the diversity of available techniques and the time required for full identification. A rapid method based on the evaluation of the gene coding for the 65-kDa heat shock protein, which contains epitopes both unique and common to various species of mycobacteria, has been reported (2). A 383-bp sequence situated at the amino terminus of this 65-kDa antigen (3) has been shown to be conserved among several species of mycobacteria. Reports based on polymerase chain reaction (PCR) amplification and DNA sequencing (4) show species-specific polymorphism at the nucleotide level within this region (5). The conserved nature of this gene allows differentiation of mycobacteria within 1 day by restriction enzyme digestion of PCR products obtained by using primers common to all mycobacteria.

From August through December 1995, postoperative wound infections developed in 45 patients in the pediatric surgery unit of Kalawati Saran Children's Hospital, New Delhi, India; 42 were day-care patients, and 3 were inpatients who had undergone major surgery. Thirty-two clinical samples (pus and exudate) were tested for acid-fast bacilli by the Ziehl-Neelson method; the same smear samples were cultured on Lowenstein-Jensen slants and examined for growth daily for 4 days and thereafter twice a week for 8 weeks. The

organism was identified biochemically (i.e., by the niacin and nitrate production test) as *M. abscessus*.

The genomic DNA from the culture of mycobacterial isolates was extracted by standardized protocol (6) and subjected to PCR-restriction enzyme pattern analysis (PRA) (2). Primers TB11 (5'-ACC AAC GAT GGG GTG TGT CCA T) and TB12 (5'-CTT GTC GAA CCG CAT ACC CT) amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence for 65-kDa heat shock protein (3). The PCR products were then digested separately by using restriction enzymes *Bst* EII and *Hae*III. The digests were fractionated on nondenaturing 10% polyacrylamide gel. The *Bst* EII pattern generated during PRA yielded two 235/210-bp bands similar to the patterns attributed to *M. chelonae* subsp. *abscessus* (2). The patterns displayed on *Hae*III digestion had distinctive 150/60-bp bands that were once again similar to the pattern attributed to *M. chelonae* subsp. *abscessus* (2). PRA results confirmed that the isolates were *M. abscessus*. The source of the outbreak was traced to the tap water in the operating room and to a defective autoclaving process (the result of a leaking vacuum pump and faulty pressure gauge in the autoclave).

This report highlights the role of rapidly growing mycobacteria in a water-related nosocomial outbreak. The PCR-PRA method promises to be a very rapid, economical, and universal system of identifying mycobacteria to the species level. This technique does not require hybridization to a panel of species-specific probes, which is a limitation of other PCR-based and hybridization methods for differentiating mycobacterial species. This method has the potential to be a useful diagnostic as well as epidemiologic marker for typing isolates of most mycobacteria during institutional outbreaks.

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Erratum Vol. 6, No. 4

In the letter "First Report of Human Granulocytic Ehrlichiosis from Southern Europe (Spain)," by José Oteo et al., there are two citation errors on p. 431, column 2. The correct citations follow.

First paragraph, second sentence: "We used a set of primers based on the published sequence of the 16s rRNA of *E. phagocytophila* (E1: 5'-GGC ATG TAG GCG GTT CGC TAA GTT-3' and E2: 5'-CCC CAC ATT CAG CAC TCA TCG TTT A-3' (10)."

Second paragraph, second sentence: "The prevalence of *E. phagocytophila* genogroup in the tick *Ixodes ricinus* is high (24.1% of nymphs, determined by PCR) in La Rioja, and evidence of HGE infection in patients at risk has been reported (11)."

We regret any confusion this error may have caused.

The Hallucinogenic Toreador

The Hallucinogenic Toreador is a complex compendium of Salvador Dalí's work and an instructive example of his method of artistic creation. The Venus de Milo on the right was engendered by a fortuitous double image seen on a common box of Venus de Milo-brand coloring pencils. Dalí's scrutiny of the negative spaces in the well-known image produced the complementary, alternate image of a bullfighter to the left of the main Venus de Milo. This supplementary image invites the eye to wander in a way that relives Dalí's visualization and introduces us to the almost hypnotic array of forms that populate this vast canvas.

The whole scene is contained in a bullfight arena. Afternoon shadows sweep to the left and engulf a panorama of memories and visual associations: the gadflies of St. Narciso, patron saint of Catalonia, swarm over the arena and form the cap, hairnet, and cape of the bullfighter and the tear in his eye, together with the shape of a dying bull in the lower left; a pool of the bull's blood and saliva transforms itself into a sheltered bay with a sunbather on an inflatable mattress; the flotsam and jetsam in the lower section of the beach take on the shape of a Dalmatian dog, its head facing the pool of water; the green tie of the toreador makes a visual twin of the shadows of Venus' garment; the form of the slain bull rises to become the sheltering mountain landscape of the Cape Creus area around Port Lligat, where this work was painted; a mountain, which in turn is mimicked on the right by the inclusion of a craggy peak with a rose near its summit, recalls the precipitous mountains around the town of Rosas, near Dalí's studio.

The unfolding, shifting spectacle of all this is watched, at bottom right, by the very young Dalí, dressed in a sailor's suit. His innocent vision seems propelled by the flies of St. Narciso, while he stands distracted, holding a child's toy hoop. On the opposite side, an artist's easel or chair, with a cubistic rendition of the Venus de Milo, harks back to Dalí's art-school experiences of drawing from plaster casts, one of which stands immediately behind the easel. The space between the student days and the childhood days is peppered with items from Dalí's mature works: his beach landscapes are suggested in the landscape at lower left; the double image of the dog recalls his interest in optics; the molecular diagram of the bull's head reminds us of his fascination with atomic science, together with his love of the architectural decorations of Antoní Gaudí; the shadows of the small, floating plaster Venus statues suggest both the silhouettes of the *Angelus* series and repetitive art-school practice; the bust of Voltaire goes back to his famous double-image work; and the floating rose brings to mind his Gradiva and bleeding-rose works. Read this way, the space bridges Dalí's artistic history. This creative chronicle is dedicated to Gala, Dalí's wife, who is seen in a cameolike apparition at the upper left; she looks disapprovingly at the bullfight scene while returning the young Dalí's gaze. Their gazes meet exactly midpoint in the canvas, fixed upon the central, obsessively painted button of the toreador's shirt collar.

In broad view, the painting is a kind of visual autobiography, prompted by the chance viewing of the Venus de Milo, the cold stone of which (in a twist of the myth of Pygmalion) is transformed into an artistic vision. The beloved landscape of the artist's birthplace, the innocence of childhood, the bullfights of youth, the legends of his adolescence, the unfolding interests of his maturity, and the faith in his wife–all are placed beneath our gaze, suffused with the red and yellow tones of the Spanish national flag.

Salvador Dalí imagined the world as being held together by a mysterious order, a ubiquitous symmetry, that only scientific thought and, by implication, his "paranoiac-critical method" could convey. The discovery of the DNA double helix by Francis Crick and James Watson in 1953 confirmed his intuition of a hidden order, encouraging his belief in the transformational power of "nuclear mysticism"–that is, in what he called the vision of matter constantly in the process of dematerialization, of disintegration, thus showing the spirituality of all substance." The paranoiac-critical method was indebted to the surrealist concept. What surrealism called for, in art as in life, was new, cohesive interaction between the phenomena of the objective, external world and the interior workings of the individual psyche.

Abstracted from Salvador Dalí: Masterpieces from the Collection of the Salvador Dalí Museum, by Kenneth Wach. Harry N. Abrams, Inc., Publishers in association with the Salvador Dalí Museum in St. Petersburg, Florida, 1996.

Editorial Policy and Call for Articles

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

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

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

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

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

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

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

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

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

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

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

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

Types of Articles

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

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

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

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

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

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

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

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

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