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Vector Interactions and Molecular Adaptations of Lyme Disease and Relapsing Fever Spirochetes Associated with Transmission by Ticks

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Pathogenic spirochetes in the genus *Borrelia* are transmitted primarily by two families of ticks. The Lyme disease spirochete, *Borrelia burgdorferi*, is transmitted by the slow-feeding ixodid tick *Ixodes scapularis,* whereas the relapsing fever spirochete, *B. hermsii*, is transmitted by *Ornithodoros hermsi*, a fast-feeding argasid tick. Lyme disease spirochetes are generally restricted to the midgut in unfed *I. scapularis*. When nymphal ticks feed, the bacteria pass through the hemocoel to the salivary glands and are transmitted to a new host in the saliva after 2 days. Relapsing fever spirochetes infect the midgut in unfed *O. hermsi* but persist in other sites including the salivary glands. Thus, relapsing fever spirochetes are efficiently transmitted in saliva by these fast-feeding ticks within minutes of their attachment to a mammalian host. We describe how *B. burgdorferi* and *B. hermsii* change their outer surface during their alternating infections in ticks and mammals, which in turn suggests biological functions for a few surface-exposed lipoproteins.

The molecular adaptations required by pathogenic spirochetes for efficient transmission by obligate, blood-feeding ticks are largely unknown. In the new era of genomics, the complete DNA sequence of two spirochetes, *Borrelia burgdorferi* and *Treponema pallidum*, have been determined (1, 2). As additional genome sequences become available for other pathogenic and free-living spirochetes, comparisons of their genomes may elucidate genes that are unique to those species of spirochetes associated with ticks. This information, along with an increased understanding of the molecular mechanisms used by tick-borne spirochetes to adapt for transmission by their tick vectors, may lead to unique disease prevention strategies.

The genus *Borrelia* currently contains 37 species of spirochetes, many of which cause diseases in humans and domestic animals (Table) (3). Except for *B. recurrentis* (which causes louse-borne relapsing fever and is transmitted by the human body louse), all known species are transmitted by ticks (4). Two groups of spirochetes stand out among these tick-borne species because of their prevalence as human pathogens: Lyme disease spirochetes, transmitted by the relatively slow-feeding ixodid (hard) ticks of the genus *Ixodes*, and relapsing fever spirochetes, transmitted by the fast-feeding argasid (soft) ticks of the genus *Ornithodoros* (Figure 1). Major observations in recent years have increased our understanding of how one species in each group adapts while infecting ticks. *B. burgdorferi*, a causative agent of Lyme disease, and *B. hermsii*, a causative agent of tick-borne relapsing fever, have received the most

attention. We describe how these two species of *Borrelia* change their outer surface during their alternating infections in ticks and mammals, which in turn suggests biological functions for a few surface-exposed lipoproteins. The dynamics of infection of these two bacteria in strikingly different types of ticks provide examples of possible adaptations for their transmission.

B. burgdorferi-Tick Interactions

Detailed studies of *B. burgdorferi* were initiated in 1982 when Burgdorfer and coworkers reported these bacteria in adult *Ixodes scapularis* ticks collected from vegetation on Shelter Island, New York (5). These researchers observed that spirochetes were commonly present in the midgut of infected ticks and occasionally seen in the hindgut and rectal ampule.

Table. Diseases caused by infection with *Borrelia* species

Disease	No. of species	Arthropod vector
Lyme disease	3[a]	Hard ticks (*Ixodes* spp.)
Tick-borne relapsing fever	21[b]	Soft ticks (*Ornithodoros* spp.)
Avian borreliosis	1[c]	Soft ticks (*Argas* spp.)
Bovine borreliosis	1[d]	Hard ticks (*Boophilus* spp.)
Louse-borne relapsing fever	1[e]	Body louse (*Pediculus*)

[a]*Borrelia burgdorferi, B. garinii,* and *B. afzelii* are known human pathogens.
[b]Some species of *Borrelia* associated with *Ornithodoros* ticks are of unknown pathogenicity.
[c]*B. anserina.*
[d]*B. theileri.*
[e]*B. recurrentis.*

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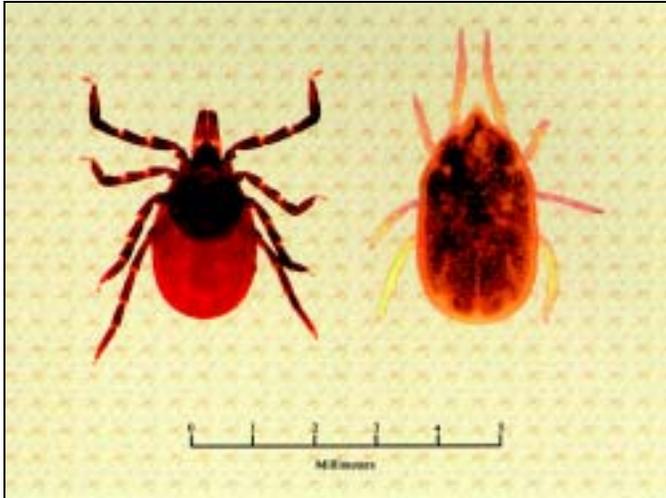
Figure 1. Dorsal view of a female *Ixodes scapularis* (family *Ixodidae*, hard ticks), a vector of *Borrelia burgdorferi* (left), and a female *Ornithodoros hermsi* (family *Argasidae*, soft ticks), the vector of *B. hermsii* (right).

No spirochetes were observed in other tissues, including the salivary glands. During this initial period of study of Lyme disease spirochetes, how vector ticks actually transmitted this new pathogen was the subject of much discussion. Because spirochetes were reported to be restricted to the digestive tract of ticks, some investigators speculated that transmission occurred by tick regurgitation or fecal contamination (6). These inefficient routes of transmission became a less likely explanation after spirochetes were described in the hemolymph (7) and salivary glands of feeding ticks (8). The hypothesis that Lyme disease spirochetes were transmitted via the salivary gland route was confirmed when spirochetes were actually identified in tick saliva (9).

Spirochete Multiplication

The principal tick vectors of Lyme disease spirochetes in North America are *I. scapularis* and *I. pacificus*; the developmental stage of the former species that transmits most human infections is the nymph. Although transmission by adult *I. scapularis* or transovarially infected larvae remains possible, our review focuses on tick-spirochete interactions within nymphal *I. scapularis*. Larval ticks ingest spirochetes from infected reservoir hosts, molt, and emerge as nymphs. When spirochetes are ingested by larvae, they rapidly multiply in the replete tick until the nymphal molt, when a precipitous drop in spirochete numbers occurs (10, 11). Thus, at the time questing nymphs are likely to contact their potential victims, spirochete numbers are at their lowest and generally restricted to the lumen of the midgut. When nymphal feeding begins, a pronounced multiplication of spirochetes takes place in the tick. Nymphal *I. scapularis* take approximately 3 to 4 days to complete feeding. Spirochete numbers are reported to increase >300-fold during this feeding period, increasing from a mean of 496 spirochetes in unfed nymphs to 166,575 at 72 hours after attachment (12). Along with this rapid multiplication,

other changes are taking place in the spirochete population that may lay the groundwork for eventual transmission to the host.

Spirochete Surface Proteins

Virtually all spirochetes in the midgut of an unfed nymph express outer surface protein (Osp) A. This protein is also the predominant surface antigen expressed by the spirochetes in vitro. As the nymphal ticks start to feed and the spirochetes in the midgut begin to multiply rapidly, most spirochetes cease expressing OspA on their surface (13,14). Simultaneous with the disappearance of OspA, the spirochete population in the midgut begins to express a different Osp, OspC (15). Upregulation of OspC begins during the first day of feeding and peaks 48 hours after attachment (14). After this time point, the proportion of spirochetes in the midgut expressing OspC decreases rapidly. Therefore, the repression of OspA synthesis and upregulation of OspC correlate with the exit of spirochetes from the midgut, dissemination through the hemolymph, and passage through the salivary glands of the feeding tick. Several researchers have hypothesized that OspA binds spirochetes to the tick midgut (14,16,17). By downregulating OspA, the spirochetes might free themselves to migrate through the midgut epithelium and out of the midgut. Recently, purified OspA of *B. burgdorferi* was shown to bind to suspensions of tick midgut cells. The binding domains apparently reside in both the central and carboxy-terminus of the OspA protein; OspA also binds to itself (16). Thus, a picture emerges of aggregates of OspA-positive spirochetes bound to the tick midgut. Also, *B. burgdorferi* and *B. afzelii* expressing OspA were shown recently to adhere to tick-cell cultures more readily than spirochetes not producing this protein (17). Thus, when OspA is rapidly cleared from the spirochete surface, a proportion of the spirochete population may be free to leave the midgut and migrate to the salivary glands for transmission in saliva. The tick midgut protein that binds to OspA has not yet been described but should be an area of future research, as will be identifying factors that modulate the dispersal of spirochetes from the tick midgut (18).

Factors that regulate the switch from expression of OspA to OspC are likely varied and complex. Temperature is clearly one factor. As the tick finds a host and starts to feed, it moves from ambient temperature to the temperature at the surface of mammalian host skin. This rapid change in temperature clearly influences the spirochete population. Shifting spirochetes in vitro from lower temperatures to 37°C induced OspC expression (14,19). Similarly, other spirochete proteins, such as the Erp lipoprotein family, are upregulated during temperature increase (20). Cell density also may regulate spirochete protein expression. In a series of experiments using anti-OspA antibody passively transferred to mice serving as hosts for infected nymphal ticks, decreased spirochete density resulting from antibody-mediated death was associated with a lack of expression of OspC (21). Similarly, growth phase affects the synthesis of various proteins, including OspC (22). A change in pH in vitro can influence the expression of many proteins,

including OspC (23,24). When ticks ingest blood, the pH of the midgut decreases from 7.4 to 6.8, which acts interdependently with increasing cell density and increased temperature to promote the reciprocal expression of OspA and OspC (25). The expression locus of the variable-like sequence (*vlsE*) may also prove to be of interest, since heterogeneity at this site appears to increase when ticks start to feed (26).

Dissemination to the Salivary Glands

Different experimental strategies have been used to visualize spirochetes as they move from the tick midgut, through the hemolymph, and into the salivary glands before delivery to the host. Spirochetes present in hemolymph (7) and salivary glands have been directly visualized by electron microscopy (8). Indirect methods for detecting spirochetes in tick salivary glands included salivary gland explant cultures in BSK medium (Sigma Chemical Co., St. Louis, MO) and an infectivity assay of salivary gland homogenates inoculated into mice (27). These experiments demonstrated that although spirochetes were occasionally detected in salivary glands early during tick feeding, sufficient numbers of infectious spirochetes were not present within the salivary glands to cause infection in experimental hosts until at least 60 hours after tick attachment. Recently, confocal microscopy has allowed direct determination of specific spirochetal proteins synthesized in salivary glands. This approach has shown that small populations of spirochetes expressing OspA are present in the tick salivary glands and the dermis at the site of tick feeding early during the tick bloodmeal, but these spirochetes do not appear to be infectious. After 2 days of feeding, large numbers of spirochetes belonging to two predominant populations appear in the salivary glands and local dermis of the feeding site: spirochetes expressing only OspC, and spirochetes expressing neither OspA nor OspC (26). Infection of the host may be determined by actual numbers of spirochetes, the particular Osp phenotype entering the host from the tick, or both.

Transmission to Host and Duration of Tick Feeding

Most persons bitten by nymphal *I. scapularis* do not become infected with *B. burgdorferi* and do not become ill with Lyme disease. Although 25% to 30% of nymphal *I. scapularis* in the northeastern United States are infected with *B. burgdorferi* sensu stricto, only approximately 1% to 2% of persons with recognized bites by nymphal *I. scapularis* become infected. One reason for this low rate of infection is that most ticks are detected and removed before they transmit infectious spirochetes. Virtually no transmission occurs during the first day of nymphal feeding, inefficient transmission takes place during the second day of tick feeding, and transmission is extremely efficient during the third day of nymphal feeding (28,29). These observations are consistent with the timing of spirochete multiplication, switching of Osps, and dispersal within the tick. A basic understanding of tick-spirochete interactions and transmission dynamics clearly has important implications in the clinical setting. The fact that prompt tick removal aborts transmission of *B. burgdorferi* sensu stricto is an important reason why most physicians in Lyme disease-endemic areas do not prophylactically treat persons bitten by ticks. In Europe and Asia, where other tick species (*I. ricinus* and *I. persulcatus*) transmit several genospecies of spirochetes, the situation may be more complex, with some risk of transmission during the first day of tick attachment (30,31).

Vaccine Implications

An important practical outgrowth of understanding how *B. burgdorferi* populations change during tick feeding is the enhanced insight into the molecular mechanisms of how the human Lyme disease vaccine based on OspA (32,33) works. Early on it was noticed that when anti-OspA antibody was present in the host at the time of tick attachment or during the first 24 hours of tick feeding, infection was prevented. If, however, antibodies were passively transferred to the host after this window of opportunity, infection was not prevented or cured with anti-OspA antibody (13,34). The implication was clear: spirochetes were killed inside the tick, before transmission to the host. Originally, it was suggested that anti-OspA antibodies actually sterilized the tick, eliminating all spirochetes present before they were transmitted (13). Subsequent studies with variable levels of anti-OspA antibody showed that ticks were only occasionally sterilized, at the highest levels of antibody in an immune host. More subtle effects were demonstrated with passively transferred anti-OspA antibody, which demonstrated that spirochete populations in the midgut were diminished but not eliminated. This diminution also prevented the switch from OspA expression to OspC expression and prevented dispersal of spirochetes to the salivary glands and transmission to the host (21). Thus, the action of anti-OspA antibody ingested by the tick had subtle effects on spirochete populations that blocked transmission to the host. Anti-OspC immunity appears to differ, acting in both the invertebrate and vertebrate host (34,35). These studies are a prime example of how insight into basic vector-pathogen interactions can lead to development of important prevention tools used to combat human disease.

Tick-Borne Relapsing Fever

In 1857, Livingstone published his observations resulting from 16 years of exploration in southern Africa. In regions that are now Angola and Mozambique, he documented an illness of humans following the bite of a tick, known regionally as the "tampan" or "carapato." This brief description was the first written account of tick-borne relapsing fever caused by *B. duttonii*. The tick was described by Murray in 1877 and named *Argas moubata*, later revised within the genus *Ornithodoros*. In 1905, Dutton and Todd (36) reported that *O. moubata* transmitted spirochetes to monkeys while feeding on them—the first demonstration that ticks were capable vectors of relapsing fever spirochetes. Also included in this landmark work was the observation that spirochetes were present in both the midgut and malpighian tubules of infected ticks.

Relatively few studies have examined the distribution of relapsing fever spirochetes in tissues of argasid ticks. Most early investigations examined *B. duttonii* in *O. moubata,* expanding on the work of Dutton and Todd, and demonstrated spirochetes in numerous tick tissues including the midgut, synganglion (central ganglion), malphigian tubules, salivary glands, ovaries, and coxal organs. Early investigators also demonstrated that *B. duttonii* is transmitted by contamination of infected coxal fluid and tick bite. Burgdorfer's study of *B. duttonii* in nymphal and adult *O. moubata* is one of the most thorough investigations of any relapsing fever spirochete (37). While confirming the infection of many tick tissues, Burgdorfer also showed that *B. duttonii* enters the hemolymph as early as 24 hours after ticks acquire spirochetes in their midgut by feeding on a spirochetemic mouse. Also, the mode of transmission varies with the stage of tick. Nymphal *O. moubata* transmit *B. duttonii* in the saliva; adults transmit primarily via the coxal fluid. This stage-dependent difference in the primary mode of transmission helped clarify earlier observations.

B. hermsii-Tick Interactions

Tick-borne relapsing fever was first reported in the United States in 1915, following the recognition of five human patients in Colorado (38). The tick vector for the causative spirochete lives in the higher elevations in western North America and was named *O. hermsi* in 1935 (39). Relapsing fever spirochetes transmitted by *O. hermsi* were first named *Spirochaeta hermsi* in 1942 and later changed to *Borrelia hermsii* in 1948. The criterion for naming the spirochete *B. hermsii* was based on the many observations in the laboratory by Davis (40). *O. hermsi* was capable of transmitting this spirochete while other species of ticks, *O. turicata* and *O. parkeri*, were not, although these other species of ticks were capable of transmitting other species of spirochetes (40). The mechanisms responsible for this strict species specificity for the transmissibility of one species of spirochete by only one species of tick are unknown.

B. hermsii infects a variety of small mammals living in coniferous forests at moderate to high elevations. The primary hosts for spirochetes and ticks are diurnal rodents such as chipmunks and tree squirrels. Ticks living in the nests or crevices nearby feed on these hosts at night, taking their bloodmeal quickly within 15 to 90 minutes, then retreating to their off-host refuge. These ticks and other species of *Ornithodoros* can fast for months to many years and retain infectious spirochetes throughout a life cycle that may take years to complete (41). Therefore, ticks have a greater potential to maintain spirochetes in nature for prolonged periods than do individual mammalian hosts that are infective to new cohorts of ticks while intermittently spirochetemic for 14 to 30 days (42).

Although other, larger species of *Ornithodoros* excrete substantial amounts of coxal fluid while feeding, *O. hermsi* excretes little or none while on the host. Therefore the only efficient mechanism for *O. hermsi* to transmit *B. hermsii* is by bite (43). The larva, three to five nymphal stages, and adult

O. hermsi are all capable of transmitting *B. hermsii* (43,44). Transovarial transmission is rare (43), so larvae in nature are unlikely to be infective during their blood meal.

The distribution of *B. hermsii* in organs and tissues of *O. hermsi* has received little attention. In 1942, Wheeler published the only study before 1998 that examined different tissues of *O. hermsi* for *B. hermsii* (45). Spirochetes were consistently found in the midgut but only occasionally seen in the hemocoel, muscles, and "esophagus" for up to 38 days after infection was initiated in ticks. No spirochetes were seen in the salivary glands. Yet, based on recent observations (46), Wheeler's negative data were most likely due to his use of thin sections and silver stain. The lack of specific immunologic stains for fluorescence microscopy 60 years ago made visualizing spirochetes much more difficult than today. Schwan and Hinnebusch (46) examined 41 *O. hermsi* from 33 to 144 days after infection with *B. hermsii* and found that the salivary glands from all ticks were infected. The midgut from 33 ticks and the synganglion from 22 ticks were also examined, and spirochetes were present in these organs from all ticks.

Antigenic Variation in Mammals and Ticks

During the last 20 years, many studies have examined the mechanism of antigenic variation of *B. hermsii,* which has been proclaimed as a spirochete adaptation to evade the mammalian host's immune response (47). The ability of the spirochete to evade immunologic destruction through antigenic variation within the mammal allows for the prolonged, recurrent bacteremias (Figure 2) that make spirochetes accessible to fast-feeding ticks and hence facilitate horizontal transmission of spirochetes in nature. A single cell of *B. hermsii* can give rise to 30 antigenic variants, each of which expresses a unique, variable major protein (Vmp) that confers a specific serotype (47,48). Other than the suggested role of immune evasion, no function has been demonstrated for these Vmps, which occur in two size classes, the variable large proteins (Vlps) and the
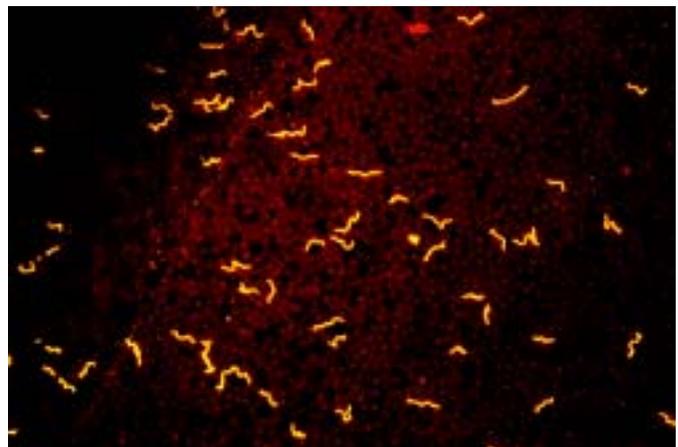
Figure 2. *Borrelia hermsii* visualized with an immunofluorescent stain in a thin blood smear of an experimentally infected mouse. Such recurrent, high densities of spirochetes circulating in the peripheral blood of small mammals allow for the acquisition of these bacteria by fast-feeding ticks that ingest a small volume of blood.

variable small proteins (Vsps) (49). During mammalian infection, *B. hermsii* produces cyclic spirochetemias that may achieve a density of 10^8 bacteria/mL of blood. Each acute phase of spirochetemia contains a population of spirochetes composed almost entirely of one serotype (48). Relapse populations are predominated by a serotype different from the population preceding or following it.

To address the influence of tick infection on the antigenic stability of spirochetes, two cohorts of *O. hermsi* were each infected with a different serotype of *B. hermsii*, serotype 7 or serotype 8 (46). The ticks were allowed to molt to the next stage and then fed individually on single mice. Eighteen (19%) of the 95 ticks transmitted spirochetes, and with every infection the first spirochetemia in mice had the same serotype ingested previously by the tick. Additional groups of ticks infected with the same serotypes were also examined. Polymerase chain reaction (PCR) and restriction fragment length polymorphism analyses of the expression locus for the *vmp* genes showed that either the *vlp7* or *vsp8* gene was present, corresponding to the serotype of *B. hermsii* ingested by the tick. Cumulatively, these observations suggested that no serotype-related antigenic changes occurred in the spirochetes during infection in ticks. However, when single salivary glands from infected ticks were examined by immunofluorescence microscopy with anti-Vlp7 or anti-Vsp8 antibody, no spirochetes were seen. Immunofluorescence staining of the other salivary glands with other antibodies showed, however, that all the glands were infected with spirochetes and that the bacteria were now expressing Vsp33, an Osp known previously from only one culture-adapted strain of *B. hermsii* (50). When blood from a mouse infected with serotype 8 of *B. hermsii* was inoculated into culture medium and incubated at either 23°C or 37°C, spirochetes continued to express Vsp8 at 37°C but switched to Vsp33 at 23°C (46). Thus, both a transfer of spirochetes from the mammalian bloodstream to tick and a drop in environmental temperature in vitro brought about an antigenic switch from the bloodstream Vmp to Vsp33. Recently, double-label immunofluorescence staining of spirochetes in the midgut and salivary glands of *O. hermsi* demonstrated that spirochetes expressing Vsp33 were detectable ≥28 days after ticks became infected (Figure 3). By 68 days after infection, essentially all the spirochetes in the salivary glands expressed Vsp33, while only a third of the spirochetes in the midgut did so.

Species Comparisons

From the observations reviewed above, one phenomenon shared by *B. burgdorferi* and *B. hermsii* is the synthesis of OspC and Vsp33 at the time these spirochetes are transmitted by tick bite. DNA and amino acid sequence analysis also shows that these proteins are homologous (51) and that antisera produced to the two proteins are cross-reactive (51-53). Several other species of *Borrelia* also contain a related gene or protein recognized with either Northern or Western blot (53). Therefore, this family of surface proteins may be shared by all species of borreliae, which are spirochetes defined, in part, by
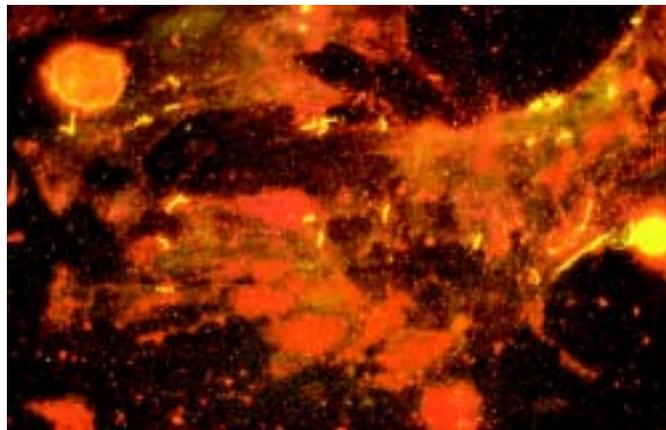
Figure 3. *Borrelia hermsii* visualized with an anti-variable small protein (Vsp) 33 antibody in a squashed salivary gland of *Ornithodoros hermsi*. Persistent infection of the salivary glands allows these spirochetes to be transmitted in only minutes while these ticks feed.

their requirement for an arthropod vector for transmission. Our hypothesis is that these proteins are involved in the transmission of these spirochetes from tick to vertebrate host.

The temporal synthesis of OspC and Vsp33 during spirochete infection in ticks is strikingly different between *B. burgdorferi* and *B. hermsii,* but appears to be adaptive to ixodid versus argasid ticks, which have considerably different feeding behaviors. Most nymphal *I. scapularis* take 3 to 4 days to feed whereas *O. hermsi* feed to repletion in only 15 to 90 minutes. In free-living, unfed *I. scapularis*, *B. burgdorferi* is usually found only in the midgut and OspC is not expressed. However, following tick attachment *B. burgdorferi* replicates, downregulates OspA, disseminates from the midgut to salivary glands, synthesizes OspC, and is transmitted via the saliva after 2 to 4 days of feeding. An increase in temperature and the ingestion of blood, environmental cues associated with a free-living tick having attached and begun to feed on a host, stimulate a sub-population of *B. burgdorferi* to transiently synthesize OspC during feeding. If OspC is required for transmission of *B. burgdorferi* by tick bite, there is ample time, because of the slow feeding behavior of *Ixodes* ticks, for both the dissemination of spirochetes from the midgut to the salivary glands and the novel synthesis of OspC. Numerous studies have shown that humans and experimental animals infected by tick bite seroconvert to OspC early, demonstrating that this protein is expressed in mammals for some undetermined period. In free-living, unfed *O. hermsii*, the distribution of *B. hermsii* in these ticks and their expression of Vsp33 are just the opposite, with spirochetes established in the salivary glands and nearly all expressing Vsp33. In the scenario with *O. hermsi* feeding for only minutes after encountering a host, there is no time for spirochetes to disseminate out of the midgut, penetrate the salivary glands to the salivary duct, and also synthesize a new Osp that may facilitate (or be required for) transmission. Hence *B. hermsii* is in a constant state of readiness for transmission that is comparable to the phenotype and localization displayed by *B. burgdorferi* only briefly during a few days of attachment by *I. scapularis* (Figure 4).

The phenotypic changes and dissemination shown by *B. burgdorferi* during transmission by tick bite point to several possible functions for OspC: dissemination from the midgut, infection of the salivary glands, or successful colonization in the mammal following delivery into the feeding lesion in the dermis. Because the changes in protein synthesis and movement of the spirochetes occur rapidly in the nymphal ticks, identifying the precise time when OspC is produced in relation to the spirochetes' movement in the tick is difficult to determine by microscopy. Quantitative reverse transcription-PCR may help increase sensitivity through detecting specific gene transcripts in different tick tissues sampled at different times. However, the events shown by *B. hermsii* during its dissemination in the tick are much more protracted and may shed some light on the function of OspC, Vsp33, or other proteins. Because it takes approximately 3 weeks or more for *B. hermsii* to disseminate and become established in the salivary glands of *O. hermsi*, immunofluorescent staining of these spirochetes in tick tissues at successive intervals after infection has recently shown that *B. hermsii* can infect the salivary glands before the upregulation of Vsp33. At least for this relapsing fever spirochete, Vsp33 does not appear to be needed either for dissemination from the gut or invasion of the salivary glands.

Additional species of *Borrelia* and their expression of Osps associated with transmission need to be examined. If OspC, Vsp33, or other proteins are required for transmission by ticks, we anticipate finding homologs to these proteins in other species associated with their transmission. Other relapsing fever spirochetes in *Ornithodoros* ticks, *B. anserina* in *Argas* ticks, and other borrelia associated with ixodid ticks will be fruitful tick-spirochete associations to study. A comparison of borre-

lial genomes will also be helpful when such sequences become available. Finally, with important advances being made recently to inactivate and introduce genes in these spirochetes (54,55), experiments to examine the importance of specific proteins in various steps of the transmission cycle are on the horizon.

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Figure 4. Schematic representation of the presence of OspC on *Borrelia burgdorferi* and variable small protein (Vsp) 33 on *B. hermsii* during infection in their respective tick vectors. Shown is the proportion of spirochetes detected with double-label immunofluorescence stains that includes either anti-outer surface protein C (Osp C) antibody (top) or anti-Vsp33 antibody (bottom). *B. burgdorferi* produces OspC in the midgut of *Ixodes scapularis* for only a few days, starting after these ticks have attached and begun to feed. During the 3 to 5 days of tick feeding, these spirochetes replicate, disseminate from the midgut, and are transmitted via saliva. In contrast, *B. hermsii* gradually upregulates the synthesis of Vsp33 after infecting *Ornithodoros hermsi*, and essentially all the spirochetes express the protein during persistent infection of the tick salivary glands.

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Traditional and Molecular Techniques for the Study of Emerging Bacterial Diseases: One Laboratory's Perspective

Pierre Houpikian and Didier Raoult

Identification of emerging bacterial pathogens generally results from a chain of events involving microscopy, serology, molecular tools, and culture. Because of the spectacular molecular techniques developed in the last decades, some authors think that these techniques will shortly supplant culture. The key steps that led to the discovery of emerging bacteria have been reviewed to determine the real contribution of each technique. Historically, microscopy has played a major role. Serology provided indirect evidence for causality. Isolation and culture were crucial, as all emerging bacteria have been grown on artificial media or cell lines or at least propagated in animals. With the use of broad-range polymerase chain reaction, some bacteria have been identified or detected in new clinical syndromes. Culture has irreplaceable advantages for studying emerging bacterial diseases, as it allows antigenic studies, antibiotic susceptibility testing, experimental models, and genetic studies to be carried out, and remains the ultimate goal of pathogen identification.

I n the last 20 years, advances in knowledge have resulted in a broad expansion of the spectrum of microorganisms regarded as human pathogens. Most advances have evolved in a series of small steps based on several techniques that have been used successively by different investigators who faced clinically suspect diseases. These include the traditional techniques of microscopy, serology, and culture, as well as more recent molecular tools (Figure 1). In addition to aiding in discovering new pathogens, these techniques also contributed to studies of the epidemiology, pathophysiology, and treatment response of the newly recognized diseases, providing further evidence for causal relationships between disease and organism (1). As a diagnostic and research laboratory specializing in fastidious, intracellular bacteria, we have been particularly interested in assessing the specific role played by culture in identifying emerging pathogens. Historical examples, such as Lyme or Legionnaires' diseases, and recent successes, such as culture of the Whipple bacillus, support the effectiveness of this technique (2). Moreover, culture provided the basis of other supplemental tools to elucidate the causes of microbial disease and to study the clinical and biological features of emerging bacterial diseases. These tools are not only antigenic and serologic assays but also in vitro and in vivo disease models for pathophysiologic studies and antimicrobial susceptibility testing, plus extensive genetic sequencing. The isolation of emerging pathogens serves, therefore, not only as a means for diagnosis but also as a route to enhance understanding of the diversity and epidemiology of emerging bacteria and the infections they cause.

Despite these unique advantages, however, culture has been challenged by the recent development of genotype-based
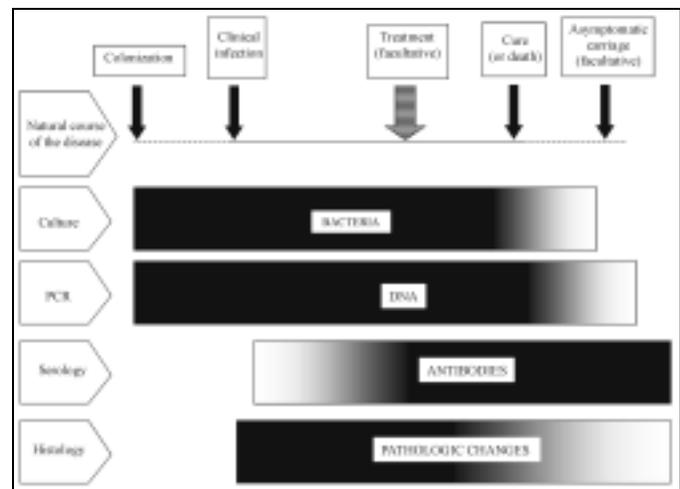
Figure 1. Diagram describing the respective places of culture-, polymerase chain reaction-, serology- and histology-based approaches for the diagnosis of acute bacterial infections, according to the natural course of the disease. Isolation and culture are possible as long as live bacteria are present in tissues, i.e., from the colonization to the treatment or to the end of the clinical manifestations (or shortly earlier). Bacterial DNA can be detected during the same period and also as far as dead microorganisms remain in tissues. Specific antibodies appear during the clinical course of the disease and persist generally for months or years. Pathologic changes can be observed soon after the contamination and, in an acute infection, will decline rapidly after elimination of the bacteria.

methods such as broad-range polymerase chain reaction (PCR) (3). Because culture as a tool is still threatened by the possible existence of uncultivatable organisms, several authors have emphasized the critical role that molecular, culture-independent techniques could play in further investigations of emerging infectious diseases, affirming that a reassessment of Koch's postulates for disease causation was required (4). What actually are the respective roles of these two techniques? Should we consider that broad-range PCR has made culture

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and traditional techniques obsolete, or is it only a step among others in the sequence of events leading to isolation of a new microorganism? To answer these questions, we examined the key steps that led to identification of most bacterial diseases that have been discovered during the last 20 years. Table 1 presents the main biological evidence that allowed emerging bacteria to be recognized and disease causation to be demonstrated. We examined the contribution of traditional and molecular techniques to understand their respective roles, and we emphasize the specific advantages of culture.

Traditional Techniques Other Than Culture: Microscopy and Serology

Optic Microscopy

Direct Detection in Smears

Historically, morphologic methods have played an important role in detecting new microorganisms, and they are still crucial for diagnosing infections caused by agents not routinely cultured, such as *Mycobacterium leprae* (40). Because microscopic examination of stained smears from biologic fluids or tissue imprints is usually rapid and easy, it has often been performed in patients who have an unexplained disease, although its interpretation is subjective and its sensitivity and specificity are generally low. The first evidence for the responsibility of *Ehrlichia* species in humans with an acute febrile illness was provided by examining blood smears stained with a Romanowsky stain, in which these as-yet-uncultivated organisms could be observed forming intracytoplasmic morulae within leukocytes (6,7). *Borrelia burgdorferi* were first observed in Giemsa-stained smears from midgut diverticula of ticks (19). Examination of smears can also be helpful when multiple organisms are cultured from a nonsterile site, as microbial culture alone, as well as molecular detection, cannot distinguish between colonization or asymptomatic shedding and tissue invasion: in such a situation, the morphology of the predominant organism visualized in the tissue sections can suggest the true causative agent (40).

Detection in Tissue Sections

Although individual bacteria generally are not detected in hematoxylin and eosin (H&E)-stained tissue sections, exceptions do exist. Clumps of finely particulate basophil material were seen in H&E-stained sections of bacillary angiomatosis and subsequently identified as *Bartonella* (41). In H&E-stained sections of gastric biopsy specimens that show acute gastritis, curved bacteria consistent with *Helicobacter pylori* may be seen in the layer of mucus on the crypt epithelium (25). Moreover, as histopathologic damage and causal microorganisms usually have a long-established association, microscopic examination of H&E-stained tissue sections during the course of an unexplained disease may lead to hypotheses about the nature of the etiologic agent (40).

Gram stain has also proven useful to routinely diagnose *H. pylori* and *H. heilmanii* in the gastric mucosa of patients with gastritis, as well as that of *B. henselae* in cardiac valves (10,24,25). Silver impregnation is among the most useful methods for detecting bacteria, especially for that stained weakly with a tissue Gram stain. Thus, bacillary angiomatosis lesions were found to contain clusters of bacilli on Warthin-Starry staining 2 years before the etiologic role of *B. henselae* was elucidated. With the same stain, this bacterium was also detected in cardiac valves of patients with endocarditis (Figure 2)(41). The first observation of Whipple agent was reported in 1907 by George Whipple in silver-stained sections of a lymph node, although the author did not link this observation with the cause of the disease (2).

Special stains have also played a role in establishing the etiologic role of new bacteria. Gimenez' and Pinkerton's stains allowed the detection of rickettsial organisms in tissue sections from patients with acute febrile disease (40). New mycobacteria were initially detected by using Ziehl-Nielsen, Kinyoun, or auramine O stains. For example, in an HIV-infected boy, examination of a retroperitoneal lymph node showed granuloma with large numbers of intracellular acid-fast bacilli that were later characterized as a new *Mycobacterium* species, *M. genavense* (34). Morphologic techniques, indeed, do not allow specific identification of the detected organisms. Despite this limitation, the approach consisting of detecting infectious lesions and agents by using cytologic and histologic examination appeared to be sometimes more valuable than the cultural or molecular techniques (40).

Electron Microscopy

Among morphologic techniques, transmission and scanning electron microscopy (EM) has substantial advantages resulting from its high flexibility and sensitivity (42). Negative staining is a rapid EM method that can be useful in patients with persisting or unexplained disease. Further, its specificity and sensitivity can be enhanced by using immunocapture assay. Thus, in patients with chronic gastritis, EM provided the first detection of *H. pylori* in the gastric mucosa (25). EM can resolve details many hundreds of time smaller than can be seen through light microscopes, and resolution of major taxonomic features can help to characterize new microorganisms (42). Thus, the agent of Whipple disease was recognized as a bacillus through ultrastructural examination of the bacilli (42). Nevertheless, limitations of EM include its availability, cost, and need for experienced staff. EM requires knowledge of histology and ultrastructure of the tissue being examined and organisms likely to be encountered and is very time-consuming, since every specimen must be examined individually (42).

Serology and Antigenic Detection

Serology

By showing rising antibody titers or seroconversion, serology can provide indirect evidence for causal relationships

Table 1. Key steps that led to identification and demonstration of disease causation for emerging bacteria[a]

Group	Species	Clinical picture	Histologic detection	Serology	Molecular detection (gene)	Culture system	Year of culture	Ref.
Alpha1 Proteobacteria								
	Ehrlichia chaffeensis	Fever, cytopenia	Smear	Antibodies to *Ehrlichia canis*	16S rRNA	Cell line (DH82)	1991	5
	E. ewingii	Fever, cytopenia	Smear	Western blot	16S rRNA	Cell line	1971	6
	Human granulocytic Ehrlichia	Fever, cytopenia	Smear	Antibodies to *E. phagocytophila, E. equis*	16S rRNA	Cell line (HeLa)	1996	7
	Rickettsia felis	Fever			gltA	Cell line (XTC-2)	2000	8
	R. japonica	Spotted fever		Antibodies to Spotted fever group rickettsiae		Cell line (Vero)	1989	9
	R. mongolotimonae	Febrile rash		Antibodies to Spotted fever group rickettsiae	rOmpA	Embryonated egg, guinea pig	1991	10
	R. slovaca	Fever, eschar, lymphadenitis		Specific antibodies	rOmpA	Cell line	1968	11
Alpha2 Proteobacteria								
	Afipia broomae	Wrist abscess				Axenic (specific)	1981	12
	A. clevelandensis	Osteitis				Axenic (specific)	1988	12
	Bartonella elizabethae	Endocarditis				Axenic (nonspecific)	1993	13
	B. grahamii	Neuro-retinitis		Antibodies to *B. henselae*	16S rRNA	Axenic (nonspecific)	1995	14
	B. henselae	Fever, cat scratch disease, bacillary angiomatosis	Tissue section	Specific antibodies	16S rRNA	Axenic (nonspecific)	1990	15,16
Beta Proteobacteria								
	Bordetella trematum	Chronic otitis				Axenic (nonspecific)	1996	17
	Neisseria weaveri	Infected wound				Axenic (nonspecific)	1993	18
Spirochetae								
	Borrelia burgdorferi sensu stricto, *B. afzelii, B. garinii*	Erythema chronicum migrans, acrodermatitis chronica atrophicans, Lyme arthritis, neuro-borreliosis		Specific antibodies		Axenic (specific)	1981	19
	B. duttonii	Relapsing fever	Smear	Specific antibodies		Axenic (specific), animal model	1999	20
	B. recurrentis	Relapsing fever	Smear			Axenic (specific)	1994	21
Delta-Xi Proteobacteria								
	Campylobacter coli, C. jejuni	Febrile diarrhea		Specific antibodies		Axenic (nonspecific)	1977	22
	Helicobacter cinaedi, H. fennelliae	Rectitis				Axenic (nonspecific)	1984	23
	H. heilmanii	Chronic gastritis	Tissue section			Mouse	1989	24
	H. pylori	Gastritis, gastroduodenal ulcer	Tissue section	Specific antibodies		Axenic (nonspecific)	1982	25
Gamma Proteobacteria								
	Escherichia coli O48:H21, O103:H2, O157:H7	Bloody diarrhea, HUS			slt	Axenic (nonspecific)	1982-1996	26
	Haemophilus influenzae biogroup *aegyptius*	Brazilian purpuric fever				Axenic (nonspecific)	1986	27

Table 1 cont'd. Key steps that led to identification and demonstration of disease causation for emerging bacteria[a]

Group	Species	Clinical picture	Histologic detection	Serology	Molecular detection (gene)	Culture system	Year of culture	Ref.
Gamma Proteobacteriae								
	Legionella anisa	Pneumonia, Pontiac fever		Specific antibodies		Axenic (specific)	1989	28
	L. bozemanii	Pneumonia	Smear	Specific antibodies		Axenic (specific)	1983	29
	L. dumoffii	Pneumonia	Smear	Specific antibodies		Axenic (specific)	1978	29
	L. feeleii	Pneumonia, Pontiac fever		Specific antibodies		Axenic (specific)	1986	30
	L. micdadei	Pneumonia		Specific antibodies		Embryonated egg, guinea pig	1979	29
	L. oakridgensis	Pneumonia	Smear	Specific antibodies		Axenic (specific)	1987	29
	L. pneumophila	Pneumonia	Tissue section	Specific antibodies		Embryonated egg, guinea pig	1947	31
	Legionella like amoebal pathogen	Pneumonia		Specific antibodies		Amoeba	1991	32
	Vibrio alginolyticus	Conjunctivitis, wound infection				Axenic (nonspecific)	1977	33
	V. cholerae O:139	Diarrhea				Axenic (nonspecific)	1992	33
	V. fluvialis	Diarrhea				Axenic (nonspecific)	1980	33
	V. furnissii	Diarrhea				Axenic (nonspecific)	1983	33
	V. metschnikovii	Cholecystitis				Axenic (nonspecific)	1981	33
	V. mimicus	Diarrhea, otitis				Axenic (nonspecific)	1981	33
Mycobacteria								
	Mycobacterium asiaticum	Pneumopathy				Axenic (specific)	1983	34
	M. celatum	Pneumopathy				Axenic (specific)	1992	34
	M. genavense	Disseminated infection, lymphadenitis	Tissue section			Axenic (specific)	1992	34
	M. malmoense	Pneumopathy, lymphadenitis				Axenic (specific)	1977	34
	M. simiae	Pneumopathy, osteitis, kidney infection				Axenic (specific)	1984	34
Mycoplasmas								
	M. fermentans	Pneumopathy, nephritis	Tissue section		Insertion sequence-like	Axenic (specific)	1993	35
	M. genitalium	Urethritis	Smear		Adhesion protein	Axenic (specific), Animal model	1981	35
Gram-positive bacteria								
	Tropheryma whipplei	Whipple disease	Tissue section	Specific antibodies	16S rRNA	Cell line (HEL)	2000	2,36
	Corynebacterium auris	Acute otitis				Axenic (nonspecific)	1995	37
	Staphylococcus lugdunensis, S. schleiferi	Skin abscess, osteoarthritis				Axenic (nonspecific)	1988	38
	Streptococcus iniae	Meningitis, endocarditis, cellulitis				Axenic (nonspecific)	1995	39

[a]Histologic detection can be performed with morphologic techniques, in blood or tissue smears, or in tissue sections. Serologic assays can detect specific antibodies to the suspected agent or to a related organism in tissues or in biological fluids. The year of the first isolation and the culture system used are indicated.
HUS = hemolytic uremic syndrome. HLE=human embryonic lung fibroblasts; ref = reference.
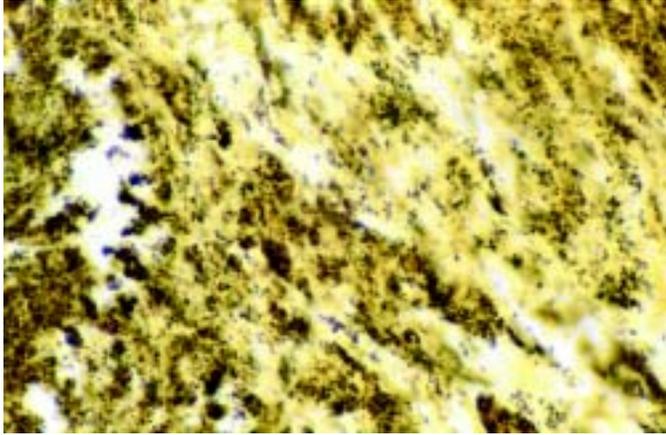
Figure 2. Demonstration of *Bartonella henselae* in cardiac valve of a patient with blood culture-negative endocarditis. The bacilli appear as black granulations (Warthin Starry, original magnification X250).

between a disease and a newly identified bacterium. Conversely, in the absence of serologic evidence, the role of a cultured organism should be interpreted cautiously, as shown by the example of *Afipia felis,* which was first thought to be the cause of cat-scratch disease, but was finally identified as a water contaminant (12,43). Serology is also useful to assess the involvement in human diseases of microorganisms that had been initially recovered from the environment, such as novel *Legionella* species, or from animal hosts, as for the tick-associated bacteria *Borellia burgdorferi* or *Rickettsia slovaca* (11,19,29). Further, serology is a valuable tool for exploring the disease spectrum of a bacterium. Thus, serologic testing contributed to the recognition of *B. henselae* as the main agent of cat-scratch disease (16), as well as implicating *Campylobacter jejuni* as a possible cause of Guillain-Barré syndrome (44).

Moreover, the contribution of serologic studies to the identification of new bacterial pathogens should not be underrated. Serologic cross-reactions are common between members of the same bacterial genus, and antibodies specific to a bacterial species can suggest the role of a closely related, still unidentified organism. Thus, specific antibodies to *Ehrlichia canis*, *E. phagocytophila*, and *E. equis*, then known only as veterinary pathogens, were detected in patients and led to description of the agents of human ehrlichioses (*E. chaffeensis*, *E. ewingii*, and human granulocytic ehrlichiosis) (5-7). Involvement of *Bartonella grahamii* in neuroretinitis was first suggested by detection of specific antibodies to *B. henselae* in the patient's blood (14). Reliable interpretation of such serologic cross-reactions, however, would not have been possible without considering other evidence, such as intraleukocytic morulae for ehrlichioses.

Antigenic Detection

Production of specific antibodies in experimental animal studies allowed immunochemical detection techniques to be developed. Direct immunofluorescence staining can be performed in smears in respiratory fluids of patients with pneumonia (29). Immunohistochemistry is useful for demonstrating

disease causation, as it provides evidence for in situ association between microorganisms and histologic structures. With this technique, *Tropheryma whipplei* was detected in a patient's mitral valve and later in intestinal mucosae (Figure 3) (2). Immunohistochemistry also suggested the role of *M. fermentans* in pulmonary infections (35). Immunologic techniques are dependent, however, on the availability of specific antibodies or antigens, which in most cases requires previous isolation of the agent; therefore, such techniques indirectly contribute to culture.

Culture: A Traditional Technique of Expanding Potential

Culture Media

Axenic Media

Broad-spectrum media allowed several previously unrecognized gram-positive bacteria, such as novel corynebacteria or *Staphylococcus* species, as well as novel beta-Proteobacteria, to be isolated, mainly from blood or pus of patients (18,37,38). The first isolation of *B. elizabethae, B. quintana*, and *B. henselae* was also achieved on blood agar (15). Use of *Campylobacter*-selective medium allowed novel *Campylobacter* and *Helicobacter* species to be grown from stools and rectal swabs, respectively (23), and provided further evidence for the association between *C. jejuni* infection and Guillain-Barré syndrome (44). For *Campylobacter* spp., selective, antibiotic-containing media could be satisfactorily replaced by nonselective blood agar, provided stool specimens had been filtered with a cellulose acetate membrane (23). Newly recognized serotypes of enterohemorrhagic *Escherichia coli* were isolated on MacConkey-sorbitol agar from stools or urine of patients with hemolytic-uremic syndrome (26). For *Vibrio cholerae* O:139 and most novel *Vibrio* species, the most convenient, highly selective medium was thiosulfate-citrate-bile salts sucrose agar (33).
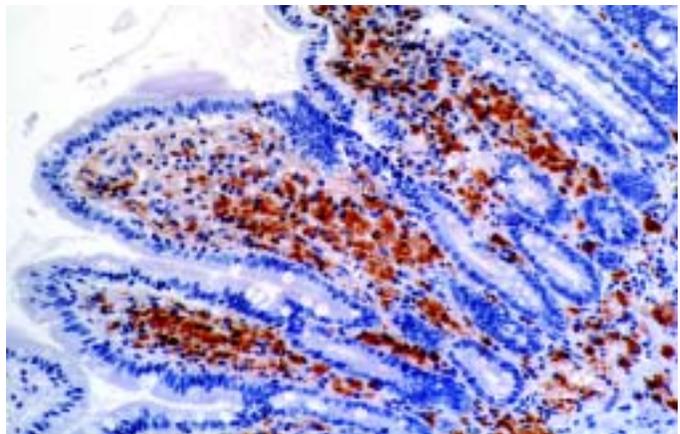
Figure 3. Demonstration of *Tropheryma whipplei* by immunohistochemistry in the lamina propria of the villous tips. Bacilli are revealed in foamy macrophage cytoplasm as red-brown deposits (polyclonal rabbit anti-*T. whipplei* antibody at a dilution of 1:500, hemalyn counterstain, original magnification X250).

The usefulness of broad-spectrum media should not obscure the fact that some emerging bacteria would not have been isolated without specific media. Buffered charcoal-yeast extract (BCYE) agar facilitated the recovery of most novel *Legionella* species, as well as *Afipia broomeae* and *A. clevelandensis,* from human respiratory sources (12,29) The first cultivation of *Borrelia burgdorferi* was achieved in 1981 in a modified Kelly medium (19). In 1994, 20 years after the first attempts, the Kelly growth medium itself allowed first cultivation of *B. recurrentis* from the blood of an Ethiopian patient with louse-borne relapsing fever, and *B. duttonii*, agent of East African tick-borne relapsing fever, was isolated for the first time in 1999 in BSK II medium (21). Generally, combining different types of medium, using both solid and liquid media, increases the effectiveness of culture, perhaps because of a preference of the bacterium for one type of medium over another or simply from the increased sensitivity obtained by culturing a large volume of specimen. For example, *B. elizabethae* and *B. henselae* were detected in BACTEC blood culture medium before inoculation in blood agar (13,15). Isolation of most novel *Mycobacterium* species required both solid- and liquid-specific media (34).

Living Systems

While more expensive and less easy to use than artificial media, animal models can provide certain advantages not available with artificial media. For example, until recently, inoculation to mice was the only means available to propagate *B. duttonii* (21). Today, animals are still necessary for isolating organisms such as *Treponema pallidum* or *Mycobacterium leprae*. Animal inoculation can help to reduce the contaminant flora. Thus, a combination of passage in guinea pigs and subsequent transfer into embryonated eggs was the key for isolating *L. pneumophila* from lung autopsy specimens (31). Embryonated eggs themselves have been recognized as a standard for rickettsial isolation, allowing, for example, the first isolation of Astrakhan fever rickettsia (45).

Cell culture is easy to use and may be very sensitive. Isolation of *T. whipplei* was obtained from valve and duodenal biopsy specimens by using human embryonic lung fibroblasts (HEL) (2,36). *Ehrlichia chaffeensis* and *R. japonica* were grown from blood samples of patients on canine macrophage cells (Figure 4) and African green monkey cells, respectively (9,46). Cultivation of facultative intracellular bacteria also was facilitated by cell culture. *L. pneumophila* has been isolated by using HEL cells while inoculated BCYE and agar plates remained sterile (47). With a bovine endothelial cell line, *B. quintana* was isolated for the first time from cutaneous biopsy material of a bacillary angiomatosis patient (48). Such enhanced sensitivity is a major advantage for an infection with low levels of bacteremia or when limited biopsy material is available (49). Indirectly, HEL cells also provided the first evidence for the role of a toxic factor in pseudomembranous colitis, which could be neutralized by clostridial antiserum. This
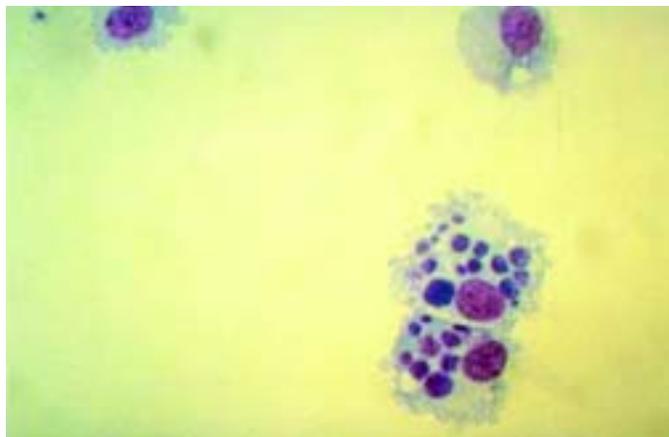
Figure 4. Canine monocytes (DH82) cultivated in vitro and heavily infected with *Ehrlichia chaffeensis*, as viewed by light microscopy after Giemsa staining. Typical ehrlichial inclusions (morulae) are observed within the cytoplasm of the infected cells (Giemsa, original magnification X600).

observation led to the discovery of *Clostridium difficile* as the responsible agent (50).

The search for appropriate media that could allow the growth of still uncultivatable or unrecognized bacteria has led us to try coculture with nonmammalian cells. Cell lines from toads (XTC-2) have been used in our laboratory to grow *Rickettsia felis*, a flea-associated *Rickettsia* pathogenic for humans (8). Coculture with arthropod cells will probably enhance our ability to detect intracellular, arthropod-transmitted bacteria. For example, tick cells (IDE8) have been used to grow the agent of human granulocytic ehrlichiosis (7). Cocultivation of samples with free-living amebae has allowed recovery of otherwise uncultivatable microorganisms from patients and the environment. This technique provided evidence for the role of several *Legionella* or *Legionella*-like species and of *Parachlamydia acanthamoeba* as etiologic agents of community-acquired pneumonia (51).

Other Critical Issues in Culture

In addition to the choice of an appropriate medium, the main critical issues in culturing concern inoculation of the specimen and incubation of the culture, both summarized in Table 2. Since successful culture usually results from the selection of a unique cultivatable clone, the quantity of injected pathogen should be as high as possible. Samples should be collected from anatomic sites that are likely to contain a high concentration of bacteria, and injection of a large volume of tissue sample is preferable. In patients with *Bartonella* endocarditis, the sensitivity of cell cultures has been shown to be higher when performed with valvular biopsy samples than with peripheral blood samples (49). These criteria, however, are not always feasible, as patients may reject, for instance, invasive explorations that are required to obtain the specimens. Arthropod-transmitted bacteria, which are often rare in infected human tissues, may be sometimes more easily recovered from samples collected from infected vectors; this

Table 2. Key issues for isolating main emerging bacteria

Group	Medium		Conditions for incubation		
	Axenic specific medium	Living system (embryonated egg, cell line)	Low temperature (<37°C)	O₂ and CO₂ conditions	Extended incubation
Alpha1 Proteobacteria		*Ehrlichia* sp. *Rickettsia* sp. *Chlamydia* sp.	ELB agent ("*Rickettsia felis*") (28°C)		*Ehrlichia* sp. *Rickettsia* sp.
Alpha2 Proteobacteria	*Afipia* sp.	*Afipia* sp. *Bartonella* sp.	*Bartonella bacilliformis* (28°C)		*Bartonella* sp.
Spirochetae	*Borrelia* sp.		*Treponema pallidum*		
Delta-Xi Proteobacteria				*Campylobacter* sp. (microaerophilic) *Helicobacter* sp. (microaerophilic)	*Helicobacter pylori*
Gamma Proteobacteria	*Legionella* sp.	*Legionella* sp.	*Yersinia pestis*		
Mycobacteria	*Mycobacterium* sp.		*Mycobacterium leprae*	*Mycobacterium malmoense* (microaerophilic)	*Mycobacterium* sp.
Mycoplasmas	*Mycoplasma* sp.				*Mycoplasma fermentans*
Gram-positive bacteria		*Tropheryma whipplei*		*Clostridium difficile* (anaerobic)	*Tropheryma whipplei*

was the key leading to the identification of *B. burgdorferi* (19). If initially such a result was insufficient in a clinical diagnostic approach, it has since led to efficient serologic and molecular tools, which would not have been available without culture. For intracellular bacteria, the use of a lysis method for eukaryotic cells before inoculation substantially enhances the ability to grow the organisms, especially when inoculation is performed in an axenic media, as for *Bartonella* or *Mycobacterium* species (15). Since low-speed centrifugation may also increase infectivity, the centrifugation-shell vial technique for isolating cytomegalovirus has been adapted to detect intracellular bacteria and used successfully to cultivate *Rickettsia* species from blood and skin biopsies and *T. whipplei* from the mitral valve of a patient with endocarditis (2).

Special attention should be accorded to the duration, temperature, and atmosphere of incubation. For some of the most important newly discovered pathogens, such as *H. pylori*, patience has been a key to successful cultivation (25). With *T. whipplei*, the first evidence of cytopathic effect and microorganisms did not occur until day 65 after inoculation (2). Isolation of *Bartonella henselae* from blood or tissue samples from infected patients required up to 33 days' incubation (15,49). Although most pathogenic bacteria have been cultured at 35°C to 37°C, which is close to the physiologic temperature of the human body, several pathogens need a lower temperature. In addition to well-known examples such as *M. leprae* and *Treponema pallidum*, several arthropod-borne pathogens, including arboviruses, *Yersinia pestis*, *B. bacilliformis*, or *R. felis* may be more easily cultivated at ≤32°C (8).

A More Recent Technique: 16S rDNA Amplification and Sequencing

With the use of universal primers that recognize highly conservative loci such as the 16S rDNA encoding gene, spe-cies-specific sequences can be amplified directly from diseased host tissues and compared with a reference-sequence database to infer phylogenetic relationships (3,4). This broad-range PCR technique has expanded the ability of laboratories to partially characterize organisms that had never been cultured. Thus, in the last decade, it has enabled two unexplained illnesses to be associated with novel etiologic agents: *B. henselae* in bacillary angiomatosis and 1 year later *T. whipplei* in patients with Whipple disease (52,53). These remarkable successes of molecular techniques, however, should not obscure the fact that a bacterial origin was previously established for both diseases on the basis of histologic studies and clinical responses to antimicrobial treatment (2,48). Further, isolation and culture were achieved at the same time as molecular identification (for *B. henselae*) or soon after (*for T. whipplei*) (2,15). In both cases, successful isolation resulted from laboratory practices generally used to enhance the detection of fastidious pathogens. Although it has been suggested that specific culture conditions could be inferred from molecular phylogenetic data, such a situation has never occurred for any bacterium (3). These examples suggest, therefore, that molecular techniques are particularly useful for taxonomic studies and identification, while traditional methods remain powerful to detect pathogens.

For viruses, several species, such as the *Sin Nombre virus* (SNV) or the *Hepatitis C virus* (HCV), were detected by reverse transcriptase PCR before any morphologic, serologic, or cultural detection. Although SNV was subsequently cultured in vitro, the HCV agent has only been cultured recently in chimeric mice (54). Because of its high sensitivity, broad-range PCR also expands the ability to detect organisms present in very low quantity and those that are difficult to grow, such as intracellular bacteria. *Ehrlichia ewingii*, previously known as a canine parasite, was detected by this technique in circulat-

ing leukocytes of four patients with febrile illness. Note, however, that morulae had been identified in neutrophils from two of the four patients, providing strong evidence for an ehrlichial origin for the disease, and that serologic evidence was reported before the PCR assays (6). Advantages of molecular techniques seem more obvious for *Bartonella grahamii* and *B. vinsonii* subsp. *berkhoffii*, which have been implicated in human disease solely on the basis of 16S rDNA amplification and sequencing (14,55). Molecular tools are also particularly useful in diseases associated with dormant or latent organisms, such as chronic Lyme arthritis, and for which the sensitivity of culture from body fluids remains very low (4,). The advantages of broad-range PCR, however, are offset by the problem of microbial DNA contamination. Even after rigorous technical precautions are taken to minimize contamination of PCR reaction, false-positive reactions can occur. Another noticeable limitation of broad-range PCR is the examination of sites that are not normally sterile, such as feces or sputum; use of family-restricted primers, in situ hybridization with specific nucleic probes, or expression library screening with immune sera may help to overcome such limitations (3,4). Another potential problem is interpretation of the microheterogeneity found in microbial sequences derived directly from host tissues, especially when these sequences become the sole basis for defining the existence of an organism. For example, attempts to characterize and classify nanobacteria using 16S rDNA sequence analysis provided doubtful results, and these organisms were later considered contamination (56). Additionally, current databases contain an insufficient number of entries with which to define species and other taxon boundaries over a wide range of microorganisms (3).

Advantages of Culture for the Study of Emerging Bacterial Diseases

Antibiotic Susceptibility Testing

When culture and isolation are achieved, susceptibility of emerging bacteria to a large panel of antimicrobial drugs can be easily tested, providing essential data to guide clinical treatment, particularly when resistant strains are reported and empiric therapy may be ineffective. This antimicrobial testing would have been difficult, if not impossible, with molecular techniques, as genetic determinants of antibiotic resistance have been identified in only a few situations (57). Thus, isolation of *H. pylori* has revolutionized the treatment of duodenal ulcers, which are now definitively healed by appropriate antimicrobial regimens. As strains resistant to either metronidazole or clarithromycin have been increasingly reported, culture of the agent is very helpful in case of proven treatment failure, to assess the antibiotic resistance pattern of local strains of *H. pylori* (58). Coculture of bacteria with cell lines has brought new insights about antibiotic susceptibility patterns for obligate and facultative intracellular organisms. For example, while patients with human ehrlichiosis have been treated for a long time, with variable results, with chloramphenicol, in vitro

studies showed that *E. chaffeensis* was resistant to this antibiotic (59).

Experimental Animal Models for Pathogenicity

With viable microorganisms, disease models can often be established in animals. Rodent models are the most commonly used. For *Legionella oakridgensis*, originally isolated from industrial cooling towers, demonstration of its pathogenicity for guinea pigs suggested for the first time, before any clinical involvement, that it might be an unrecognized human pathogen (29). For assessing the capability of various *Vibrio* species to elaborate an enterotoxin, rabbit and mouse intestinal models were used (33). Human tissues can also now be maintained in immunodeficient mice (SCID-hu), which can then serve as useful models for human host-specific pathogens (56,60). Although less accessible, primate models supported, for example, the implication of *Mycoplasma genitalium* in genital tract infections (35). Finally, experimental animal models are useful for immunization studies, as for *H. pylori* in mouse and primate models. Following culture, immunodominant antigens can be cloned, expressed, and inoculated to animals to identify candidate vaccines (61).

Genetic Studies
Isolated Genes

For noncultured organisms, molecular techniques have been proposed to identify isolated bacterial genes directly from clinical specimens. These techniques, however, are quite difficult to use and can identify only a few, short genetic fragments (3). On the other hand, by providing pure microbial cell mass, culture enables genes to be identified in high numbers through recombinant chromosomal libraries built from the extracted DNA. Genes identified in this fashion can then be utilized as more refined diagnostic tools. For example, *Rickettsia mongolotimonae* and *R. slovaca* were associated with human disease on the basis of amplification of a species-specific *rOmpA* gene fragment from skin biopsy specimens (11,12). DNA probes developed after isolation of *Chlamydia pneumoniae* enabled this organism to be detected by in situ hybridization in coronary atherosclerotic plaques (62). Further, molecular subtyping of cultured strains has offered new perspectives for epidemiologic studies. Thus, comparison of nucleotide sequences of 16S rDNA, *OspA,* and *Fla* genes for different strains of *B. burgdorferi* provided phylogenetic data that consistently supported the division of *B. burgdorferi* sensu lato into three geographically distinct genotypes, which were subsequently shown to have different pathogenic potentials (63). Correlation between genotypes and biologic characters is a key to understanding the pathophysiology of bacterial diseases.

Complete Genome Sequence

Because of the importance of organisms such as *H. pylori, M. genitalium,* and *C. pneumoniae* as emerging human pathogens and the value of complete genome sequence information for drug discovery and vaccine development, the complete

nucleotide sequences of these three organisms has been determined by the whole-genome random sequencing method as described initially for *Haemophilus influenzae*. Sequence analyses allowed identification of several predicted coding regions that included genes required for DNA replication, transcription and translation, DNA repair, cellular transport, and energy metabolism (64). With the availability of complete genome sequences, further assessment of microbial genetic diversity is possible; based on the large number of sequence-related genes encoding outer membrane proteins, *H. pylori* was predicted to use recombination as a mechanism for antigenic variation and adaptative evolution (65). As the genome sequences of new bacterial species or strains are determined, comparative genomics will be an increasingly useful method to provide insights into physiologic differences among microorganisms (64).

Conclusion

A comprehensive study of the histories of emerging bacterial diseases provided new insights into the respective roles played by the different identification techniques. Because of the spectacular development of molecular methods, traditional techniques have been prematurely considered obsolescent. We hope to have shown, however, that such a statement does not reflect the real contribution of these techniques. The undoubted value of novel molecular methods, especially for rapid bacterial detection and phylogenetic studies, should not hide the crucial role that traditional techniques have historically played. Moreover, these traditional techniques have never stopped evolving towards increased sensitivity and specificity. Today, these techniques appear complementary. If broad-range PCR was helpful in determining the taxonomic position of new, still uncultured organisms, most of the novel infectious diseases were finally described after culture and isolation of the responsible agents. In the current, fast-changing world of emerging infections, fulfillment of Koch's postulates, which requires culture, remains a very necessary model of rigorous proof and scientific thinking (1). Culture is still an irreplaceable key for studying emerging bacterial diseases, even if routine diagnosis can be efficiently achieved by using other (although generally culture-derived) tools, including genetic amplification. The history of infectious diseases shows that no human bacterial pathogen is uncultivable so far: the real issue seems to be whether we are able to determine the environmental conditions required by prokaryotic agents for growth (2).

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Current Status of Antimicrobial Resistance in Taiwan

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While some trends in antimicrobial resistance rates are universal, others appear to be unique for specific regions. In Taiwan, the strikingly high prevalence of resistance to macrolides and streptogramin in clinical isolates of gram-positive bacteria correlates with the widespread use of these agents in the medical and farming communities, respectively. The relatively low rate of enterococci that are resistant to glycopeptide does not parallel the high use of glycopeptides and extended-spectrum beta-lactams in hospitals. The evolving problem of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates is substantial, and some unique enzymes have been found. Recently, some gram-negative bacteria (e.g., *Pseudomonas aeruginosa* and *Acinetobacter baumannii)* that are resistant to all available antimicrobial agents including carbapenems have emerged.

Antimicrobial resistance has become a major health problem worldwide, affecting every country to some degree. It is an inevitable consequence of the inappropriate use of antibiotics in humans and animals. In Europe and North America, methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-nonsusceptible *Streptococcus pneumoniae* (PNSSP), vancomycin-resistant enterococci (VRE), and extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* have emerged and spread into communities and hospitals. In Taiwan, the widespread use of antimicrobial agents in primary care clinics and animal husbandry has allowed the rapid emergence of resistant bacteria. During the last 2 decades, many antimicrobial agents—such as extended-spectrum cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides—have been introduced and empirically used as first-line drugs to treat these resistant bacteria (1,2). This has further accelerated the development and dissemination of drug-resistant bacteria. Previous studies in Taiwan have clearly demonstrated the remarkably high prevalence of some critically resistant bacteria, such as MRSA, PNSSP, and macrolide-resistant streptococci (1,2). In addition, several multidrug-resistant bacteria, including ones resistant to carbapenems and fluoroquinolones and pan-drug-resistant gram-negative bacilli, have been isolated from different hospitals (3-6).

Approval of Antibiotics

Table 1 shows the years that selected antibiotics were approved in Taiwan. These antibiotics are now widely used to treat various infections, including community-acquired and nosocomial infections. Until now, two gylcopeptides (vancomycin and teicoplanin), two carbapenems (imipenem and meropenem), four macrolides (erythromycin, roxithromycin, clarithromycin, and azithromycin), and six quinolones (nalidixic acid, norfloxacin, ofloxacin, lomefloxacin, ciprofloxacin, and levofloxacin) have been available for clinical use in Tai-

wan. Most of these drugs were also readily available at drugstores without prescription before 1995.

Drug-Resistant Bacteria

The following drug-resistance data were collected from a nationwide resistance survey (Surveillance from Multicenter Antimicrobial Resistance in Taiwan) of clinical isolates (including those recovered from hospitals and outpatients) from 12 major hospitals as well as isolates causing nosocomial infections from National Taiwan University Hospital (NTUH) in 2000 in Taiwan. These hospitals are located in different parts of the country. The number of beds in these hospitals ranged from 800 to 3,200. All these data were derived by using the disk-diffusion method (7).

Some dilution antimicrobial susceptibility and epidemiology studies, including ≥100 strains published in English-language journals from January 1995 through 2001, were also included. Rather than provide a comprehensive review of all resistance problems in Taiwan, our aim was to point out some of the more critical resistance problems threatening the treatment of infections caused by *Staphylococcus* species, *S. pyogenes*, *Streptococcus pneumoniae*, *Enterococcus* species, and

Table 1. Year of approval of selected antimicrobial agents in Taiwan

Antimicrobial agent	Year of approval
Erythromycin	1968
Oxacillin	1970
Gentamicin	1981
Cefotaxime	1983
Amikacin	1986
Ceftazidime	1988
Imipenem	1988
Vancomycin	1983
Ciprofloxacin	1990
Cefepime	1997

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Mycobacterium tuberculosis among the gram-positive pathogens, and *Haemophilus influenzae, Escherichia coli, Klebsiella pneumoniae, Enterobacter* species, *Salmonella* species, *Campylobacter* species, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* among the gram-negative pathogens. Resistance rates included in this review reflect both intermediate and fully resistant populations. Table 2 summarizes the prevalence of antimicrobial resistance among clinical isolates (12 hospitals, including NTUH) and nosocomial isolates (from NTUH only) of some selected bacterial species. The ranges in numbers of clinical isolates of select bacteria (Table 2) recovered from these hospitals were as follows: *Staphylococcus aureus*, 1,889 to 7,516 isolates; beta-hemolytic streptococci, 335 to 1,102; *S. pneumoniae*, 138 to 461; enterococci, 509 to 3,676; *H. influenzae*, 427 to 602; *E. coli*, 1,734 to 9,553; *K. pneumoniae*, 950 to 3,226; *E. cloacae*, 427 to 1,426; nontyphoid *Salmonella*, 94 to 626; *P. aeruginosa*, 1,741 to 4,896; and *A. baumannii*, 896 to 2,434.

Gram-Positive Bacteria

MRSA

MRSA was first documented in Taiwan in the early 1980s (8). Since then, there has been a remarkable increase in prevalence of MRSA in nosocomial infections (from 26.7% in 1990 to 75% to 84% in 1998-2000) (9). Several dominant clones have been documented in hospitals (9). The prevalence of MRSA in community-acquired infections remains unclear, although the incidence of MRSA among patients of outpatient departments is estimated to be 40% (1). Data from a survey of >5,000 clinical isolates of *S. aureus* at the NTUH from January 1999 to June 2001 using brain-heart-infusion agar plus 4 mg/L of vancomycin showed results negative for vancomycin-intermediate or -resistant strains.

PNSSP and Multidrug-Resistant *Streptococcus pneumoniae* (MDRSP)

The overall prevalence of clinical isolates of PNSSP in 1999-2000 was 60% to 80%, including 20% to 30% penicillin-intermediate and 40% to 50% penicillin-resistant strains (10-16). This prevalence of PNSSP was slightly lower than that in Korea and higher than that in most other geographic areas (15,16). All PNSSP were resistant to multiple antibiotics (13,16). This resistance was higher among nasopharyngeal isolates from children (12). Approximately 60% of the PNSSP isolates were also not susceptible to extended-spectrum cephalosporins and carbapenems (13). Most of these PNSSP belong to serotypes 23F, 19F, 6B, and 14 (13,15). Wide dissemination of multiple high-level penicillin-, extended-spectrum cephalosporin-, and macrolide-resistant clones as well as the Spain 23F clone contributes to the high rates of resistance to these drugs in clinical isolates of *S. pneumoniae* (14,17). Only one clinical isolate was reported to be resistant to fluoroquinolones (18).

Table 2. Prevalence of antimicrobial resistance in selected bacteria (all clinical isolates) isolated from 12 major hospitals, including National Taiwan University Hospital (NTUH), in Taiwan in 2000 and in all clinical isolates and isolates causing nosocomial infections from NTUH in 2000[a]

Resistant pathogen	% of isolates	
	2,000 (12 hospitals) (clinical)	2,000 (NTUH) (clinical/nosocomial)
Methicillin-resistant *Staphylococcus aureus*	53-83	65/74
Erythromycin-resistant beta-hemolytic streptococci	30-51	34/–
Penicillin-nonsusceptible *Streptococcus pneumoniae*	60-84	77/–
Erythromycin-resistant *S. pneumoniae*	67-100	89/–
Gentamicin-resistant (high-level) enterococci	36-54	48/54
Vancomycin-resistant enterococci	1-3	3/2
Ampicillin-resistant *H. influenzae*	45-73	61/–
Cefotaxime-resistant *Escherichia coli*	5-19	12/19
Ciprofloxacin-resistant *E. coli*	11-33	20/29
Cefotaxime-resistant *Klebsiella pneumoniae*	4-34	9/18
Ciprofloxacin-resistant *K. pneumoniae*	5-33	9/16
Cefotaxime-resistant *E. cloacae*	36-68	45/49
Ampicillin-resistant nontyphoid *Salmonella*	44-69	56/–
Cefotaxime-resistant nontyphoid *Salmonella*	1-4	2/–
Quinolone resistant non-typhi *Salmonella*	0-16	0/–
Ceftazidime-resistant *Pseudomonas aeruginosa*	4-21	13/10
Imipenem-resistant *P. aeruginosa*	3-16	14/10
Ciprofloxacin-resistant *P. aeruginosa*	10-36	15/10
Imipenem-resistant *Acinetobacter baumannii*	0-19	19/16
Ciprofloxacin-resistant *A. baumannii*	54-74	54/42

[a]Susceptibility of these bacteria was determined by the standard disk-diffusion method.

VRE and Glycopeptide-Resistant Staphylococci

The first clinical isolate of Van-A-phenotype VRE (*E. faecalis*) was found in 1995 (19). Since then, isolation of VRE remains rare and accounts for <3% of all clinical isolates of enterococci (20,21). The proportion of *Enterococcus* hospital isolates resistant to vancomycin in Taiwan is low compared with those in North America and Europe (22), a finding that needs further investigation. However, an increase in VRE

isolation associated with the continuous widespread use of glycopeptides in a Taiwanese university hospital was observed (23). Furthermore, interhospital and nosocomial spread of some VRE clones, particularly one *vanB2 E. faecium* clone, or long-term persistence of multiple clones in hospitalized patients still exists (21,24). Although avoparcin has been approved for veterinary use since 1977, this agent has been banned in the farming industry since 2000 (24). Glycopeptide resistance has been found in some isolates of coagulase-negative staphylococci, particularly in *S. simulans* and *S. warneri* (25).

Macrolide-Resistant Streptococci

Under the increasing and highly selective pressure of macrolide usage in Taiwan, the prevalence of macrolide resistance and distribution of M-phenotype (*mef* gene-positive) among macrolide-resistant isolates vary among different streptococcal species (Figure) (26-31). More than 90% of the *S. pneumoniae* isolates were resistant to macrolides, and approximately two thirds exhibited high-level resistance (MLS$_B$ phenotype-*erm*
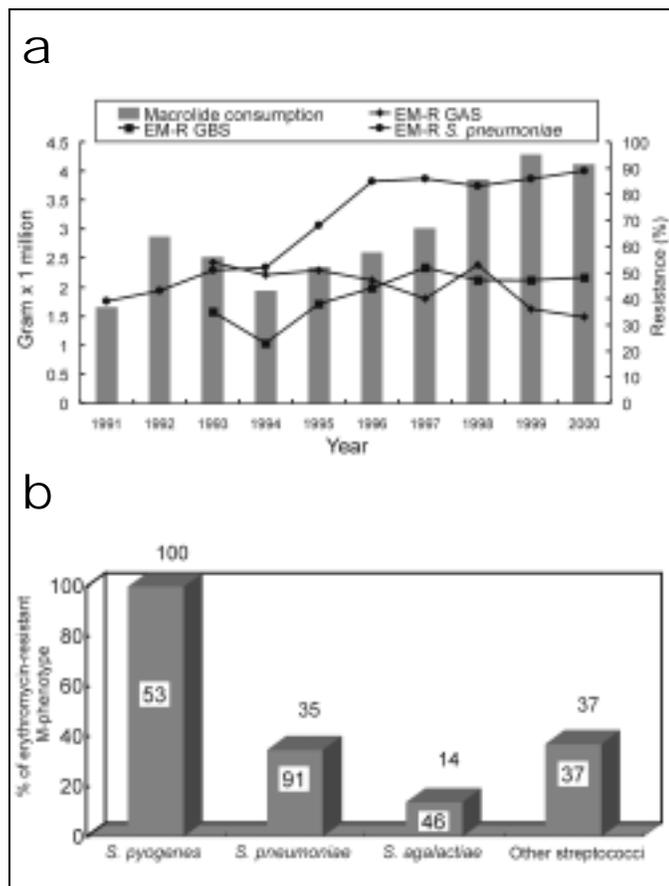
Figure. A, Macrolides consumption (grams x 1,000,000) in Taiwan and the trends of erythromycin-resistant group A *Streptococcus* (EM-R GAS), group B *Streptococcus* (EM-R GBS), and *S. pneumoniae* in National Taiwan University Hospital from 1991 to 2000. Macrolides include intravenous and oral forms of erythromycin and oral forms of clarithromycin, roxithromycin, and azithromycin. B,. Distribution of erythromycin-resistant M-phenotype among isolates of streptococci. Other streptococci include Groups C, F, and G, and viridans group streptococci. Number in each bar indicates the percentage of erythromycin-resistant isolates. Number above each bar indicates the percentage of M-phenotype among erythromycin-resistant isolates.

gene-positive) (29). However, macrolide resistance accounted for 50% to 60% of all clinical isolates of *S. pyogenes,* and a stepwise increase of proportion of M phenotype was clearly demonstrated (29).

Streptogramin-Resistant Gram-Positive Cocci

Quinupristin-dalfopristin is not available for clinical use in Taiwan; nevertheless, the incidence of resistance to this agent was high (51%) in vancomycin-resistant *E. faecium* (25). Three resistant *E. faecium* isolates were recovered from animal sources (pigs) in Taiwan. Restricted use of virginiamycin, which has been widely used in animal feed for >20 years in this country, might be required to alleviate quinupristin-dalfopristin resistance among bacteria from human sources (25).

Multidrug-Resistant *Mycobacterium tuberculosis* (MDRTB)

The prevalence of pulmonary tuberculosis (TB) in adults was 0.65% in 1993, and the associated death rate was 6.93 per 100,000 in 1998 (32). The overall incidence of isoniazid-resistant *M. tuberculosis* was 31.5%. The incidence of primary resistance (isolates from patients with newly diagnosed TB who had no prior history of anti-TB therapy or from patients whose anti-TB therapy was begun <2 weeks) was 12.0%; the incidence of acquired resistance (isolates from patients who had a prior history of anti-TB medication) was 63.0%. The overall incidence of MDRTB was 17.3% (primary resistance 1.6%; acquired resistance 46%) (33). An aggressive intervention program, such as expanded use of directly observed therapy, short course, is ongoing to improve the cure rate of TB and to decrease the resistance rate.

Gram-Negative Bacilli

H. influenzae and *Moraxella catarrhalis*

The annual incidence of invasive *H. influenzae* type b disease in children <5 years old was 1.6 to 1.9 per 100,000 population per year before the introduction of conjugated Hib vaccine in 1995 (34). Beta-lactamase production was found in 50% to 60% of *H. influenzae* and in >95% of *M. catarrhalis.* BRO-1 isoform accounts for 88% of all beta-lactamase producers of *M. catarrhalis* (16,35,36). Among amoxicillin-resistant *H. influenzae* isolates, beta-lactamase nonproducers were rare (<2%) (16). A continuing upsurge of *H. influenzae* isolates resistant to macrolide (30%) and to trimethoprim-sulfamethoxazole (50%) during the last decade has become evident (16,35).

Enterobacteriaceae

The proportion of isolates of *K. pneumoniae* exhibiting the ESBL phenotype has increased progressively from 3.4% in 1993 to 10.3% in 1997 in NTUH (37). Approximately one fifth of the ESBL-producing *K. pneumoniae* were also resistant to ciprofloxacin (37). From 1998 through 2000, several reports from different hospitals showed that ESBL production

accounts for 8% to 30% of clinical isolates of *K. pneumoniae*. Those producing SHV-5 and SHV-12 predominated. In addition, four novel beta-lactamases (CMY-8, SHV-25, SHV-26, and IMP-8) were identified in 2000 in Taiwan (38-42). Among the ESBL-producing *E. coli* isolates, which accounted for 1.6% to 6.7%, strains having CTX-M-3 and CMY-M-2 were disseminated in Taiwan (39,43). In Taiwan, the previous belief that characteristically susceptible strains (uniformly susceptible to cephalosporins) of *K. pneumoniae* caused primary liver abscess, an endemic disease entity in patients with diabetes mellitus, has now been disproved because two cephalosporin-resistant *K. pneumoniae* strains causing primary liver abscess have been found (44-46).

More than 40% of clinical isolates of nontyphoid *Salmonella* species were resistant to multiple antibiotics (ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole). Resistance to cefotaxime and fluoroquinolones was estimated to be low (1% to 3%) (47).

P. aeruginosa, A. baumannii, and Other Bacteria

P. aeruginosa, A. baumannii, and other nonfermentative gram-negative bacilli are usually resistant to various antimicrobial agents. A high proportion of clinical isolates, particularly those recovered from patients in intensive-care units, that are resistant to some last-line agents (ceftazidime, amikacin, ciprofloxacin, and carbapenems) have now been found in Taiwan (3-6,48,49). A small outbreak of infections (three patients) caused by a pan-drug-resistant *P. aeruginosa* (serogroup O:4) clone in an intensive-care burn unit from April 1997 to May 1997 has been identified (3) This clone had been isolated from a patient on the same unit 5 months before the outbreak (3). Among *P. aeruginosa* isolates with reduced susceptibilities to imipenem, VIM-2 and VIM-3 are the predominant metallo-beta-lactamases (50). Furthermore, clonal dissemination of VIM-3-producing *P. aeruginosa* has been found among hospitals in Taiwan (50). Strains of ceftazidime- and ciprofloxacin-resistant *A. baumannii* causing severe community-acquired pneumonia have emerged (49). Infections caused by *Chryseobacterium indologenes*, a multidrug-resistant nosocomial pathogen, appear to be another emerging problem in Taiwan (5). Isolates of the *Chryseobacterium* genus have remarkable discrepancies of susceptibility results by the disk-diffusion and dilution method. Vancomycin is not recommended as a drug of choice for treating *C. meningosepticum* meningitis or other infections caused by *Chryseobacterium* species because these isolates are highly resistant to vancomycin when the standard agar dilution method is used (4).

Several multidrug-resistant (extended-spectrum cephalosporins, ciprofloxacin, or carbapenem resistance) *Aeromonas* species have been reported (51,52). A derepressed mutant of *A. hydrophila,* which overexpresses beta-lactamases and shows resistance to extended-spectrum cephalosporins, is used if treatment with cefotaxime for *Aeromonas* bacteremia fails (52). High prevalence of ciprofloxacin resistance for human isolates of *Campylobacter jejuni* (52%) and *C. coli* (75%) may be attributable to the widespread use of quinolones in poultry in Taiwan (53,54).

Strategy for Resistance Control in the 21st Century

By the end of the 20th century, many measures to control resistance problems had been instituted in Taiwan. Antibiotics had been removed from the list of available nonprescription drugs at drugstores. Antibiotic interventions had been implemented in many hospitals, particularly in intensive-care units, to alleviate the high prevalence of resistance among nosocomial pathogens. In 2000, the Council of Agriculture in Taiwan prohibited the use of several antimicrobial agents (such as avoparcin, kanamycin, kitasamycin, lasalocid, spiramycin, salinomycin, and streptomycin), which had been widely used as growth promoters or prophylactic agents in animal husbandry in Taiwan during the past 2 to 3 decades, because they may select for critical forms of resistance in human pathogens in food-producing animals (54). Further research is ongoing to reduce the risk for increasing resistance in human pathogens caused by antibiotic use in animal husbandry. In the new millennium, the Center for Disease Control, Department of Health, in Taiwan, has made control of antimicrobial resistance a major goal. The two main tasks are to restrict use of antibiotics for trivial upper respiratory tract infections and to avoid inappropriate use of antibiotics for surgical prophylaxis.

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An Outbreak of Rift Valley Fever in Northeastern Kenya, 1997–98

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In December 1997, 170 hemorrhagic fever-associated deaths were reported in Garissa District, Kenya. Laboratory testing identified evidence of acute *Rift Valley fever virus* (RVFV). Of the 171 persons enrolled in a cross-sectional study, 31(18%) were anti-RVFV immunoglobulin (Ig) M positive. An age-adjusted IgM antibody prevalence of 14% was estimated for the district. We estimate approximately 27,500 infections occurred in Garissa District, making this the largest recorded outbreak of RVFV in East Africa. In multivariable analysis, contact with sheep body fluids and sheltering livestock in one's home were significantly associated with infection. Direct contact with animals, particularly contact with sheep body fluids, was the most important modifiable risk factor for RVFV infection. Public education during epizootics may reduce human illness and deaths associated with future outbreaks.

Rift Valley fever virus (RVFV) is a zoonosis that can cause epizootics and associated human epidemics in Africa (1). RVFV is a member of the family *Bunyaviridae*, genus *Phlebovirus*. Epizootics of RVFV occur periodically after heavy rains that flood natural depressions in the grasslands of sub-Saharan Africa (2). Flooding allows for the hatching of the primary vector and reservoir, multiple species of mosquitoes known as floodwater *Aedes*, which feed on nearby mammals (3,4). High levels of viremia in these animals lead to infection of secondary arthropod vector species and to subsequent infection of other mammals and livestock, in which it causes abortions and death in susceptible animals (5-7).

Human infection with RVFV was first reported soon after Daubney and colleagues isolated the virus in 1930 (8). Extensive human disease outbreaks were not reported until 1951, however, when an estimated 20,000 persons were infected during an epizootic of cattle and sheep in South Africa (9). Outbreaks were reported exclusively from sub-Saharan Africa until 1977-78, when 18,000 persons were infected and 598 deaths were reported in Egypt (10). Transmission of the virus to humans is thought to occur by arthropod vectors, aerosols of blood or amniotic fluid, or other direct contact with infected animals. RVFV in humans manifests a broad spectrum of disease, from asymptomatic infection to a benign febrile illness, to a severe illness in approximately 1%-3% of cases that can include retinitis, encephalitis, and hemorrhagic fever (11,12).

In addition to the human illness, disability, and suffering, RVFV outbreaks can result in devastating economic losses when livestock in an agricultural society are affected (13,14).

The normalized difference vegetation index has a linear relationship to rainfall in semiarid regions such as East Africa. Figure 1 depicts the vegetation development (as sensed by orbiting satellites) in late 1997, indicating elevated rainfall and the potential for RVFV outbreaks (15). In December 1997, the Kenyan Ministry of Health (MOH) and the World Health Organization (WHO) in Nairobi received reports of unexplained deaths in the North Eastern Province of Kenya and southern Somalia. Clinical features included an acute onset of fever and headache often associated with hemorrhage (hematochezia, hematemesis, and bleeding from other mucosal sites). Farmers and local veterinary health officials also reported high rates of spontaneous abortion and death among domestic livestock. Active surveillance conducted by the WHO, the Kenya MOH, and international relief organizations during December 22 to 28 in 18 villages in Garissa District (population 231,022), North Eastern Province, Kenya, identified 170 human deaths, reportedly from a hemorrhagic fever. No clinical specimens were available from the persons who

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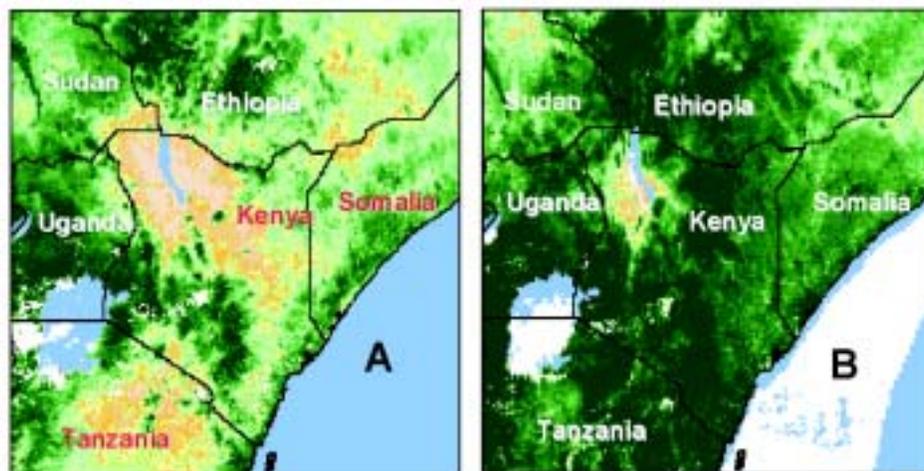
Figure 1. Images from advanced, very high resolution radiometer instrument on a National Oceanic and Atmospheric Administration satellite comparing normalized difference vegetation index data (as a surrogate for rainfall), from December 1996 (A) and December 1997 (B). Increasing vegetation is depicted from tan to yellow [predominating in part (a)], to light and dark green [predominating in (b)].

had died. However, of 36 blood samples obtained from other ill persons in these villages that were tested at the National Institute for Virology and at the Centers for Disease Control and Prevention (CDC), 17 (47%) had evidence of acute infection with RVFV by detection of IgM antibodies, virus isolation, or reverse-transcriptase-polymerase chain reaction (RT-PCR) for viral nucleic acid.

After RVFV was identified as the primary cause of this outbreak, an international task force led by the Kenya MOH established surveillance for hemorrhagic fever and other severe manifestations of infection. Because the surveillance system identified only those with severe symptoms, the task force also conducted a cross-sectional study in the human population of the Garissa District to determine the incidence of recent infection with RVFV and to evaluate risk factors for infection.

Methods

Site and Population

The investigation was conducted in the Garissa District of the North Eastern Province of Kenya, which borders southern Somalia. In 1998, an estimated 231,022 residents living in Garissa District were distributed among 12 divisions and 84 sublocations (Office of the President, Garissa District Development Plan: 1997-2001, unpub. data). In addition to the Kenyan residents, 120,000 Somali refugees lived in the district in 1998. Garissa has an arid climate with a predominantly flat and grassy landscape. Precipitation in the district averages 250 mm to 500 mm annually, varies considerably from year to year, and occurs in a bimodal pattern with two peaks, in March through May and October through December. Although a number of settled towns are dispersed throughout the region, the rural population is principally composed of nomadic herdsmen.

Two events hampered surveillance. By January 1998, floodwater had damaged or covered most of the already poor roadways in the Garissa District, making them impassable. Investigators required air transportation to perform surveillance. Additionally, a nationwide nurses' strike in December

and January greatly decreased the medical personnel in hospitals and clinics, which contributed to the difficulties in providing patient care and collecting data.

Hemorrhagic Fever Surveillance

Case Definition

A probable case was defined as someone presenting with fever and bleeding from their gums, nose, eyes, rectum, lungs, or gastrointestinal tract, between October 1997 (the onset of flooding) and February 8, 1998. If specimens were available, diagnostic laboratory tests for RVFV were performed.

Case Ascertainment

In Garissa, active case-finding for hemorrhagic fever was performed by members of local law enforcement, village chiefs, and other government officials. Periodic reports were provided to the district health officers and the task force. We disseminated a case-report form to all district medical officers and international relief agencies involved in medical care in the area. When possible, reports of possible cases were investigated to obtain demographic and clinical information, as well as blood, stool, or tissue specimens. The WHO Hemorrhagic Fever Task Force was conducting similar surveillance throughout Kenya and in southern Somalia and northern Tanzania.

Laboratory

Initially, specimens were transported to the Africa Medical Research Foundation in Nairobi, where serum specimens were centrifuged, divided into aliquots, and forwarded to the Viral Research Center/Kenyan Medical Research Institute. Subsequently, a laboratory operated by Médècins du Monde was established in Garissa to process clinical samples. Within 12 hours of collection, serum specimens were stored at 4°C until packed and shipped to National Institute for Virology and CDC for RVFV-specific IgG and IgM antibody enzyme-linked immunosorbent assay (ELISA), virus isolation, RT-PCR, and sequencing of the DNA product, or immunohistochemistry using previously described methods (16-19).

Cross-Sectional Survey

Case Definition

For the cross-sectional survey, a laboratory-confirmed case definition was used. A case of recent infection was defined as presence of RVFV-specific IgM antibodies by ELISA. Persons with RVFV-specific IgG antibodies by ELISA and no IgM antibody were considered to have been infected before this outbreak. In our initial calculations, we considered persons with only anti-RVFV IgG antibodies to be protected against infection. The background prevalence of past RVFV infection in Kenya is not known, but IgG positivity as high as 40% has been shown in populations at high risk after epizootics (20). In contrast, a 1983 study for serologic prevalence of hemorrhagic fever viruses in Kenya demonstrated that <1% of persons sampled had anti-RVFV IgG, but a less sensitive immunofluorescent antibody test was used (21). This study did not include the North Eastern Province. Additionally, the duration of IgM antibodies is not well documented. Therefore, a second analysis included persons with either anti-RVFV IgG or IgM antibodies.

Sampling

We selected participants for the cross-sectional survey from the non-refugee population of Garissa District using a modified multistage cluster design (22). In Kenya, each province is divided into divisions that are made up of locations and then sublocations. We used sublocations as the units for choosing clusters. Thirty clusters (sublocations) were selected from 12 divisions. The number of clusters per division was weighted to represent population density. Clusters were then chosen randomly. Seven households were chosen in each cluster by systematic random sampling for a total sample size of 210 persons. The investigating team identified one person in each household for recruitment into the study. To reflect the age distribution of the RVFV infection and hemorrhagic fever cases already identified in the outbreak, we included one child between 2 and 9 years old, five persons between 10 and 49 years old, and one person ≥50 years in each cluster. Between February 8 and 14, 1998, three field teams interviewed and obtained samples from 202 persons from 29 clusters. Three of the selected cluster sites had been destroyed by the flood and could not be located. Replacement sublocations were sampled for two of the three destroyed clusters. In one cluster, only six persons were sampled. In four clusters, children <10 years of age were not sampled and were replaced by adults.

Data Collection

After informed consent was obtained, a blood specimen was collected and each participant was interviewed. We used a standardized questionnaire that included demographic characteristics (age, gender, family size), exposure information (e.g., slaughtering practices, butchering, consuming raw meat and milk), environmental factors (displacement by flood, type of settlement, loss of livestock), and history of illness between the start of the floods and the date of the interview. Exposure

to mosquitoes was evaluated through questions about attempts to reduce bites (i.e., mosquito nets, fires, other methods). Otherwise, all persons were assumed to share a similar risk for insect bites. Local health workers fluent in English, Kiswahili, and Somali were trained to administer the questionnaire and were supervised by an epidemiologist. Interviewers recorded information in English.

Laboratory

Blood specimens were kept at ambient temperature for <6 hours. Specimens were processed at the Médècins du Monde laboratory as noted above. RVFV-specific IgG ELISA, as well as IgM ELISA, was performed on all blood specimens. At the time of the cross-sectional survey, there were no reports of severe manifestations of illness and we believed that virus transmission was not ongoing. Therefore, virus isolation and PCR were not performed on these specimens.

Data Management and Analysis

Data from completed questionnaires were double-entered into databases by using Epi Info version 6.0 (CDC, Atlanta, GA). Univariate and multivariable analyses were performed by using SAS (version 6.12, Cary, NC). Poisson regression was used with a generalized estimating equations algorithm and controlled for the clustered nature of the data (23).

Results

Hemorrhagic Fever Surveillance

The hemorrhagic fever surveillance system identified 77 persons with severe febrile illness in Garissa District whose onset of fever was between November 10, 1997, and February 8, 1998. Fifty-three persons (57% male; median age 28 years, from 3 to 85 years) met the case definition for hemorrhagic fever (Figure 2). Of these 53 patients, 10 (19%) had evidence of acute RVFV infection (Table 1), and another 10 were positive by the anti-RVFV IgG ELISA. Because few cases were available for follow-up, case-fatality proportions were not calculated. Of the 24 persons whose illness did not meet the case definition for hemorrhagic fever, six (25%) had evidence of acute RVFV infection; one of these six had encephalitis and another had retinitis. The limited surveillance system also confirmed human disease in Tanzania and in Somalia (Figure 3).

Cross-Sectional Survey

Antibody Prevalence

Of the 202 persons enrolled in the cross-sectional study, 31 (15%) were positive only for anti-RVFV IgG (i.e., previously infected). Although persons having only IgG antibody were widely dispersed geographically, the highest prevalence was found in the Hulugho Division (32%) and the Masalani Division (29%). The highest percentage of previous infection by age group was for persons ≥65 years of age (Table 2). Characteristics of the sampled population are provided in Table 2.
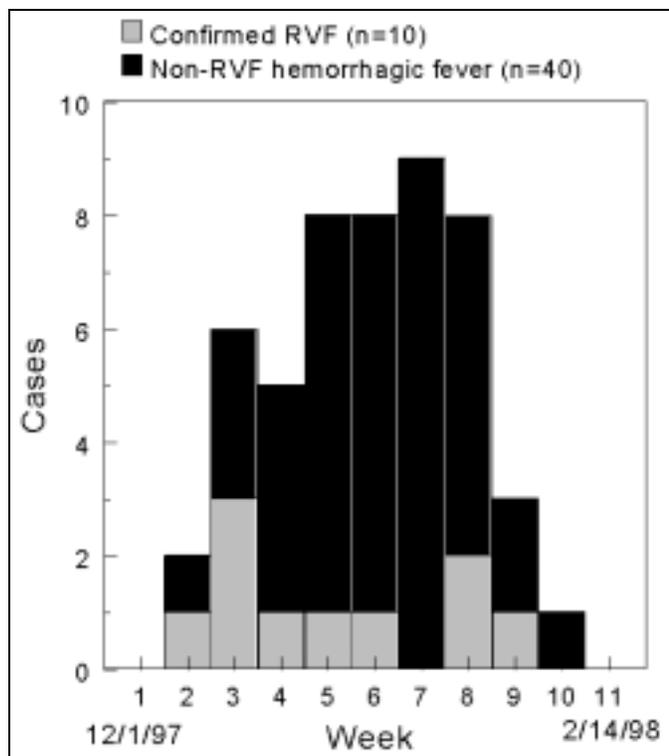
Figure 2. Temporal distribution of hemorrhagic fever cases, by date of onset, Garissa District, Kenya, December 1, 1997, to February 14, 1998. Source: CDC, Morbidity and Mortality Weekly Report 1998;47:261-4.

Table 1. Results of testing for laboratory-confirmed cases of Rift Valley fever, Garissa District, Kenya, 1997–98

Onset date	Collection date	Virus isolation	RT-PCR	IgM ELISA	IgG ELISA
12/9/97	12/23/97	+	+	-	-
12/18/97	12/25/97	+	+	+	-
12/18/97	12/26/97	-	Not done	+	+
12/19/97	12/26/97	+	+	+	+
12/21/97	12/26/97	+	+	+	-
12/22/97	01/22/98	Not done	Not done	+	+
12/30/97	01/23/98	Not done	+	+	+
1/18/98	01/22/98	Not done	-	+	+
1/28/98	02/02/98	Not done	Not done	+	+
2/7/98	02/09/98	Not done	Not done	+	-

RT-PCR=reverse transcription-polymerase chain reaction; IG=immunoglobulin; ELISA=enzyme-linked immunosorbent assay.

ELISA, including rural habitation (p=0.02) and household size of less than four persons (p=0.001). Age <15 years was associated with a lower rate of recent infection (p=0.05). A large number of animal contact activities, including herding, milking, slaughtering, and sheltering animals in the home, were statistically associated with recent RVFV infection (Table 3). The association was greatest with sheep-related activities, especially for those resulting in contact with sheep blood or body fluids.

Many of the animal contact variables were highly associated with infection in univariate analysis, but were also highly correlated. Multivariable analysis of composite variables for species-specific activities that resulted in similar exposures and potential confounders demonstrated a significant association between recent RVFV infection and persons who had contact with sheep blood, amniotic fluid, or milk (not including milk consumption; RR 3.0, 95% CI 1.3-6.7) (Table 4). Sheltering any domestic livestock (mostly sheep and goats) in one's home during the flood also remained independently and significantly associated with infection in the multivariable analysis

Of the 171 susceptible persons in the sample, 31 (18%) were positive for anti-RVFV IgM. The percentage of those with detectable IgM antibody varied with age; this antibody was detected in 5% of children <15 years of age, 23% of persons 15 to 65 years of age, and 13% of persons >65 (Table 2). The age-adjusted prevalence of IgM antibody positivity standardized to the population of Garissa District was 14% (95% CI 11-16). Sixteen clusters, representing all 12 of the administrative divisions in Garissa District, had persons with anti-RVFV IgM antibody, indicating recent viral transmission. Of the 31 persons with serologic evidence of recent infection, 30 (97%) reported having a recent illness, compared with 116 (83%) of the antibody-negative persons (p=0.06); no symptoms distinguished recent RVFV-associated illness from other illness.

Assuming an age-adjusted IgM antibody prevalence of 14% in the susceptible population, we estimate that approximately 27,500 persons were infected with RVFV during this outbreak in Garissa District alone. If the presence of IgG antibody were included in the case definition for recent infection, then the standardized prevalence would increase to 23% (95% CI 20-26) and would represent approximately 53,000 infected residents.

Risk Factor Assessment

Certain demographic characteristics (Table 2) were associated with higher rates of infection as determined by IgM

Figure 3. Geographic distribution of Rift Valley fever outbreak, East Africa, 1997–98. (Number of confirmed cases / number of cases with severe febrile illness reported to surveillance system). Source: CDC, Morbidity and Mortality Weekly Report 1998;47:261-4.

Table 2. Demographic characteristics of persons enrolled in Rift Valley fever cross-sectional survey, Garissa District, Kenya, 1997–98

Characteristic	Total (%) n = 202	Prior[a] infection (% of total) n=31	Susceptible[b] n=171	Acute[c] infection (% of susceptible) n=31
Age group (years)				
<15	40 (20)	2 (5)	38	2 (5)
15 to 65	150 (74)	25 (17)	125	28 (22)
>65	12 (6)	4 (33)	8	1 (13)
Sex				
Male	103 (51)	16 (16)	87	18 (21)
Female	99 (49)	15 (15)	84	13 (15)
Rural habitation	161 (80)	25 (16)	136	30 (22)
Household >4 persons	74 (37)	10 (14)	64	21 (33)

[a]Anti-RVF IgG antibody-positive (no immunoglobulin M [IgM]).
[b]Total screened minus those with prior infection.
[c]Anti-*Rift Valley fever virus* IgM antibody-positive.

(RR 3.5, 95% CI 1.3-9.1). Although not significant in univariate analysis, being male also was significantly associated with infection (RR 1.6, 95% CI 1.0-2.8). Age <15 years was associated with a reduced risk for infection when controlling for the other risk factors (RR 0.3, 95% CI 0.06-1.0). Drinking raw sheep milk was independently associated with infection, but did not reach statistical significance (RR 1.6, 95% CI 0.9-2.9).

When the analysis was repeated using detection of either anti-RVFV IgM or IgG as the outcome variable, which effectively doubled the number of cases of RVFV, only contact with sheep blood or body fluids and sheltering animals in the home remained significantly associated with illness in the multivariable model (data not shown).

Discussion

After heavy rainfall in late 1997, an epidemic of Rift Valley fever among humans accompanied an epizootic among ungulates in East Africa. From the cross-sectional investigation in the Garissa District of Kenya, we estimated 27,500 recent human infections with the virus occurred during this period, making this the largest outbreak of RVFV infection ever recorded in sub-Saharan Africa. In addition to Garissa District in the North Eastern Province, we identified recent human infection associated with hemorrhagic fever or encephalitis in four of Kenya's six provinces during the 1997-1998 outbreak (CDC, unpub. data). Surveillance also confirmed human disease in Tanzania, and, for the first time, in Somalia. Risk factors for human infection identified by this study included a broad array of activities associated with animal exposures, but most significantly, contact with sheep (particularly contact with sheep blood or other body fluids), male gender, and housing animals indoors with household members. Children <15 years of age were significantly less likely to have had recent RVFV infection.

Persons identified by the surveillance system did not undergo thorough clinical and laboratory investigations, and most persons who reported hemorrhaging were not directly observed by a clinician. Laboratory testing also found evidence of infection with other viral agents (*Dengue* and *Bunyamwera* viruses), malaria, shigella dysentery, and leptospirosis as explanations for some of the persons whose illness met the hemorrhagic fever case definition, but who were negative for RVFV infection (CDC, unpub. data). Additionally, the epidemic appears to have preceded a large outbreak of malaria reported from the same region (24). Other possible explanations for the persons with fever and hemorrhage who had no laboratory evidence of RVFV infection include an overly sensitive case definition; improper collection, labeling, handling, and transport of samples; other pathogens or toxins; and complications of malnutrition.

In the cross-sectional study, the case definition for recent infection was based on the detection of IgM antibody. Unfortunately, the kinetics of the IgM response to RVFV are not well

Table 3. Exposures during previous 90 days, Rift Valley fever (RVF) cross-sectional survey, Garissa District, Kenya, 1997–98

	Acute[a] infection (%) n = 31	No infection (%) n = 140	Relative risk	95% CI
Animal exposures				
Sheltered livestock in home after flood	27 (87)	63 (45)	5.3	2.3-12.6
Killed an animal	20 (64)	47 (34)	2.4	1.3-4.3
Butchered an animal	14 (45)	33 (24)	2.0	1.1-3.6
Skinned an animal	20 (65)	38 (27)	2.4	1.6-3.5
Cooked with meat	20 (65)	48 (34)	2.3	1.1-4.9
Milked animals	25 (80)	59 (42)	3.8	1.9-7.7
Drank raw animal milk	30 (97)	89 (64)	8.6	2.0-36.0
Care of animal during birth	21 (68)	46 (33)	2.6	1.4-4.9
Disposal of aborted fetus	19 (61)	36 (26)	2.8	1.5-5.5
Sheep contact[b]	25 (81)	48 (29)	6.3	2.9-14.0
Goat contact[b]	28 (90)	91 (65)	3.1	1.6-6.4
Cow contact[b]	20 (65)	49 (35)	2.4	1.3-4.5
Camel contact[b]	5 (16)	17 (12)	1.3	0.5-3.8
Non-animal exposures				
Home flooded since November 1997	25 (81)	103 (73)	1.3	0.8-2.1
Ill family member	7 (23)	20 (14)	1.6	0.8-3.1
Contact with a dead human body	6 (21)	10 (7)	2.2	1.0-4.6
Use mosquito nets	19 (61)	102 (73)	0.7	0.3-1.4

[a]Anti-*Rift valley fever virus* immunoglobulin M antibody-positive.
[b]Contact includes herding, cooking, slaughtering or other body fluid contact (except consumption), drinking raw milk.

Table 4. Multivariable risk factor analysis—cross-sectional survey, Garissa District, Kenya, 1997–98

Exposure	Relative risk	95% CI
Contact with sheep blood or body fluids	3.0	1.3-6.7
Sheltering animals in the home	3.5	1.3-9.1
Male gender	1.6	1.0-2.8
Age <15 years	0.3	0.06-1.0
Drinking raw sheep milk	1.6	0.9-2.9

described. After natural infection, domestic animals lose a detectable amount of IgM antibody within 6 months of infection (25). In a large percentage of humans, experimental inoculation with a killed vaccine results in an early IgM response that wanes and is undetectable by 4 to 6 weeks, but this is not a model for natural infection (26). Of the few clinical infections that have been followed closely for serologic conversion, IgM antibody appears around day 5, is absent in 50% by day 45, and is undetectable 4 months later (12), whereas IgG appears about day 4 and may persist indefinitely at high titer.

All blood specimens for our cross-sectional study were obtained within 12 weeks of the first reported case of hemorrhagic fever in Garissa District. Therefore, the IgM antibodies probably represent recent infection related to the outbreak. If the IgM antibodies disappear quickly, however, the persons in whom we detected only IgG antibody may actually have been infected recently. If we include those persons in the analysis, the incidence of infection could be as high as 23%, representing an additional 25,000 infected persons in Garissa District alone.

Human suffering from RVFV is compounded by the loss of domestic animals. Livestock owners reported losses of approximately 70% of their animals, with the greatest losses among sheep and goats. Other infections thought to contribute to illness among livestock during the flooding included nonspecific pneumonia, pasteurellosis, contagious caprine pleuropneumonia, contagious pustular dermatitis, bluetongue, foot rot, and complications of mange (Field Mission of the Food and Agriculture Organization of the United Nations, unpub. data).

Because direct contact with blood or body fluids from viremic animals is an important risk factor for human infection identified by this study, the high number of domestic animal abortions and deaths may have increased the risk for humans developing illness. Many of the early cases were in persons who had recently been involved in the dissection, slaughter, or care of sick animals (12,27). A high attack rate has been demonstrated for abattoir workers (28), herdsmen (19), and veterinary personnel (9), all of whom have extensive contact with animal blood or other body fluids in the course of their work. In a retrospective investigation in Senegal based on IgG antibody positivity, men who assisted with animal births or abortions, and women who treated ill animals were found to be at increased risk for infection (29). Additionally, the virus has

been isolated from raw milk, and ingestion of raw milk has been suggested as a risk factor in previous studies (30,31).

Although any animal that develops a high level of viremia can pose a certain risk for animal-to-human transmission of virus, we found the greatest association with sheep. In past RVFV epizootics, sheep have been the most susceptible domestic animals (1,9,32). After a 2- to 4-day incubation period, young lambs become listless, and fever and bloody diarrhea occur; the case-fatality of 90% to 100% is attributed to hepatic liquefaction (33,34). The disease manifests similarly in adult sheep, although the case-fatality rate is much lower (20%-30%). As many as 80% of pregnant ewes abort after infection (9). The more severe manifestations in sheep are possibly the result of higher levels of viremia (10^{10} suckling mouse intra-cerebral [SMIC] 50% lethal dose/mL), which can exceed those documented in cows and goats (10^{8-10} SMIC 50% lethal dose/mL) (14). Higher levels of viremia with a high rate of abortion and deaths result in an increased likelihood of human contact with an infectious inoculum. If the animal survives acute illness, the virus can be isolated in lower titers for as long as 3 weeks after illness, making it potentially dangerous to slaughter an animal, even after the epizootic appears to be over (33,35).

From this study it is not possible to identify which cases were infected by mosquitoes and which through direct contact with animals because we did not gather data on the numbers, species, prevalence of RVFV infection, or biting rates of mosquitos at the time of the outbreak. During February 1998, however, 3,180 mosquitoes were collected from three trapping sites in Garissa District. Of the nine captured species, three have been previously implicated in RVFV transmission to humans (*Anopheles coustani, Mansonia africana,* and *M. uniformis*). In our multivariable model, we assumed that the high mosquito density led to an equal mosquito exposure rate for the entire population. The association of infection with being male and >15 years indicates that persons who are more likely than other groups to perform high-risk behaviors (i.e., adult men) are more likely to be exposed to the virus. All but one (97%) of the persons with acute RVFV infection, as identified by IgM antibody, had recent exposure to either blood, milk, or abortive materials of a sheep or goat. Most of these people (87%) also sheltered animals in their home.

The probability of recurring outbreaks in East Africa and the potential for spread, by either natural or intentional means, to non-disease–endemic areas emphasize the necessity of developing and validating methods to predict, prevent, detect, and treat Rift Valley fever. Remote sensing satellite technology, which can predict rainfall patterns likely to result in disease emergence, has been suggested as a means to monitor RVFV activity (15,36). Longitudinal studies using satellite data to target areas for animal vaccination, enhance surveillance activities for RVFV in animals and humans, and conduct prospective entomologic studies are in progress. However, until these methods are validated, simple public health inter-

ventions may greatly reduce transmission of the virus. Control programs aimed at protecting persons during epizootics should include education about the risks of having contact with infected animal body fluids. Although this report highlights the importance of direct animal contact in the transmission of RVFV to humans, the role of arthropod vectors, particularly in the virus life cycle in epizootics, cannot be discounted. Control of mosquito populations during or after heavy rains should be pursued to prevent animal and human infection.

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Surveillance for Unexplained Deaths and Critical Illnesses Due to Possibly Infectious Causes, United States, 1995–1998

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Population-based surveillance for unexplained death and critical illness possibly due to infectious causes (UNEX) was conducted in four U.S. Emerging Infections Program sites (population 7.7 million) from May 1, 1995, to December 31, 1998, to define the incidence, epidemiologic features, and etiology of this syndrome. A case was defined as death or critical illness in a hospitalized, previously healthy person, 1 to 49 years of age, with infection hallmarks but no cause identified after routine testing. A total of 137 cases were identified (incidence rate 0.5 per 100,000 per year). Patients' median age was 20 years, 72 (53%) were female, 112 (82%) were white, and 41 (30%) died. The most common clinical presentations were neurologic (29%), respiratory (27%), and cardiac (21%). Infectious causes were identified for 34 cases (28% of the 122 cases with clinical specimens); 23 (68%) were diagnosed by reference serologic tests, and 11 (32%) by polymerase chain reaction-based methods. The UNEX network model would improve U.S. diagnostic capacities and preparedness for emerging infections.

The 1992 Institute of Medicine report—Emerging Infections, Microbial Threats to Health in the United States (1)—highlighted the need for a more effective means to detect emerging infectious diseases. In response to this report and as part of the Emerging Infections Program (EIP) (2), the Centers for Disease Control and Prevention (CDC) collaborated with state health departments and academic institutions to develop a pilot surveillance strategy for early detection of new and unrecognized infectious diseases in the United States. This project—Surveillance for Unexplained Deaths and Critical Illnesses Due to Possibly Infectious Causes—was developed on the basis of two observations. The first was the realization that supposedly new infectious diseases identified in the United States in recent decades had been occurring long before they were recognized and identified. The second was important progress in molecular diagnostic methods, which in some instances has allowed new infectious agents to be identified and characterized with molecular probes, making in vitro cultivation unnecessary.

In 1995, we initiated population-based surveillance for unexplained deaths and critical illnesses due to possibly infectious etiologies (UNEX) at four U.S. sites. The objectives of this effort were to define the incidence, epidemiologic features, and possible causes of these deaths and illnesses; create a bank of clinical specimens for future testing as new pathogens and methods are identified; and assist in building U.S. capacity for detecting and responding to uncommon and previously unrecognized pathogens. This report describes the methods we developed to reach these goals and the results of the first 3.5 years of surveillance.

Methods

Surveillance Sites

Population-based surveillance for UNEX was initiated on May 1, 1995, among persons 1 to 49 years of age residing in the San Francisco Bay area (Alameda, Contra Costa, and San Francisco Counties) of California (n=2,168,810); in New Haven County, Connecticut (n=556,592); in the entire state of Minnesota (n=3,419,760); and among persons 1 to 39 years of age residing in Oregon (n=1,544,466).[2] All these sites were participants in the EIP, and the total population targeted for

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surveillance was 7.7 million. We report the results of surveillance for cumulative cases through December 31, 1998.

Case Definition

An UNEX case was defined as illness in a previously healthy resident of a surveillance area who was 1 to 49 years old (1 to 39 years old in Oregon) and who died or was hospitalized with a life-threatening illness with hallmarks of an infectious disease for which no cause was identified through routine testing initiated by health-care providers. A previously healthy person was defined as a patient without a preexisting known systemic, chronic medical illness diagnosed before the acute onset of the UNEX. Such preexisting conditions included malignancy; HIV infection; chronic cardiac, pulmonary, renal, hepatic, or rheumatologic disease; or diabetes mellitus. Patients were also excluded from the study if they had received any immunosuppressive therapy, had evidence of toxic ingestion or exposure, had trauma before their illness, or acquired their illness $\geq$48 hours after hospital admission (Appendix I).

A life-threatening illness was defined as any illness requiring admission to an intensive-care unit (ICU). Hallmarks of an infectious disease were defined as the following: fever or history of fever, leukocytosis, histopathologic evidence of an acute infectious process, or a physician-diagnosed syndrome consistent with an infectious etiology, including encephalitis or meningitis, fulminant hepatitis or hepatic failure, myocarditis, adult respiratory distress syndrome, respiratory failure, or sepsis.

Case Finding and Ascertainment

Patients meeting the case definition were sought at surveillance sites through various mechanisms. Practicing clinicians in all surveillance sites were informed about the project through letters and bulletins and presentations at local and regional professional society meetings. Personnel at some surveillance sites attempted to identify cases more actively through regular communications with persons working in ICUs and local medical examiners or through routine review of ICU admission records. Physicians and other health professionals were asked to report suspected cases by telephone to local surveillance site personnel. When a case was reported, a screening form was completed to determine if the patient met the case definition. This surveillance system was not designed to provide timely reporting or testing.

Surveillance Audit

To evaluate the sensitivity of the surveillance system, personnel at all surveillance sites conducted a retrospective review of death records from their surveillance areas, and three sites (California, Connecticut, and Oregon) reviewed all hospital discharge data in their areas for a period of at least 1 year. All death certificates for the age groups included in the surveillance were reviewed for the presence of specific International Classification of Disease codes (ICD-9), selected for

their potential to identify unexplained deaths due to possibly infectious causes (3). Persons whose death records included ICD-9 codes indicating a disqualifying underlying medical condition were excluded. Once potential cases were identified, the patients' medical records were reviewed. If the records were not available, the primary physician was contacted to determine if the patient met the surveillance case definition. The sensitivity of the surveillance system for detecting deaths (S_D) was calculated by dividing the number of deaths (D1) detected through surveillance alone by the total number of deaths (D1+D2) detected through both surveillance and death record review (D2): S_D=D1/D1+D2. The sensitivity of the surveillance system (S_C) for detecting critical illness cases was calculated by dividing the number of such cases (C1) detected through surveillance alone by the total number of cases (C1+C2) found through both surveillance and hospital discharge review (C2): S_C=C1/C1+C2.

Collection of Clinical Information and Specimens

For patients meeting the case definition, surveillance site personnel completed a case report form that included demographic, epidemiologic, and clinical information. This information was collected through interview of physicians caring for the patient, review of the medical record, and contact with the patient or the patient's family. Cases were assigned a clinical syndrome depending on the predominant system involved, on the basis of information provided by the physician. These syndromes included neurologic (encephalitis, meningitis), cardiac (myocarditis, pericarditis, endocarditis), respiratory (pneumonitis), and hepatic (hepatitis). Syndromes such as sepsis, in which no predominant organ system was involved, were classified as "other." The hospital laboratories were requested to save all remaining clinical specimens obtained as part of routine clinical management, including biopsies and autopsies.

Laboratory Testing

For the first 2 years of the study, the project investigators selected diagnostic tests individually for each case. Decisions were made on the basis of clinical, epidemiologic, and histologic data; previous laboratory testing ordered by the health-care providers; and availability, timing, quality, and quantity of clinical specimens. In the third year of the project, based on information gained to date, a set of standardized syndrome-specific laboratory testing protocols was developed for respiratory, neurologic, cardiac, and hepatic syndromes (Appendix II: available online at URL: http://www.cdc.gov/ncidod/EID/vol8no2/pdf/01-0165-app2.pdf). These protocols prioritized testing based on available clinical and epidemiologic information and a differential diagnosis; they guided a first round of laboratory testing which, if negative, prompted a customized second round of testing. Cases that did not fit any of these four syndromes were discussed by the project investigators on an individual basis.

Histopathologic Testing

Whenever possible, in addition to initial examination by local pathologists, tissue specimens were examined by CDC pathologists to help guide further laboratory testing decisions. CDC pathologists have available a unique set of antibodies and probes for immunohistochemistry (IHC) and in-situ hybridization (ISH);[3] these and other special studies, such as chemical stains, were selected based on all available case information. IHC tests were performed by a two-step indirect immunoalkaline phosphatase technique with various antibodies (4). ISH tests used digoxigenin-labeled probes with an immunoalkaline phosphatase staining protocol (5). Positive and negative controls were run in parallel with case specimens.

Testing for Viral Pathogens

The California Department of Health Services (CDHS) Viral and Rickettsial Diseases Laboratory was the primary testing site for viral pathogens other than the hepatitis viruses. Serologic tests were available for immunoglobulin (Ig) G directed against 19 viral pathogens and for IgM directed against 14 of these.[4] When only a single serum specimen was available, the presence of both IgM and IgG was assessed, either by enzyme immunosorbent assay (EIA), indirect immunofluorescence assay (IFA), or both (6). Paired sera were tested by EIA or IFA for increase in IgG titer. Additional testing included nucleic acid amplification by polymerase chain reaction (PCR) for selected viral pathogens if adequate specimens were available (7-9). An increase in IgG titer by EIA was interpreted as evidence of recent or current infection if the ratio of convalescent- to acute-phase indices was ≥1.5; an index is determined by the equation (optical density [OD]-positive antigen - OD-negative antigen)/predetermined positive threshold OD (usually 0.1). The CDHS diagnostic assays for IgM to B19V, *Cytomegalovirus*, *Hantavirus* (SNV), herpes simplex virus, MeV, MuV, RUBV, SLEV, VZV, and WEEV have varying minimum positive values, with indices from 1.0 and 2.0. For the enterovirus IgM assay, which detects the presence of enterovirus group antibody in serum, a ratio of OD-positive antigen to OD-negative antigen ≥2.26 was considered positive. Agents tested by IFA were considered positive if a fourfold or greater rise in titer was detected. IFA IgM assays were considered positive if the staining pattern was distinct for that agent at the appropriate serum dilution.

Bacterial Broad-Range Ribosomal DNA (rDNA) PCR

DNA extraction from clinical specimens was performed as described (10,11). All clinical specimens tested with the broad-range bacterial rDNA PCR were analyzed by using at least one of the three following primer pairs: fD1mod (positions 8-27 in *Escherichia coli* 16S rRNA gene) (12) and 16S1RR-B (575-556) (13); 8F2 (8-27) and 806R (806-787); and 515F (515-533) and 13R (1390-1371). PCR products were characterized by direct sequencing or cloning and sequencing,

followed by comparison with rDNA sequences available in GenBank (11).

Criteria for Causation

Cases were defined as having definite, probable, possible, or no microbial etiology (Table 1). These levels of certainty for the causal role of an infectious agent reflected the integration of several factors, including the relationship of anatomic site of detection to site of disease, reliability of the method, and whether the putative agent was a known cause of the clinical syndrome under investigation. Cases were classified as explained if results showed a definite or probable disease cause and as unexplained if results indicated a possible infectious cause or none at all.

Statistical Analysis

Analysis was performed by SAS 6.12 (SAS Institute, Cary, NC). Denominators for the population under surveillance, obtained from the 1992 intercensus (14), included all persons in the age groups under surveillance at the various sites; denominators including only previously healthy persons are not available, and no attempt was made to estimate this frac-

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[3]IHC was available at CDC for the following pathogens: *Acanthamoeba culbertzoni*; adenovirus; *Bacillus anthracis; Balamuthia* spp.; *Bartonella henselae, Bartonella quintana; Brucella* spp.; *Chlamydia* spp.; *Coccidiodes* spp; *Coxiella burnetii; Crimean-Congo hemorrhagic fever virus; Cryptococcus* spp.; *Cytomegalovirus; Dengue virus; Eastern equine encephalitis virus*; Ebola virus; *Ehrlichia chaffeensis; Enterovirus* (Panenterovirus); human enterovirus 71; *Flavivirus*; Japanese encephalitis serocomplex group (*West Nile virus, St. Louis encephalitis virus* [SLEV], *Japanese encephalitis virus*); *Francisella tularensis*; Group A streptococci; *Guanarito virus* (Venezuelan hemorrhagic fever virus); *Hantavirus; Helicobacter pylori*; Hendra virus; herpes simplex viruses 1 and 2; *Histoplasma* spp.; human granulocytic ehrlichiosis; *Human herpesvirus 6*; HIV-1; HIV-2; *B19 virus* (B19V); *Influenza A virus* (FLUA); *Influenza B virus* (FLUB); *Junin virus* (Argentine hemorrhagic fever); La Crosse virus; *Lassa virus; Legionella pneumophila* serogroups 1, 5, 6; *Leptospira* spp.; *Listeria monocytogenes; Lymphocytic choriomeningitis virus* (LCMV); *Machupo virus* (Bolivian hemorrhagic fever); *Marburg virus; measles (Edmonston) virus* (MeV); *Mycobacterium* spp.; *Mycoplasma pneumoniae; Naegleria fowleri; Neisseria meningitidis* C; Nipah virus; *Human parainfluenza virus types 1 and 3* (HPIV 1,3); *Rabies virus* (RABV); *Human respiratory syncytial virus* (HRSV); *Rickettsia* spp. Orientia group; *Rickettsia* spp. spotted fever group; *Rickettsia* spp. Typhus group; *Rift Valley fever virus; Rotavirus; Streptococcus pneumoniae; Toxoplasma gondii; Treponema pallidum; Trypanosoma cruzi*; varicella-zoster virus (VZV); *Venezuelan equine encephalitis virus; Western equine encephalomyelitis virus* (WEEV); *Yellow fever virus*; and *Yersinia pestis*.

[4]The following viral tests were used at the CDHS Viral and Rickettsial Diseases Laboratory: IgG was detected by both EIA and IFA for adenovirus, HHV-6, HHV-8, herpes simplex virus, FLUAV, FLUBV, MeV, *Mumps virus* (MuV), HPIV-1-4, HRSV, *Rubella virus* (RUBV), VZV, SLEV, and WEEV. IgG was detected by EIA only for *Hantavirus* (*Sin Nombre virus* [SNV]) and B-19. IgG was detected by IFA only for Epstein-Barr virus (viral capsid antigen), LCV, and RABV. IgM was detected by both EIA and IFA for HHV-6, herpes simplex virus, MeV, MuV, HPIV-1-4, HRSV, RUBV, and VZV. IgM was detected by EIA only for enterovirus, hantavirus (SNV), and B19V. IgM was detected by IFA only for Epstein-Barr virus. PCR tests performed were Herpesvirus consensus PCR (6); enterovirus PCR, modified from (7) [Antisense primer (1R): 5'-ATT GTC ACC ATA AGC AGC CA, sense primer (1L): 5'-CCT CCG GCC CCT GAA TGC GGC TAA T]; and adenovirus PCR (8).

Table 1. Classification of laboratory test results and cases,[a] surveillance for unexplained death and critical illness possibly due to infectious causes (UNEX), 1995–1998

A	B	C
1. Detection of organism by culture from involved site[b]	1. Detection of organism by culture, IF, IHC, IEM, ISH, or PCR[c] in blood or clinically relevant site[d]	1. Detection of organism by culture, IF, IHC, IEM, ISH, or PCR from uninvolved, but nonmucosal, noncutaneous site
2. Detection of organism by direct immunologic staining (i.e., IF, IHC,IEM) at involved site	2. Positive serology: $\geq$4-fold change in IgG/IgA titer or significantly elevated IgM titer	
3. Detection of organism by DNA/RNA ISH at involved site	3. Detection of organism by EM[e] at involved site	
4. Detection of organism by PCR[f] at involved site	4. Detection of other specific microbial antigen at characteristic site (e.g., urine, CSF)	

[a]Case classification: A case was considered to have a definite explanation if the organism was a well-recognized cause of syndrome and there was one test from column A or 2 from column B. A case was considered to have a probable explanation if the organism was a well-recognized cause of the syndrome and there was one test from column B, or if the organism was not a well-recognized cause of the syndrome and there was one test from column A or 2 from column B. A case was considered to have a possible explanation if the organism was not a well-recognized cause of the syndrome and there was one test from column B, or if there was one test from column C, regardless of whether organism is known to cause the syndrome.
[b]"Involved" refers to the presence of typical pathology.
[c]IF = immunofluorescence, IHC = immunohistochemistry, IEM = immunoelectronmicroscopy, ISH = in situ hybridization, PCR = polymerase chain reaction, Ig = immunoglobulin, EM = electron microscopy, CSF = cerebrospinal fluid.
[d]For example, bronchoalveolar lavage in respiratory syndrome.
[e]EM is often nonspecific and may not permit reliable microbial identification without further characterization (e.g., IEM).
[f]Specific or broad range PCR/reverse transcriptase-PCR; product must be characterized beyond size determination (e.g., sequencing, single-strand conformation polymorphism, restriction fragment-length polymorphism, or probe hybridization).

tion. Data from the surveillance population were standardized to the U.S. population by race and age to project the number of cases occurring nationally. The chi-square test was used to compare the distribution of characteristics between explained and unexplained cases. A p value $\leq$0.05 was considered statistically significant.

Results

Epidemiology

From May 1, 1995, to December 31, 1998, 525 possible cases were reported to UNEX personnel; 388 of these reports were excluded. The three most common reasons for exclusion were the presence of a preexisting medical condition (33%), residence outside the surveillance area (17%), and cause identified by local health-care providers on further testing (26%). Among cases excluded for the last reason, 72% had an infectious cause identified.

A total of 137 cases met the case definition, for a minimum overall annual rate of 0.5 per 100,000 population. After data

were adjusted for age and race, this rate translates into 920 cases in the United States each year. The overall annual incidence rates remained stable over time, but varied among the different sites from 0.3 to 2.3 per 100,000 per year. The highest rate was in Connecticut, where active surveillance was conducted in a well-defined population of approximately 500,000 persons. Forty-one (30%) of the case-patients died, of whom 30 (73%) had autopsies performed, reflecting a rate much higher than the national autopsy rate of <11% (15). Cases were reported a median of 6 days from time of admission to the hospital (0 to 289 days).

The median age of case-patients was 20 years; 20 (15%) were 1 to 4 years of age, 53% were female, and 82% were white. The incidence rates varied by age group (Figure 1) but did not differ by sex and race. No differences were observed in the seasonal distribution of cases, nor was there clustering of cases by time or place. As for exposures, 54% of all cases were reported to have pets, which is similar to national rates of pet ownership: 54% to 64% (American Veterinary Medical Association U.S. Pet Ownership and Demographics Sourcebook); 8% had traveled outside the United States in the year before hospitalization, and 4% had received transfusions at least once in their lifetime.

Clinical Features

Table 2 summarizes the distribution of cases and the proportion explained by syndrome, as well as the syndrome-specific case-death ratios. The largest proportion of cases presented as a neurologic syndrome, followed closely by respiratory syndrome. The highest syndrome-specific case-death ratio was seen among cases with cardiac syndrome (46%) and the lowest among cases with neurologic syndrome (18%). An example of a case is described in Appendix III.

Surveillance Audit

Table 3 summarizes the results of the surveillance audits. The site-specific sensitivity (S_D) of our prospective surveillance for detecting unexplained deaths ranged from 38% in California to 100% in both Connecticut and Minnesota. Retrospective death record review identified 25% to 100% of all deaths detected through surveillance. Cases detected through surveillance but not by death record review were missed by the
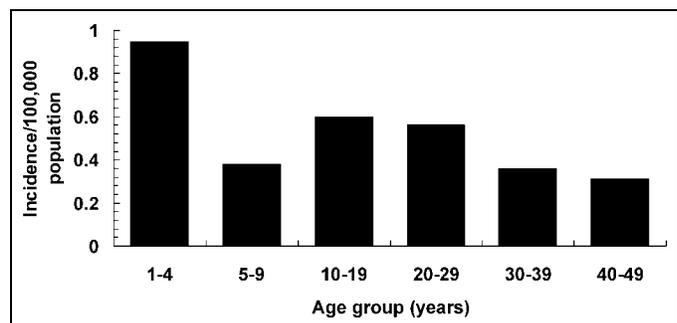
Figure 1. The incidence of cases by age group, 1995–1998, the Surveillance for Unexplained Deaths and Critical Illnesses Due to Possibly Infectious Causes Project (UNEX).

Table 2. Distribution of unexplained deaths and critical illness cases by syndrome, with proportion explained

Syndrome	No. (%)	No. of deaths (%)	No. of explained /cases with specimens (%)
Neurologic	39 (29)	7 (18)	15/37 (41)
Respiratory	36 (26)	11 (31)	13/33 (39)
Cardiac	28 (20)	13 (46)	3/22 (14)
Multisystem	18 (13)	4 (22)	3/15 (20)
Hepatic	9 (7)	4 (44)	0/8 (0)
Other	7 (5)	2 (29)	0/7 (0)
All cases	137	41 (30)	34/122 (28)

latter because the death certificates did not have the specified ICD-9 codes. The review of hospital discharge data focused on one tertiary-care referral hospital under surveillance in California and the one in Oregon, but included the entire surveillance area in Connecticut (16). Of potential cases identified by the selected ICD-9 codes, 90% to 96% were excluded, indicating the lack of specificity of these codes. The sensitivity of our prospective surveillance to detect only critical illnesses due to potentially infectious causes (S_C) was 13% to 73%. Retrospectively, the hospital discharge review was able to identify 41% to 81% of all cases detected prospectively through our surveillance.

Search for Etiologic Agents

Of the 137 UNEX case-patients, 122 had specimens available for testing; 10 of these had tissue specimens only. Of the 122 cases, 34 (28%) could be attributed to a specific infectious agent; these agents were classified as definite or probable causes of the illness, based on our criteria (Table 1). Specific infectious causes and the laboratory methods used for diagnosis are listed in Table 4. Table 5 lists additional infectious causes for possible cases that did not meet our criteria for definite or probable causation. All the infectious agents identified in this study were previously recognized bacterial and viral pathogens. One patient, admitted because of syncope, was found to have a complete heart block and had evidence of simultaneous infection with *Borrelia burgdorferi* and *Ehrlichia chaffeensis,* which has been previously reported (17). A number of cases met the clinical definition for various infectious diseases syndromes, including toxic shock syndrome (five cases), but did not meet our definition for an explained case. In addition, four cases had evidence of polyclonal serologic response to multiple infectious agents and therefore could not be attributed to a specific etiology. The proportion of explained cases was largest among those with neurologic syndromes, followed by those with respiratory syndromes; it was higher among surviving patients (29%) than among patients who died (15%), although this difference was not statistically significant (p=0.2) (Figure 2). Explained cases were similar to unexplained cases in terms of patient age, sex, and race, but were reported sooner after admission than unexplained cases

(median 4 vs. 7.5 days, respectively; p=0.1). The proportion of explained cases during 1998 (7 [17%] of 41), when laboratory testing protocols were used routinely for first-round testing, did not differ significantly from the same proportion for cases enrolled during 1995-1997 (27 [28%] of 96) when no such protocol was used (p>0.05).

Clinical specimens from each enrolled patient underwent an average of 28 laboratory tests (up to 103 tests). The mean number of tests performed did not differ substantially for explained and unexplained cases (30 vs. 27, respectively). None of the cases with only histologic specimens available had an infectious cause identified. Of the 34 explained cases, 23 (68%) were explained by using serologic tests, 7 (21%) by specific primer PCR, and 4 (12%) by 16S rDNA PCR. Among the 122 cases with specimens, serologic testing provided the highest yield in identifying infectious causes (23 [22%] of 104), followed by specific primer PCR (7 [10%] of 70) and 16S rDNA PCR (4 [8%] of 48). An infectious etiology was more likely to be identified in cases with paired serum specimens (14 [23%] of 62) than in those with single serum specimens (2 [5%] of 42) (p=0.05).

Discussion

This study is the first to measure the population burden of unexplained deaths and critical illness from possibly infectious causes in the United States. To our knowledge, this is the first public health attempt to describe the features of this problem, in spite of its clinical complexities. This project established the infrastructure needed to detect UNEX cases, attempt to identify their etiology, and ultimately identify new infectious agents. However, since this project was a pilot study, it was difficult to standardize many of its aspects. Many lessons were learned during this project, whether related to the best surveillance methods to use or the laboratory testing process. In

Table 3. Sensitivity of methods to identify cases of unexplained deaths and critical illnesses of possible infectious etiology, including the prospective surveillance conducted during this project and retrospective record reviews

	California	Oregon	Connecticut	Minnesota[a]
Sensitivity of prospective surveillance for unexplained deaths (%)	38	72	100	100
Proportion of all unexplained deaths identified retrospectively through death record review (%)	63	100	25	83
Sensitivity of prospective surveillance for critical illnesses (%)	25	13	73	—
Proportion of critical illnesses identified retrospectively by hospital discharge data review (%)	75	81	41	—

[a]Minnesota did not review hospital discharge records.

Table 4. Infectious disease causes for explained cases, UNEX,1995–1998, California, Oregon, Connecticut, and Minnesota (n=34)

Syndrome	Etiology (n)	Tests (n)
Neurologic (n=15)	*Neisseria meningitidis* (4)	16S rDNA PCR (2), PCR (1), EIA IgM (1)[a]
	Bartonella henselae (1)	PCR, IFA IgG
	Bartonella spp. (2)	IFA IgG
	Chlamydia pneumoniae (1)	MIF IgG
	Mycoplasma pneumoniae (1)	EIA IgM/IgG
	Cytomegalovirus (1)	EIA & IFA IgG
	Coxsackie B (1)	EIA IgM, viral culture
	Enterovirus (1)	EIA IgM
	Epstein-Barr virus[b] (1)	IFA IgG (VCA and EA)
	Human herpes virus 6 (1)	IFA and EIA (IgM and IgG)
	Mumps virus (1)	IFA IgM, IFA and EIA IgG
Respiratory (n=13)	*Chlamydia pneumoniae* (2)	MIF IgG (2), IFA IgM
	Mycoplasma pneumoniae (4)	PCR (blood), EIA IgM/IgG
	Streptococcus pneumoniae (2)	16S rDNA PCR (pleural fluid)
	Legionella spp. (1)	PCR (from lung)
	Adenovirus (1)	EIA and IFA IgG
	Influenza B virus (1)	EIA and IFA IgG
	Influenza A virus (1)	EIA and IFA IgM, EIA (IgG)
	Human parainfluenza virus types 1 and 3 (1)	EIA and IFA IgG
Cardiac (n=3)	*Borrelia burgdorferi/ Ehrlichia chaffeensis* (1)	EIA/ IFA flagella IgG, Western blot (IgG, IgM)
	Enterovirus (1)	EIA IgM
	Legionella spp. (1)	PCR (heart)
Multisystem (n=3)	*Neisseria meningitidis* (1)	PCR (CSF)
	Adenovirus (1)	PCR (blood)
	Enterovirus (1)	IgM EIA

[a]EIA = enzyme immunosorbent assay, IFA = indirect immunofluorescent assay, IG = immunoglobulin, MIF = microimmunofluorescence, PCR = polymerase chain reaction.
[b]See Appendix III for a detailed description of this case.

addition, data obtained in the first 3.5 years of this project suggest that UNEX occur in previously healthy persons at rates similar to those of other conditions of clear public health concern and priority (18). Of obvious concern is also the large proportion of these deaths and severe illnesses that remains unexplained after extensive laboratory testing. Our findings highlight the substantial limitations of available diagnostic tests for infectious diseases and the need for improved tests and novel approaches to identify infectious disease agents.

Our surveillance estimated the burden of disease only among previously healthy persons 1 to 49 years of age. Since a different age cut-off was used in Oregon, the final rates of disease were adjusted for age and race. The lower age limit was chosen to avoid confusion with congenital problems seen in infants but to include most children in day care, where infec-

tious diseases are common and new infectious diseases might spread rapidly. The upper age limit was intended to exclude an expected increased proportion of unexplained deaths due to noninfectious causes among persons $\geq$50 years of age. Although immunocompromised patients are more susceptible to a variety of infectious diseases, available resources and a concern that the clinical relevance of novel microbial findings would be more difficult to interpret in immunocompromised persons compelled us to focus on previously healthy persons. In addition, many of the new infectious diseases first identified in these persons have subsequently been found to affect persons with normal immune systems (19,20).

The surveillance methods adopted during this project were customized to meet the objectives of this study, taking into consideration the limitations of local resources; therefore UNEX cannot be easily compared with other classical surveillance systems. The different methods of surveillance used at the four sites allowed us, through the surveillance audits and validation, to determine how these differences affected case-finding. For example, investigators in Connecticut were able to detect most UNEX cases largely because they conducted more active surveillance in a smaller population base; in this site, surveillance focused on all seven hospitals in New Haven County. At the academic tertiary-care hospital, EIP staff reviewed ICU admission logs and communicated with clinicians daily. At the other six hospitals, a stimulated passive surveillance system was used in which physicians and infection control practitioners were given reminders several times per year. The active prospective method captured a greater proportion of total cases (86% of cases at the single hospital) then did the passive methods (50% of total cases at the six remaining hospitals).

If this surveillance is to be expanded, different methods may be chosen, depending on availability of resources and overall objectives. Less resource-intensive passive surveillance may be used if the goal is to monitor trends in disease occurrence. For example, although analyzing all death certifi-

Table 5. Infectious causes for "possibly" explained cases, UNEX, 1995–98, California, Oregon, Connecticut, and Minnesota (n=34)

Syndrome	Etiology (n)	Tests (n)
Neurologic	*Mycoplasma pneumoniae*	Remel EIA (IgM/IgG)[a]
	Influenza B virus (FLUBV)	Nasopharyngeal culture
	Varicella-zoster virus (reactivation)	EIA/IFA IgG
Respiratory	*Enterovirus*	EIA IgM
Cardiac	*Chlamydia pneumoniae*	MIF IgG
	Adenovirus	EIA IgM
	FLUBV	IFA IgM
Other[b]	Enterovirus (2)	IgM EIA

[a]EIA = enzyme immunosorbent assay, IFA = indirect immunofluorescent assay, IG = immunoglobulin, MIF = microimmunofluorescence, PCR = polymerase chain reaction.
[b]Other syndromes included one case with thrombotic thrombocytopenic purpura and one with hemolytic uremic syndrome.
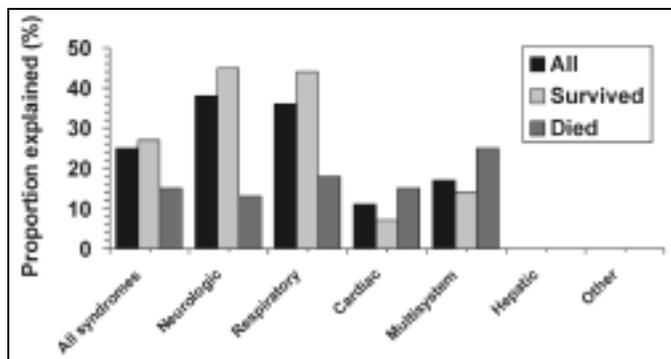
Figure 2. The explained proportions of cases by syndrome and survival status, 1995–1998, Surveillance for Unexplained Deaths and Critical Illnesses Due to Possibly Infectious Causes (UNEX).

cates for UNEX cases would be prohibitively time-consuming, electronically searching only the certificates in which the manner of death was recorded as natural, undetermined, or pending investigation could substantially decrease the workload. Under a passive system, maintaining good communication between study staff and clinical staff (clinicians, pathologists, and infection control practitioners) is critical and aided by the provision of diagnostic testing not locally available (such as serologic testing for hantavirus or toxin testing for botulism) and timely feedback of study results. Such collaboration may be critical to early diagnosis of diseases that produce characteristic clinical syndromes (e.g., potential bioterrorist agents such as botulism) or that are not readily confirmed by clinical laboratories.

Before initiating this project, we had reviewed multiple cause-of-death data for the United States to estimate the number of unexplained deaths from possibly infectious causes at these EIP sites (3). In 1992, the rate of unexplained deaths among healthy persons 1 to 49 years of age was 8.9 per 100,000 population. The discrepancy between this rate and that found in our study (0.5 per 100,000) is likely due to the low specificity of ICD-9 codes in excluding persons with previous health problems, as well as the problems related to retrospective analysis in general. For at least two reasons, we expect that the incidence of UNEX found in this study represents only a minimal estimate of the true burden of this problem. First, the denominator in our calculations included all persons in our designated groups, since we chose not to estimate the fraction of previously healthy persons in the surveillance populations at the four sites. Second, the differences in incidence rates between the four surveillance sites and results of the surveillance audits support the assumption that the overall rate detected was a minimal estimate of overall disease.

An important unresolved issue from our study is the large proportion of cases that remained unexplained, even after extensive laboratory testing. Although a standardized protocol for testing was used only during 1998, the proportion of explained cases before and after this protocol was used did not differ substantially. Some illnesses may have noninfectious causes, especially given the lack of specificity in our clinical criteria for case inclusion and in the features of infection in general. In cardiac syndromes, for example, myocarditis and myocardial infarction can have very similar presentations. Some cases may have been caused by microbial products such as toxins without the presence of the organism or substantial amounts of its nucleic acids. Laboratory methods for screening and detection of toxins remain inadequate. For some patients, specimens were not available from the primary site of disease, were severely limited in quantity, or were only available from late in the course of the disease; in many cases, multiple serum specimens were not available, autopsies were incomplete, and tissue specimens were obtained only from unaffected organs. Finally, the breadth of our testing methods may not have been adequate. Since broad-range PCR methods were applied only to bacteria and a limited range of viruses, many other potential agents may have been missed. Our approach to the detection of viral pathogens relied more heavily on serologic and immunohistochemical techniques, in part because of the difficulty in designing a comprehensive set of consensus PCR primers for all known viral families (21). In our study, viral testing was also constrained by limited experience with certain IgM assays. The development, testing, and application of comprehensive broad-range viral and fungal consensus primers for use in PCR assays may be helpful. Through this project, we created a population-based bank of clinical specimens that may prove valuable in the search for newly recognized etiologic agents, the development of diagnostic tests, and the standardization of nucleic acid-based techniques for identifying previously unknown etiologic agents.

This project represents an attempt to build capacity for early detection and response to emerging infectious diseases threats in the United States and elsewhere. The usefulness of this surveillance system for UNEX was recently illustrated during an outbreak of *West Nile virus* encephalitis in the northeastern United States (22) and an outbreak of unexplained illness among injecting drug users in Scotland and Ireland (23); initial reports of illness from both these investigations were received through the UNEX surveillance project, and initial testing was conducted through the infrastructure developed for this project. Future surveillance for UNEX may benefit from simplified case-finding methods, improved specimen quality, and more focused syndrome-specific surveillance. Once validated, surveillance methods may be adopted by the broader public health community. Such surveillance approaches will strengthen the collaboration between clinicians, laboratorians, and public health professionals, leading to improved detection of unexplained deaths and critical illnesses, including possible bioterrorism events, and better monitoring of emerging infectious diseases.

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Appendix I

Case Definition, Surveillance for Unexplained Deaths and Critical Illnesses Due to Possibly Infectious Causes, United States, 1995–1998

Previously Healthy

Patients are considered previously healthy who had no known preexisting chronic medical condition before the onset of the illness resulting in hospitalization or death, including malignancy; HIV infection; chronic cardiac; pulmonary, renal, hepatic, or rheumatologic disease; or diabetes mellitus. These patients have no history of immunosuppressive therapy, trauma thought to be related to illness, evidence of toxic ingestion or exposure, or nosocomial infection.

Reasons for Exclusion

1. A history of a malignancy other than nonmelanoma skin cancer
2. HIV infection identified during hospitalization, previously or after discharge
3. History of physician-confirmed myocardial infarction, angina with known coronary artery disease, or congestive heart failure
4. Any history of hospital admission for asthma or other pulmonary diseases except for uncomplicated pneumonia
5. History of dialysis or chronically elevated blood urea nitrogen and creatinine
6. Biopsy-proven liver disease of any kind or chronic coagulopathy or chronic *Hepatitis B* or *C virus* infection as a result of hepatic insufficiency
7. Physician-confirmed rheumatologic conditions requiring chronic or intermittent medical therapy with oral steroids or other immunosuppressive drugs
8. Any known physician-confirmed diabetes mellitus previously or during hospitalization
9. Development of hallmarks of infection >48 hours after hospital admission
10. Any mention of a history of excessive alcohol use, alcohol abuse, or alcoholism is a reason for exclusion (e.g., delirium tremens, withdrawal seizures, alcoholic neuropathy, persistent liver function test abnormalities, gastrointestinal bleeding, coagulopathy, or hypoalbuminemia).
11. Any mention of injecting drug use
12. Any history of neurologic disease, including seizures,
13. Obesity, defined as body mass index ≥30 or "obese" noted in medical chart
14. Physician-confirmed diagnosis of anorexia

Not Reasons for Exclusion

1. Hypertension or a history of hypertension
2. Any history of inhaler use
3. Pyelonephritis or nephrolithiasis or a history of either of these conditions in the absence of a chronically elevated blood urea nitrogen and creatinine
4. History of hepatitis
5. Pregnancy

Appendix III

Algorithm for Meningo-Encephalitis available online only at URL: http://www.cdc.gov/ncidod/EID/vol8no2/pdf/01-0165-app2.pdf

Appendix III

An Example of a Clinical Case Surveillance for Unexplained Deaths and Critical Illnesses Due to Possibly Infectious Causes, United States, 1995–1998

A 22-month-old boy from Oregon was healthy except for previous bouts of otitis media, for which tympanostomy tubes had been placed. Three days before admission, in May 1997, tactile fever was noted, and one day before admission, the patient had decreased activity and rhinorrhea. On the day of admission, he vomited twice. In the emergency room, he had a temperature of 39.2°C, was irritable and lethargic, and had nuchal rigidity. A complete blood count showed a total leukocyte count (WBC) of 18,300 (69% segmented cells, 8% bands). Cerebrospinal fluid (CSF) analysis showed a WBC of 54 (25% segmented cells, 75% monocytes), protein 38 mg/dL, and glucose 70 mg/dL. The patient was hospitalized and initially treated with ceftriaxone. On the next day, he became less responsive, and abnormal posturing developed in the left upper and lower extremities. A computed tomography scan of the head (without and with contrast) was normal. He was transferred to a tertiary-care center, where an electroencephalogram showed moderate generalized slowing and recurrent right hemispheric electrographic seizures. A magnetic resonance imaging scan done on the same day showed a diffusely increased white matter signal consistent with viral encephalitis or acute disseminated encephalomyelitis. The patient received acyclovir for 3 days. His responsiveness and clinical condition gradually improved, and he was transferred to a rehabilitation service 17 days after admission. Initial work-up at the hospital revealed negative blood cultures and negative bacterial and viral cultures of the CSF. PCR for Epstein-Barr virus in the blood and CSF was negative, as was PCR for herpes simplex virus in CSF.

The patient was enrolled in the UNEX project and evaluated. Specimens available for testing included acute- and convalescent-phase serum and CSF specimens. A variety of tests were conducted (see neurologic syndrome testing protocol in Appendix II). Because the quantities of specimens available were limited, testing was prioritized. First-round testing was negative for *Cytomegalovirus*, HHV-6, and arboviruses. However, testing for IgG antibodies (by IFA) for Epstein-Barr viral capsid antibodies showed a fourfold rise in titer between acute- and convalescent-phase serum specimens; testing for IgG antibodies (also by IFA) to Epstein-Barr early antigen revealed a fourfold decrease in titer between convalescent- and acute-phase serum specimens, indicating acute Epstein-Barr infection.

Lack of Evidence for Human-to-Human Transmission of Avian Influenza A (H9N2) Viruses in Hong Kong, China, 1999[1]

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In April 1999, isolation of avian influenza A (H9N2) viruses from humans was confirmed for the first time. H9N2 viruses were isolated from nasopharyngeal aspirate specimens collected from two children who were hospitalized with uncomplicated, febrile, upper respiratory tract illnesses in Hong Kong during March 1999. Novel influenza viruses have the potential to initiate global pandemics if they are sufficiently transmissible among humans. We conducted four retrospective cohort studies of persons exposed to these two H9N2 patients to assess whether human-to-human transmission of avian H9N2 viruses had occurred. No serologic evidence of H9N2 infection was found in family members or health-care workers who had close contact with the H9N2-infected children, suggesting that these H9N2 viruses were not easily transmitted from person to person.

In April 1999, two World Health Organization reference laboratories independently confirmed the isolation of avian influenza A (H9N2) viruses for the first time in humans (1). H9N2 viruses were isolated from nasopharyngeal aspirate specimens collected from two young children who were hospitalized in Hong Kong during March 1999 (2). The children were not related, were hospitalized at different facilities, did not have any known contact with or link to each other, and had not traveled outside Hong Kong (2). Both children had uncomplicated, febrile, upper respiratory tract illnesses and fully recovered (Table 1) (2). Evidence for five additional human illnesses attributed to H9N2 in Guangdong Province, China, during 1998 has been reported (3). Detection of antibody to H9N2 has been reported from persons in northern and southern China (3,4) and poultry workers in Hong Kong (5), suggesting that additional unrecognized human H9N2 infections have occurred.

H9N2 viruses have been prevalent in domestic poultry (chickens, ducks, geese, quail, and pigeons) throughout Asia since the early 1990s and were also isolated from swine in Hong Kong in 1998 (6). H9N2 viruses circulating in Asia have been classified into three antigenically and phylogenetically distinct sublineages (7). Two of these Asian H9N2 virus sublineages, influenza A/Quail/Hong Kong/G1/97 (G1-like lineage) and influenza A/Chicken/Hong Kong/G9/97 (G9-like lineage), were isolated from poultry in Hong Kong (6). The two Hong Kong children were infected by G1-like viruses, influenza A/Hong Kong/1073/99 and A/Hong Kong/1074/99 (8). The H9N2 viruses that have been isolated from poultry in Hong Kong are not highly pathogenic in chickens (8), whereas antigenic analysis of the H9N2 viruses isolated from humans in southern China suggested that they were more closely related to the G9-like viruses (9). However, the G1-like viruses contain internal genes that are highly homologous to those of highly pathogenic influenza A (H5N1) viruses isolated from chickens and humans in Hong Kong in 1997 (7).

The first and only documented human outbreak of highly pathogenic avian influenza A (H5N1) virus infections resulted in 18 hospitalizations and six deaths among Hong Kong residents during 1997 (10-12). A case-control study identified recent exposure to live poultry as an important risk factor for H5N1 infection (13), and cohort studies suggested that human-to-human transmission of H5N1 virus was limited (14,15). The poor transmissibility of these H5N1 viruses among humans and the elimination of approximately 1.5 million chickens appear to have been key factors that stopped this outbreak (12).

Avian populations, including domestic poultry and waterfowl, are the natural reservoir for all 15 known *Influenza A virus* (FLUAV) hemagglutinin (HA) subtypes, including H5 and H9 viruses (16). Viruses with novel HA can emerge when animal and human FLUAV genes undergo reassortment in the same host or when viruses from an animal host, such as swine or poultry, directly infect susceptible persons who lack protective immunity against the novel HA (17,18). In addition to ability to infect humans, the transmissibility of a novel *Influenzavirus* is a key factor influencing whether the novel virus can cause an influenza pandemic (19). The emergence of novel

Table 1. Clinical characteristics of two children infected with influenza A (H9N2) viruses, Hong Kong, 1999[a]

Patient characteristics	History, symptoms, signs on admission	Treatment received	Laboratory studies	Clinical course	Outcome
13-month-old girl; possible failure to thrive; no recent travel	Fever 39.5°C (1 day), poor appetite, vomiting, inflamed oropharynx	Cefuroxime Paracetomol Chloropheniramine Pseudoephedrine - Triprolidine (No antiviral medications)	CRP[b] 0.12 (mg/dL) (normal $\leq$0.8 mg/dL); WBC 2.22 x 10^9; AST 66 IU/L; CXR normal; U/A normal; NP aspirate for influenza A EIA: pos; NP aspirate for viral culture: pos for influenza A (H9N2), adenovirus type 3	Uneventful No fever at discharge. Duration of hospitalization March 5-7, 1999	Recovered, no sequelae
4-year-old girl, mild eczema, asthma, no recent travel	Fever 38.9°C (1 day), malaise, sore throat, headache, vomiting, abdominal pain, diarrhea inflamed oropharynx	Cefuroxime Cefotaxime Beclomethasone Paracetamol (No antiviral medications)	CRP 0.25 (mg/dL); (normal $\leq$0.8 mg/dL); WBC 12.5 x 10^9 (82%^{N}, 10%^{L}, 7%^{M}); CXR: normal; blood culture neg; stool culture neg; U/A normal; NP aspirate for influenza A EIA pos; NP aspirate for viral culture pos for influenza A (H9N2)	Persistent fever, no fever at discharge. Duration of hospitalization March 1-8, 1999	Recovered, no sequelae

[a]Source: Epidemiologic investigation by the Hong Kong Department of Health and review of medical records.
[b]CRP = C-reactive protein; WBC = leukocytes; AST = aspartate aminotransferase; CXR = chest X-ray; U/A = urinalysis; NP = nasopharyngeal; EIA = enzyme immunoassay

influenza A (H1N1), A (H2N2), and A (H3N2) viruses led to three influenza pandemics during the 20th century (19).

The identification of two children who had acute infection with novel H9N2 virus strains provided the first opportunity to investigate their transmissibility and pandemic potential among humans. We report the results of four retrospective cohort studies designed to detect serologic evidence of H9N2 virus infection among family members and health-care workers (HCWs) exposed to the two H9N2 patients, as well as unexposed controls.

Methods

The target populations included HCWs at the two hospitals where the H9N2-infected patients received care, as well as family and household members of the patients. The infectious period for an H9N2 patient was defined as a 15-day period beginning from the day before illness onset to the 14th day after illness onset (patient 1: February 27 to March 13, 1999; patient 2: March 3 to 17, 1999). The infectious period was defined conservatively to reflect the potential for prolonged viral shedding, especially since children can shed influenza viruses for longer periods than adults. Close contact was defined as coming within 3 m of an H9N2-infected patient. Participants were defined as exposed if they had close contact with an H9N2 patient during the infectious period. An unexposed person was defined as having had no contact with the H9N2 patients during the infectious periods. Unexposed subjects included family members and relatives who did not live in the same household as and had no contact with an H9N2 patient, and HCWs who worked on hospital units different from those where the H9N2 patients were located and who denied exposure to the H9N2 patients.

Study Design

We conducted four retrospective cohort studies of either HCWs or family and household members of the H9N2

patients. During face-to-face interviews conducted in either English or Cantonese, staff from the Hong Kong Department of Health administered a detailed questionnaire to a group of household members, family members, and relatives of each H9N2-infected child. The questionnaire assessed the level of exposure and contact with the H9N2-infected patient during the infectious period, along with other suspected risk factors for H9N2 infection, such as recent contact with poultry and swine. A similar questionnaire administered to HCWs asked about contact with each H9N2-infected patient during the patient's hospitalizations (patient 1: March 1-8, 1999; patient 2: March 5-7, 1999), and recent exposure to poultry and swine. All participants provided written, signed informed consent. Approximately 10 cc of blood was provided by each participant approximately 5 to 6 weeks (except where indicated) after the onset of the H9N2 patients' illnesses to test for antibody to H9N2.

Serologic Testing

Serum samples from all study participants and the two H9N2 patients were tested for antibody to FLUAV H9N2 by a microneutralization assay at both the Centers for Disease Control and Prevention (CDC), Atlanta, and the Hong Kong Department of Health Government Virus Unit Laboratory, as described (20), except that A/Hong Kong/1073/99 (HK/1073; H9N2) virus, isolated from patient 1, was used in the assay. Specimens from H9N2 patients were single serum samples collected 35 days (patient 2) and 39 days (patient 1) after illness onset. The virus isolated from patient 2 (A/Hong Kong/1074/99) was antigenically indistinguishable from HK/1073. Sera were considered positive by microneutralization if anti-H9 titers $\geq$80 were obtained in at least two independent assays.

At CDC, a Western blot assay with bromelain-purified or baculovirus-expressed recombinant hemagglutinin (rHA; Protein Sciences, Inc., Meriden, CT) from HK/1073 virus was used to confirm each positive microneutralization result, as

described (14). Microneutralization-positive sera were adsorbed with FLUAV H3N2 viruses to remove antibodies that were cross-reactive among FLUAV subtypes before retesting by microneutralization assay. Serum (50 µL) mixed with 100 µg of purified virus was incubated 45 min at 20°C and then 2 h at 4°C. Virus was pelleted by ultracentrifugation (30 min at 45,000 rpm and 4°C). To remove residual virus, serum was further adsorbed twice with 10% v/v turkey red blood cells (RBC) (30 min at 4°C) and then centrifuged to remove RBC (2 min at 12,000 rpm).

At the Government Virus Unit in Hong Kong, microneutralization-positive sera were confirmed by a single radial hemolysis assay for H9N2 antibodies, based on a modified, previously described protocol (21). HK/1073 virus-turkey RBC complexes, cross-linked by chromium, and complement were suspended in an agarose matrix. Sera were added to 2-mm diameter agarose wells. After overnight incubation at 35°C, a zone of hemolysis around the wells indicated the presence of anti-H9N2 antibodies. Sera producing hemolysis were absorbed with HK/1073 virus concentrate by mixing 15 µL of sera with 5 µL of virus concentrate, followed by a 1-h incubation at room temperature. The mixture was then retested as described. The absence of hemolysis confirmed the presence of H9N2 antibody. Absorption with A/Sydney/05/97 (H3N2)-like and A/Beijing/262/95 (H1N1)-like viruses was done to remove the nonspecific zones so only H9N2 antibody reacted on the single radial hemolysis plates. Sera were considered positive for H9N2 antibodies if the microneutralization assay and all confirmatory tests were positive in both laboratories.

Sera from the two H9N2-infected children were also tested by enzyme-linked immunosorbent assay (ELISA) to detect immunoglobulin (Ig) G and IgM antibodies to H9 as described (14), except that HK/1073 rHA (1 µg/mL) was used as the antigen. ELISA titers were calculated as the reciprocal of the highest dilution of sera that gave an A_{490} value greater than the mean A_{490} plus 3 standard deviations of six to seven negative age-matched controls at an equivalent dilution of sera. A titer $\geq 1,600$ was considered positive.

Statistical Analysis

Univariate analysis of associations between exposure variables and antibodies to H9N2 virus results were done by SAS 6.12 (SAS Institute Inc., Cary, NC).

Results

Serologic Response to H9N2 Virus Infection

Patient 1 was positive for antibodies to H9 by all serologic assays and had substantial titers of H9 HA-specific IgG and IgM antibodies (Table 2). Low titers of H9 HA-specific IgG and IgM antibodies were detected by ELISA in serum from patient 2, but no neutralizing antibody response was detected.

Study Participants

The demographic characteristics of the study participants are shown in Table 3. For H9N2 patient 1, exposed and unexposed family members or HCWs did not differ significantly by age or sex. For H9N2 patient 2, exposed and unexposed HCWs did not differ by age or sex. For family members of H9N2 patient 2, the unexposed participant was older than the exposed participants, but the number of study participants was very small. In the HCW cohorts of both H9N2 patients, more participants were women than men.

Family Member Cohort Studies

Fourteen of 15 eligible persons were enrolled in the family cohort study (3 exposed immediate family members and 11 unexposed relatives) of H9N2 patient 1. One exposed participant reported respiratory symptoms within the 2 weeks after onset of illness in the patient. This participant's serum was obtained 3 weeks after H9N2 patient 1's illness onset and was seronegative for H9N2 antibodies. No other participant reported respiratory illness. All 14 study participants tested seronegative for H9N2 antibodies (Table 3).

All seven family and household members eligible for the family cohort study of H9N2 patient 2 were enrolled (six exposed and one unexposed family and household members). Two exposed participants reported respiratory symptoms within 2 weeks after onset of illness in H9N2 patient 2. The unexposed participant reported no respiratory illness. All seven study participants tested seronegative for H9N2 antibodies (Table 3).

HCW Cohort Studies

The HCW study population for H9N2 patient 1 consisted of 30 exposed HCWs from 4 hospital units and 75 unexposed HCWs from 14 hospital units. Three exposed and three unexposed HCWs reported respiratory symptoms (cough, sore throat, or rhinorrhea) during H9N2 patient 1's hospitalization or within 5 days of the date of hospital discharge. All 30 exposed study participants were seronegative for H9N2 antibodies. One of the 75 unexposed HCWs was seropositive (Table 3). The HCW who tested seropositive for antibodies to H9N2 had no known exposure to a confirmed H9N2-infected patient and reported no contact with poultry or swine.

The HCW study population for H9N2 patient 2 was 15 exposed and 23 unexposed HCWs from four hospital units. One exposed HCW declined to participate. Four exposed HCWs reported respiratory symptoms beginning 2 to 5 weeks after contact with the patient. All 38 study participants tested seronegative for H9N2 antibodies (Table 3).

Discussion

These cohort studies suggest that influenza A (H9N2) viruses were not transmitted from the two H9N2-infected children to family and household members or HCWs who were

Table 2. Serologic responses of two patients from Hong Kong infected with influenza A (H9N2) virus

Patient	Age (years)	Sex	Serologic anti-H9 response				
			Days post symptom onset	Neutralizing antibody titer[a]	Western blot[b]	ELISA IgG[c]	ELISA IgM[c]
1	4	Female	39	135	Positive	51200	18100
2	1	Female	35	40	Positive	6400	1600

[a]Titers expressed as the geometric mean of four replicate titers; titers ≥ 80 were considered positive for anti-H9 antibodies.
[b]Western blots were performed by using a purified baculovirus-expressed recombinant HK/1073 HA as antigen.
[c]Enzyme-linked immunosorbent assay (ELISA) immunoglobulin (Ig) G and IgM antibodies were detected on plates coated with purified baculovirus-expressed recombinant HK/1073 HA (1 µg/mL). Titers are expressed as the geometric mean of duplicate endpoint titers estimated as described in Methods. A titer $\geq 1,600$ was considered positive for anti-H9 antibodies.

exposed to the H9N2 patients during their acute illness infectious periods. As described for the avian influenza A (H5N1) viruses (15,20), a combination of serologic assays was effective in detecting H9 virus-specific antibodies in two pediatric cases of H9N2 infection. However, the same serologic assays did not detect H9 antibodies in family members or HCWs exposed to the H9N2 patients. Only two known exposed persons, an HCW and a family member of one H9N2 patient, declined to participate in the studies. The HCW who tested seropositive for antibodies to H9N2 had no known exposure to a patient with confirmed H9N2 infection or contact with poultry or swine. The timing of H9N2 infection in this HCW could not be determined.

Evidence for influenza A (H9N2) infection as the cause of acute illness in the two patients includes the direct isolation of H9N2 viruses from nasopharyngeal aspirate specimens during the acute phase of illness (1) and the detection of H9-specific IgM antibodies, suggesting recent infection with an H9 virus. No other bacterial or viral pathogens were identified except for isolation of adenovirus type 3 from patient 1. The significance of the latter finding is unknown since this patient did not have typical signs of adenovirus type 3 infection, such as conjunctivitis. Isolation of adenovirus in this patient could represent acute atypical infection, acute subclinical infection, or persistent viral shedding from previous adenovirus infection.

The apparent lack of human-to-human transmission of avian H9N2 viruses and the low transmissibility of avian H5N1 viruses among humans have several possible explanations (14,15). The genomes of the H9N2 and H5N1 strains that were isolated from humans were derived entirely from avian influenza viruses; no reassortment with circulating human influenza A viruses had occurred. It is possible that the avian virus genome limits viral spread among humans. The molecular basis of influenza virus transmission among humans and other species remains poorly understood. However, following the introduction of an avian virus into humans, alterations in receptor-binding specificity of the HA are likely necessary for effective human-to-human transmission (22). Alternatively, the children may not have shed H9N2 virus in titers sufficient to facilitate transmission to other persons. Neither H9N2-infected child had coughing or sneezing that would have enhanced transmission to persons who had close contact with them.

To improve specificity for detecting antibody for H9N2 over that of the hemagglutination-inhibition antibody assays used previously (3), we used a combination of confirmatory tests and an adsorption step to reduce cross-reactivity with antibodies to other influenza viruses. Sera testing positive by neutralization test were then tested by Western blot assay. Sera positive for both these assays were further tested by neutralization assay following adsorption of sera with influenza A (H3N2) viruses. Sera that were negative for antibodies to H9N2 by neutralization assay were not tested by Western blot because of resource limitations. However, all sera from children who were contacts of the H9N2 patients, as well as the

Table 3. H9N2 serologic results of cohort studies involving family members and health-care workers, Hong Kong, 1999

	Patient 1		Patient 2	
Family members	Exposed (n=3)	Unexposed (n=11)	Exposed (n=6)	Unexposed (n=1)
Median age in year (range)	30 (2 to 31)	31 (<1 to 39)	31.5 (2 to 55)	68
Male:female	1:2	1:0.8	1:1	1:0
Seropositive	0	0	0	0
Health-care workers	Exposed (n=30)	Unexposed (n=75)	Exposed (n=15)	Unexposed (n=23)
Median age in year (range)	29.5 (19 to 51)	28 (19 to 59)	36 (24 to 56)	36 (25 to 50)
Male:female	1:4	1:3.4	1:14	0:23
Seropositive	0	1	0	0

patients themselves, were also tested by an H9-specific ELISA. Both patients but none of the exposed children tested positive for H9 antibodies.

Because of insufficient sera, the H9N2 patients were not tested for antibodies to neuraminidase (NA). The N2 NA of the H9N2 viruses isolated from patients is antigenically distinct from that of recent H3N2 human viruses, although some cross-reactivity with human H2N2 and early H3N2 viruses has been reported (8). However, additional studies from our laboratory indicate that the apparent cross-reactive antibodies that could be removed from some human sera by adsorption with H3N2 viruses was not due to cross-reactivity between the N2 NAs, since these sera also reacted with a reassortment H9N7 virus (CDC, unpub. data).

Because only two H9N2 cases were identified, we did not conduct a case-control study to identify risk factors for H9N2 infection. Thus, the sources and modes of acquisition of H9N2 for the two infected children are unknown. The Hong Kong Department of Health found that one H9N2 patient had very brief exposure to live chickens 11 days before onset of illness but did not directly touch the birds. No other contacts with live poultry, swine, or other animals for either H9N2 patient were found. There was no known contact or common exposure between the two H9N2 patients.

During the 1997 FLUAV (H5N1) outbreak in Hong Kong, a case-control study found that visiting a poultry stall or market with live poultry during the week preceding illness was the main risk factor for H5N1 infection (12). During that outbreak, the Hong Kong Department of Health enhanced its active surveillance for influenzalike illness and influenza viruses in hospitals, general outpatient clinics, and physicians' offices. This enhanced surveillance system detected the two novel H9N2 infections.

We were able to obtain only one convalescent-phase blood specimen from study participants, which limited our ability to document seroconversion. However, none of the exposed persons were seropositive for H9N2. Currently, there are no seroprevalence data on rates of H9N2 infection in children or the general population. One study of a cohort of poultry workers in Hong Kong found that approximately 30% were seropositive for antibodies to H9N2 (5). Ongoing surveillance and availability of H9N2-specific reagents should facilitate timely identification of H9N2 infection and allow collection of paired sera for further studies of person-to-person transmission.

In addition to H9N2, other avian influenza viruses have been isolated from specimens collected from Hong Kong poultry since 1997, including H6, H4, and H11 viruses (23). During April and May 2001, highly pathogenic avian influenza A (H5N1) viruses were again isolated from live poultry in Hong Kong markets (24). After chicken deaths were observed in some markets, the Hong Kong government temporarily closed all wholesale and retail live poultry markets for cleaning, stopped importing poultry from China, and slaughtered approximately 1.3 million birds during May 2001. The poultry markets reopened in June 2001. No human illnesses attributed to avian influenza viruses have been identified since the two H9N2 cases in 1999. However, these recent events have heightened the need to understand the public health risk of H5N1, H9N2, and other avian influenza viruses.

These limited studies suggest that avian influenza A (H9N2) viruses were not transmitted from the two infected children to exposed household members, relatives, or HCWs in Hong Kong. However, H9N2 viruses are widely distributed in avian populations, can infect humans, and could evolve or undergo genetic reassortment with potential for increased pathogenicity and transmissibility in humans. The recent emergence of human infections with avian influenza A (H9N2) and (H5N1) viruses highlights the need to improve surveillance for influenza viruses in poultry, swine, and humans, especially in Asia. Further studies to assess the health risks posed by H9N2 and other avian influenza viruses are warranted.

Acknowledgments

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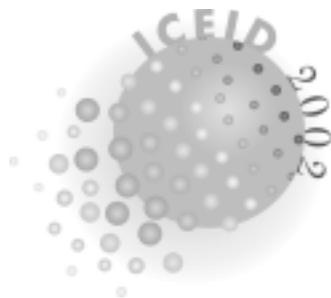
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Community-Acquired *Klebsiella pneumoniae* Bacteremia: Global Differences in Clinical Patterns

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We initiated a worldwide collaborative study, including 455 episodes of bacteremia, to elucidate the clinical patterns of *Klebsiella pneumoniae*. Historically, community-acquired pneumonia has been consistently associated with *K. pneumoniae*. Only four cases of community-acquired bacteremic *K. pneumoniae* pneumonia were seen in the 2-year study period in the United States, Argentina, Europe, or Australia; none were in alcoholics. In contrast, 53 cases of bacteremic *K. pneumoniae* pneumonia were observed in South Africa and Taiwan, where an association with alcoholism persisted (p=0.007). Twenty-five cases of a distinctive syndrome consisting of *K. pneumoniae* bacteremia in conjunction with community-acquired liver abscess, meningitis, or endophthalmitis were observed. A distinctive form of *K. pneumoniae* infection, often causing liver abscess, was identified, almost exclusively in Taiwan.

*K*lebsiella pneumoniae is among the most common gram-negative bacteria encountered by physicians worldwide. It is a common hospital-acquired pathogen, causing urinary tract infections, nosocomial pneumonia, and intraabdominal infections. *K. pneumoniae* is also a potential community-acquired pathogen. In this international collaborative study, we evaluated geographic differences and trends in three prominent presentations of community-acquired *Klebsiella* infection.

First, *K. pneumoniae* has been a recognized pulmonary pathogen since its discovery >100 years ago. The classic clinical presentation is dramatic: toxic presentation with sudden onset, high fever, and hemoptysis (currant jelly sputum). Chest radiographic abnormalities such as bulging interlobar fissure and cavitary abscesses are prominent. However, the incidence of community-acquired *Klebsiella* pneumonia has apparently declined in the United States (1,2). In studies from the 1920s to the 1960s, *K. pneumoniae* was considered an important cause of community-acquired pneumonia (2); however, in the last decade *K. pneumoniae* accounted for <1% of cases of pneumonia requiring hospitalization in North America (3,4).

Second, a striking clinical finding concerning a new manifestation of community-acquired *K. pneumoniae* infections has been documented. An unusual invasive presentation of *K. pneumoniae* infection, primary bacteremic liver abscess, has been described by numerous investigators in Asia; >900 patients with *Klebsiella* liver abscess have been reported from Taiwan in the last 10 years (5-22). In addition, case reports and small series from Korea, Singapore, Japan, India, and Thailand have been published (23-32). The Taiwanese patients with *K. pneumoniae* liver abscess have no history of hepatobiliary disease. Seventy percent of such patients have diabetes mellitus (5,13,18,19); 11% to 12% of the reported patients with *Klebsiella* liver abscess have other septic metastatic lesions, including pulmonary emboli or abscess, brain abscess, pyogenic meningitis, endophthalmitis, prostatic abscess, osteomyelitis, septic arthritis, or psoas abscess (5,13,19).

The third striking clinical observation is the preponderance of *K. pneumoniae* as a cause of community-acquired bacterial meningitis in adults in Taiwan, even in the absence of liver abscess or other sites of infection. The proportion of cases of culture-proven bacterial meningitis due to *K. pneumoniae* in one Taiwanese hospital increased from 8% during 1981 and 1986 to 18% during 1987 to 1995 (33). In contrast, in a recent large review only 3 (1.2%) of 253 cases of community-acquired bacterial meningitis from the Massachusetts General Hospital were due to *K. pneumoniae* (34).

Given these empiric observations, we established an international collaboration of researchers from each of the world's populated continents. These investigators worked in large tertiary-care hospitals or hospitals serving veterans. One of our aims was to delineate in a single time period, with a consistent set of definitions, global differences in the clinical manifestations of serious *K. pneumoniae* infections. We also examined the influence of prior antibiotic use on these differences in *K. pneumoniae* infections.

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Methods

A prospective study of consecutive patients with community-acquired *K. pneumoniae* bacteremia was performed in 12 hospitals.[1] The study period was January 1, 1996, to December 31, 1997. Records of patients >16 years of age with positive blood cultures for *K. pneumoniae* were reviewed, and a 188-item study form was completed. All items on the form were objective criteria, allowing standardization among medical centers. The study was observational in that administration of antimicrobial agents and other therapeutic management were controlled by the patient's physician, not the investigators.

Community-acquired bacteremia was defined as a positive blood culture taken on or within 48 hours of admission. Severity of acute illness at the time of positive blood cultures was assessed by a previously validated scoring system, based on mental status, vital signs, need for mechanical ventilation, and recent cardiac arrest (Pitt bacteremia score) (35). Type of infection was defined as pneumonia, urinary tract infection, meningitis, incisional wound infection, other soft tissue infection, intraabdominal infection, and primary bloodstream infection, according to Centers for Disease Control and Prevention definitions (36). In addition, distinctive sites of *K. pneumoniae* bacteremia were further defined as liver abscess, meningitis, or endophthalmitis. Liver abscesses were defined by the coexistence of blood cultures positive for *K. pneumoniae* and evidence of an intrahepatic abscess cavity by ultrasonography or computed tomography. Meningitis was defined as culture of *K. pneumoniae* from cerebrospinal fluid, and endophthalmitis was defined as decreased visual acuity, pain, hypyon, or severe anterior uveitis in a patient concurrently bacteremic with *K. pneumoniae*. Death was defined as including deaths from all causes within 14 days of the date the first positive blood culture for *K. pneumoniae* was obtained.

Blood cultures of *K. pneumoniae* were sent by participating hospitals on nutrient agar slants to a central study laboratory in Pittsburgh, where the identity of each isolate as *K. pneumoniae* was confirmed by the Vitek GNI system (Biomerieux Vitek, Hazelwood, MO). Extended-spectrum beta-lactamase (ESBL) production was defined phenotypically by broth dilution as a ≥ 3 twofold concentration decrease in MIC for either cefotaxime or ceftazidime tested in combination with clavulanic acid compared with the MIC when tested alone. The protocol was reviewed and approved by Institutional Review Boards according to local requirements.

All data were entered into a central database (PROPHET version 5.1; BBN Systems and Technologies Corporation, Cambridge, MA). Contingency data were analyzed by two-tailed chi-square or Fisher's exact tests, and continuous data were analyzed by Student *t* test or Mann-Whitney U test.

Results

Two hundred two (44.4%) of 455 episodes of *K. pneumoniae* bacteremia during the study period were community-acquired cases. The percentage of cases of *K. pneumoniae* bacteremia that were community acquired in each study country differed strikingly: 96 (68%) of 142 in Taiwan, 25 (43%) of 68 in the United States, 28 (39%) of 71 in Australia, 40 (34%) of 116 in South Africa, 6 (22%) of 27 in Europe, and 7 (17%) of 41 in Argentina. *K. pneumoniae* bacteremia in Taiwan was significantly more likely to be community acquired than was bacteremia in the other countries combined (68% vs. 36%, p=0.0001).

The characteristics of patients with community-acquired *K. pneumoniae* bacteremia from Taiwan, South Africa, and the rest of the world were compared (Table 1). The source of bacteremia in community-acquired cases was geographically distinctive (Table 2). Pneumonia was the most common infection worldwide, accounting for 57 (28%) of 202 cases. However, 53 (93%) of 57 of all cases of community-acquired *K. pneumoniae* pneumonia occurred in Taiwan and South Africa.

Antibiotics had been used for >24 hours before admission in 21 (10%) of 202 patients. Prior antibiotic use was significantly lower in Taiwan (4 [4%] of 96 patients) and South Africa (2 [5%] of 40) than in the other countries (15 [23%] of

Table 1. Clinical characteristics of patients with community-acquired *Klebsiella pneumoniae* bacteremia from Taiwan, South Africa, and other countries

Clinical characteristics	Taiwan (n=96)	South Africa (n=40)	Other countries (n=66)	p value[a]
Age (mean, years)	58.8	47.2	59.9	0.014
Female, n (%)	35 (38%)	17 (42%)	24 (36%)	NS
Underlying diseases, n (%)				
Diabetes mellitus	38 (40%)	8 (20%)	19 (29%)	0.06
Liver disease	33 (34%)	6 (15%)	12 (18%)	0.02
Alcoholism	12 (12%)	3 (8%)	1 (2%)	0.08
Malignancy	15 (16%)	3 (8%)	25 (38%)	0.0002
HIV infection	0 (0%)	7 (18%)	0 (0%)	0.0001
Chronic renal failure	6 (6%)	2 (5%)	6 (9%)	NS
Organ transplant	0 (0%)	0 (0%)	7 (11%)	0.0006
Corticosteroid use	5 (5%)	1 (3%)	7 (11%)	NS
No underlying disease	22 (23%)	21 (52%)	24 (36%)	0.003
Critically ill[b]	30 (31%)	12 (30%)	2 (3%)	0.0001
Death rate at 14 days, n (%)	30 (31%)	24 (60%)	8 (12%)	0.0001

[a]p values refer to differences between the three regions; NS = not significant at p >0.20.
[b]Critically ill defined as Pitt bacteremia score ≥ 4.

Table 2. Worldwide differences in the sites of infection associated with community-acquired *Klebsiella pneumoniae* bacteremia

Infection site	Taiwan[a] (n=96)	South Africa (n=40)	Other countries (n=66)	p value[b]
Pneumonia	28 (29%)	25 (62%)	4 (6%)	0.0001
Liver abscess	17 (18%)	0	1 (2%)	0.0002
Endophthalmitis	1 (1%)	0	0	NS
Meningitis	5 (5%)	2 (5%)	0	0.06
Urinary tract infections	14 (15%)	4 (10%)	25 (38%)	0.0003
Acute cholangitis	13 (14%)	0	12 (18%)	0.02
Intravascular catheter-related infections	0	2 (5%)	11 (17%)	0
Skin and soft tissue infections	5 (5%)	1 (3%)	4 (6%)	NS
Spontaneous bacterial peritonitis	7 (7%)	0	1 (2%)	0.06
Intraabdominal abscess	2 (2%)	2 (5%)	2 (3%)	NS
Other	1 (1%)	0	2 (3%)	NS
No primary site evident	7 (7%)	5 (12%)	4 (6%)	NS

[a]Four Taiwanese patients had more than one site of infection: pneumonia and liver abscess (2), liver abscess and meningitis (1) and pneumonia and endophthalmitis (1). One South African patient had both pneumonia and meningitis.
[b]p values refer to the differences between the three regions; NS = not significant at p >0.20.

66) (p=0.0003). In four patients, the prior antibiotic was an oxyimino-containing cephalosporin (two in South Africa, one in Argentina, and one in Taiwan).

ESBL production was detected in 7 (3.5%) of 202 community-acquired strains compared with 78 (30.8%) of 253 hospital-acquired strains (p<0.00001). None of the patients with community-acquired ESBL-producing strains had recently received an oxyimino-containing cephalosporin. However, of the seven patients with community-acquired ESBL-producing *K. pneumoniae* bacteremia, only one (a patient with pneumonia from Africa) had no recent hospital exposure. The countries from which community-acquired ESBL-producing *K. pneumoniae* isolates were collected included Africa (three isolates), Turkey (two), the United States (one), and Australia (one). Twenty-five bloodstream isolates were ciprofloxacin resistant, of which 7 (28%) of 25 were community acquired. All these seven patients had serious underlying disease and frequent hospitalizations or nursing home admissions, and none had received a quinolone in the 14 days before hospital admission. Five of seven patients with community-acquired, ciprofloxacin-resistant *K. pneumoniae* bacteremia were from Taiwan.

Community-Acquired Pneumonia

Community-acquired pneumonia due to *K. pneumoniae* was significantly associated with alcoholism (p=0.007); 18%

of patients with pneumonia were alcoholics as defined by their physicians, compared with 4% with other sources of *K. pneumoniae* bacteremia (Table 3). However, no patient with community-acquired *K. pneumoniae* bacteremic pneumonia outside South Africa or Taiwan was an alcoholic; of these patients, one was neutropenic, two were nursing home residents with neurologic impairment (ages 81 and 90), and one was a Vietnamese immigrant to Australia with no underlying illness.

Community-acquired pneumonia due to *K. pneumoniae* was significantly associated with HIV infection on univariate evaluation (p=0.002). Of the seven patients with HIV infection and *K. pneumoniae* bacteremia (all from Africa), six had community-acquired pneumonia. Community-acquired pneumonia due to *K. pneumoniae* was not associated with underlying liver disease, chronic renal failure, receipt of chemotherapy for malignant disease, or receipt of corticosteroids.

Multivariate analysis showed that residing in Africa (p=0.0001) or Taiwan (p=0.0046) and being an alcoholic (p=0.04) were significantly associated with community-

Table 3. Comparison of the characteristics of patients with community-acquired bacteremic pneumonia due to *Klebsiella pneumoniae* and other patients with community-acquired *K. pneumoniae* bacteremia: association between pneumonia and alcoholism and residence in South Africa

Characteristic	Bacteremic pneumonia (n=57)	Bacteremia without pneumonia (n=145)	p value[a]
Resides in South Africa	25 (44%)	15 (10%)	<0.001
Age (years)	53.6	58.6	0.07
Serum creatinine[b] (mg/dL)	2.1	2.3	0.2
Blood urea nitrogen[b] (mg/dL)	34.8	37.9	NS
Liver function tests[b,c]			
Serum albumin (g/mL)	2.8	3.1	0.05
Serum bilirubin (mg/dL)	2.8	2.9	NS
AST (IU/mL)	174	303	NS
ALT (IU/mL)	115	189	NS
Underlying disease			
Diabetes mellitus (%)	12 (21%)	53 (37%)	0.03
Alcoholism (%)	10 (18%)	6 (4%)	0.007
Malignancy (%)	7 (12%)	36 (25%)	0.05
HIV infection (%)	6 (10%)	1 (1%)	0.002
No underlying disease	23 (40%)	44 (30%)	NS (0.17)
Critically ill	21 (37%)	23 (16%)	0.001
Death rate at 14 days (%)	31 (54%)	32 (22%)	0.0001

[a]NS = not significant at p >0.20; AST = aspartate aminotransferase; ALT = alanine aminotransferase.
[b]Laboratory values are those taken on first visit to a health-care provider; for continuous variables, the figures in the table are mean values.
[c]Bacteremic patients with liver cirrhosis, acute cholangitis, and liver abscess were excluded from the analysis of liver function tests.

acquired *K. pneumoniae* pneumonia. HIV infection was not independently associated with pneumonia (p=0.23). The death rates from community-acquired pneumonia due to *K. pneumoniae* were 54% in Taiwan and 56% in South Africa.

A Distinctive *K. pneumoniae* Bacteremia Syndrome

Twenty-five patients had a distinctive syndrome of *K. pneumoniae* bacteremia, which was defined by the presence of *K. pneumoniae* bacteremia in conjunction with liver abscess, endophthalmitis, or meningitis. Of these patients, 88% (16 with liver abscess, 4 with meningitis, 1 with liver abscess and meningitis, and 1 with endophthalmitis) were from Taiwan, compared with 12% from the other countries combined (2 with meningitis from South Africa and 1 with liver abscess from Belgium) (p=0.0001).

Twelve (67%) of 18 patients with liver abscess had diabetes mellitus. On univariate analysis, residing in Taiwan (p=0.0001) and having diabetes mellitus (p=0.001) were significantly associated with community-acquired *K. pneumoniae* liver abscess. Patients with liver abscess were more likely to have renal failure, but this association was not statistically significant (p=0.09). There was no association between liver abscess and gender, age, previous antibiotic use, or presence of underlying liver disease. Multivariate analysis showed that residence in Taiwan (p=0.0034), diabetes mellitus (p=0.0058), and renal failure (p=0.0178) were significantly associated with the presence of liver abscess.

Patients with any of the distinctive manifestations of *K. pneumoniae* (liver abscess, meningitis, or endophthalmitis) were compared with patients with other community-acquired infections (Table 4). These complications were significantly associated with diabetes mellitus (60% vs. 28%, p=0.0015) and living in Taiwan (88% vs. 42%, p=0.0001).

Discussion

When pneumonia due to *Klebsiella* was first described by Friedlander in 1882, he believed it to be the most common cause of bacterial pneumonia (37). Although this concept was soon refuted in favor of pneumococcus, from the 1930s through the 1960s, 10 to 50 cases of *Klebsiella* pneumonia were reported each year by large hospitals in the United States (2). U.S. textbooks of medicine continue to list *K. pneumoniae* as an important cause of community-acquired pneumonia (38,39).

In our prospective study, we found only four cases of community-acquired *K. pneumoniae* pneumonia in 2 years in nine large hospitals from the United States, Australia, Europe, and Argentina. The hospitals surveyed included an inner-city veterans hospital in the United States and two large inner-city public hospitals in Australia. These three centers care for large numbers of indigent and alcoholic patients.

Recently published reports from the United States, Israel, and Europe support our observations. Neither Vergis et al. (40) from the United States nor Lieberman et al. (41) from Israel found a single case of *K. pneumoniae* pneumonia in large multicenter studies of community-acquired pneumonia in the

Table 4. Comparison of the characteristics of patients with distinctive infections associated with community-acquired *Klebsiella pneumoniae* bacteremia (liver abscess, meningitis, and endophthalmitis) and other patients with community-acquired *K. pneumoniae* bacteremia: association with diabetes mellitus and residence in Taiwan

Characteristic	Liver abscess, endophthalmitis or meningitis (n=25)	Other community-acquired bacteremia (n=177)	p-value[a]
Resides in Taiwan	22 (88%)	74 (42%)	0.0001
Age (years)	55.5	57.4	NS
Serum creatinine[b] (mg/dL)	2.2	2.2	NS
Blood urea nitrogen (mg/dL)	35.7	37.2	NS
Underlying disease			
Diabetes mellitus (%)	15 (60%)	50 (28%)	0.0015
Chronic renal failure (%)	3 (12%)	11 (6%)	NS
Underlying liver disease (%)	5 (20%)	46 (26%)	NS
Chronic *hepatitis B virus* infection	0	9 (5%)	NS
Hepatitis C virus infection (%)	1 (4%)	8 (5%)	NS
Alcoholism (%)	2 (8%)	14 (8%)	NS
Malignancy (%)	0	43 (24%)	0.006
No underlying disease	6 (24%)	61 (34%)	NS
Critically ill[c]	9 (36%)	35 (20%)	0.07
Death rate at 14 days (%)	8 (32%)	54 (31%)	NS

[a]NS = p >0.20.
[b]Laboratory values are those taken on first visit to a health-care provider; for continuous variables, the figures in the table are mean values.
[c]Critically ill defined as Pitt bacteremia score ≥4.

1990s. Nine European studies published since 1990 show that only 14 (2.3%) of 621 patients admitted with severe community-acquired pneumonia requiring intensive-care unit admission had *K. pneumoniae* as the presumptive etiologic agent (42-50). In contrast, *K. pneumoniae* continues to be associated with community-acquired pneumonia in Africa and Asia. In our study, we observed 28 cases in Taiwan and 25 in South Africa, accounting for 29% and 62% of all cases of community-acquired *K. pneumoniae* bacteremia in Taiwan and South Africa, respectively (Table 2). Recent studies from Taiwan, Singapore, and South Africa corroborate these findings. In a Taiwanese study, *K. pneumoniae* accounted for 34% of 41 cases of community-acquired bacteremic pneumonia (51). *K. pneumoniae* was the cause of 15% of community-acquired pneumonia requiring intensive-care unit admission in Singapore (52). *K. pneumoniae* was found to be the cause of pneumonia in 32% of African patients with severe community-acquired pneumonia requiring intensive-care unit admission in Johannesburg (53) and 11% of patients requiring intensive-care unit admission in Cape Town (54).

In our study, 18% of patients with community-acquired *K. pneumoniae* pneumonia were alcoholics (p=0.007) (Table 3).

Alcoholics in Africa and Asia may have limited access to health care (perhaps including reduced access to antibiotics) compared with those in the Americas, Europe, and Australia, and may have respiratory symptoms later. A weakness of our study is that we were not able to ascertain the duration of symptoms before each patient was hospitalized. However, a recent study of aborigines from rural northern Australia (35% of whom were alcoholics and most of whom had suboptimal access to health-care facilities) showed that none of 90 admitted to hospital with community-acquired pneumonia had *K. pneumoniae* infection (55). The hypothesis that *Klebsiella* pneumonia is related to poor primary health care for alcoholics may therefore be less plausible.

Bacteremic *K. pneumoniae* liver abscess occurred almost exclusively in patients from Taiwan (Table 2), consistent with a growing number of reports from Asia describing this distinctive type of infection. *K. pneumoniae* was the most common cause of liver abscesses in Taiwan, Singapore, and Korea in reports from 1990 to 1999 (5,9,24,31,32); similarly, numerous reports of liver abscess have recently been published from Hong Kong, Thailand, and Japan (23,25-30). In total, >900 patients with *K. pneumoniae* liver abscess have been reported from Asian countries in the last 10 years; in contrast, reports of only 23 patients with this condition have been published from regions outside Asia in this same period (56-63).

K. pneumoniae meningitis in adults has also been infrequently reported from North America, Europe, and Australia, in contrast to Taiwan. In our study, five cases of bacteremic *K. pneumoniae* meningitis were in Taiwanese patients and two in African patients (Table 2). Four (57%) of 7 patients with meningitis had prior diabetes mellitus. Meningitis caused by *K. pneumoniae* in the United States, Australia, and Europe is most often hospital acquired and associated with prior neurosurgical procedures or instrumentation. However, of 115 cases of *K. pneumoniae* meningitis reported from Taiwan (33,64,65), 84% were community acquired, and 64% of cases had concurrent *Klebsiella* bacteremia. Unlike pyogenic liver abscess, the clinical course was fulminant, with a death rate of 57% (33,64,65). The death rate from bacteremic *K. pneumoniae* meningitis in our series was 71%.

We found only one patient (an alcoholic from Taiwan) with *K. pneumoniae* bacteremia and endophthalmitis. *K. pneumoniae* endophthalmitis is also likely to be far more common in Asia than elsewhere; >50 cases have been reported in the last 10 years from Asia (6,10,11,19,27,66-70) compared with only 10 from the United States, Europe, and Australia (57,60,62,71-76). More than 50% of previously reported Asian patients with *K. pneumoniae* endophthalmitis have had concurrent liver abscess (6,10,11,19,27,66-70).

The reason for the geographic preponderance of these severe manifestations of *K. pneumoniae* infections in Asia is unknown. The geographic diversity of *Klebsiella* infections possibly results from interaction between bacterial variables, host variables (for example, defects in host defense caused by diabetes mellitus or alcoholism), socioeconomic factors, and possibly genetic susceptibility in different racial groups. We are studying the phenotypic and genotypic differences in *K. pneumoniae* causing different disease manifestations in different countries. Because no more than three hospitals from each country were included in our study, our results may not necessarily be generalizable to hospitals in other regions. In addition, other countries in the same continent (e.g., other countries in Asia or eastern Europe) were not studied but may have different clinical patterns compared with the study country.

In summary, our results challenge the classic view of serious *Klebsiella* infections. In the United States, Europe, Argentina, and Australia, we have observed that hospital-acquired *K. pneumoniae* infections predominate, with community-acquired bacteremia being caused by urinary tract infection, vascular catheter infection, and cholangitis. Classic community-acquired pneumonia is no longer an important entity in these regions. In South Africa, pneumonia (especially in alcoholics) continues to be an important community-acquired infection. In Taiwan, community-acquired pneumonia persists, and distinctive infections such as liver abscess, endophthalmitis, and meningitis have emerged as substantial public health problems.

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Buruli Ulcer in Ghana: Results of a National Case Search

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A national search for cases of Buruli ulcer in Ghana identified 5,619 patients, with 6,332 clinical lesions at various stages. The overall crude national prevalence rate of active lesions was 20.7 per 100,000, but the rate was 150.8 per 100,000 in the most disease-endemic district. The case search demonstrated widespread disease and gross underreporting compared with the routine reporting system. The epidemiologic information gathered will contribute to the design of control programs for Buruli ulcer.

Buruli ulcer disease is assuming public health importance in many countries, prompting the establishment of a Global Buruli Ulcer Initiative by the World Health Organization (WHO) in early 1998. Ever since *Mycobacterium ulcerans* infection was first described in Australia in 1948 (1) and later named Buruli ulcer in Uganda (2), cases have been reported throughout the tropical and subtropical world. In the African WHO region, at least 16 of 46 member countries report cases, especially in West Africa and parts of eastern and central Africa (3).

One characteristic of the disease is its apparent association with bodies of water worldwide (4,5). The recent identification of *M. ulcerans* in certain water insects has raised the possibility of mechanical transmission of the infection (6).

Buruli ulcer commonly affects the young, even though cases are reported in all age groups (7,8). Oluwasanmi et al. (5) and van der Werf (8) did not find any sex difference in their series, but Barker reported prevalence to be higher among women than men and among boys than girls. The disease is characteristically found more often on the extremities than on the trunk (9). The infection is usually restricted to relatively small areas and patchy in its distribution (10,11).

The first probable case of Buruli ulcer in Ghana was reported in the Greater Accra Region in 1971; the presence of additional cases along the tributaries of the Densu River in the area was considered likely (12). In 1989, van der Werf et al. described 96 cases in the Asante Akim North District of Ashanti Region (8). This report was followed by the description of a major endemic focus in Amansie West District in the same region (13). Since then, isolated cases have been found in scattered communities in many parts of the country, generating much political and media concern and interest.

In 1993, a passive surveillance system for reporting Buruli ulcer was initiated in Ghana. By the end of 1998, approximately 1,200 cases had been reported from four regions. Gross underreporting was suspected, however, as the media continued to report cases in remote rural communities. Because most cases were known to be in relatively deprived, inaccessible

areas, the routine reporting system was judged inadequate to provide a true picture of the extent of disease and the geographic distribution of cases for design of a national control program. In addition, a case search would provide baseline data against which intervention measures could be assessed.

The main objective of the national case search was to establish the extent of the disease in Ghana to facilitate development of a national program for its control. The specific objectives were to determine the epidemiologic characteristics of Buruli ulcer in Ghana and determine physical accessibility of disease-endemic communities to health-care services.

Methods

Definition of Variables

Geographic distribution was defined in terms of regional, district, subdistrict, and community distribution of cases. The burden of disease was considered in terms of number of cases affected, age and sex distribution, clinical presentation (preulcerative, ulcer, or deformity), and site of lesion. Preulcerative lesions include nodular, plaque, papular, and nonulcerative edematous forms, as described by the WHO Global Buruli Ulcer Programme (3). Deformities include scars, constriction of limbs, ankylosis of joints, or amputations.

The case search covered every district and known community in Ghana from June to July 1999. A team of 20 national facilitators was trained in the use of the survey instruments and in the clinical presentation of the disease in an endemic focus. Two facilitators were then sent to each region to train regional teams (three from the regional level and two from each district). Seven teams of two persons each from the subdistrict and communities performed the case search.

The permission of the local political and traditional authorities was sought in advance, and the purpose of the search was explained to them and to all participants. The data collectors used a pictorial document designed by the WHO Global Buruli Ulcer Initiative (3). At each village and community, they showed the pictures of Buruli ulcer disease at different stages of development to as many people as possible and asked whether anyone in the village had a similar condition. All persons with lesions that met the WHO standard case definition were interviewed with a simple questionnaire. There was no

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laboratory confirmation of the cases. A prepackaged dressing was given to each person identified as having ulcers, and the particulars of all the cases were provided to local health authorities for follow-up. The process was repeated in each village until the whole district was covered.

A team from the national level, including a dermatologist familiar with the disease, later validated findings in two randomly selected districts from a region where the disease had not previously been endemic. All cases reported there were found to be consistent with the clinical case definitions used.

Data Entry and Analysis

Data from all the regions were entered centrally and later cross-checked and edited by EpiInfo 6 software. Data analysis was done both manually and by EpiInfo 6, as appropriate. Yates corrected chi-square and Cornfield 95% confidence interval tests were used as the statistical tests.

Information on regional population distribution was taken from the 2000 national census. About 200 forms from Atwima District of Ashanti Region had to be excluded from the data analysis because information was incomplete on almost all variables. Records with missing data for a particular variable were also excluded from analysis of that variable.

Results

We identified 5,619 patients with 6,332 suspected Buruli lesions at various stages of development. Approximately 48.5% of the lesions were at the ulcerative stage (Figure 1) and 12.5% at the preulcerative stage; 36.3% had formed scars (Figure 2), and 2.7% of lesions were associated with other forms of deformities.

Of the 5,619 patients, 46% had ulcers only, 10.8% had preulcerative lesions only, and 33.4% had healed scars only. Lesions at both ulcerative and preulcerative stages were seen in 2.5% of the patients; the rest had various combinations of lesions. If only active lesions (preulcerative or ulcerative) were used to calculate the prevalence of the disease in Ghana, 3,725 of the patients (66.3%) had active lesions and 1,894 (33.7%) had healed lesions, for a national crude prevalence rate of 20.7 per l00,000.

Age and Sex Distribution

Approximately 49% of all patients for whom data were available (n=5,596) were female. The ages of those with active lesions ranged from 0.8 to 100 years (median 25 years; first quartile 12 years; third quartile 50 years; and mean 32 years.)

Among patients with active lesions, age is significantly associated with sex. There are significantly more females than males among patients ≥20 years than those <20 years old (chi square = 14.9; p=0.0001; odds ratio [OR]=1.3; 95% confidence interval [CI] 1.13-1.48). The age-specific odds ratio for male likelihood of having an active Buruli lesion is 0.58 (CI 0.5-0.68; p=0) while that for females is 0.73 (CI 0.62-0.68; p=0.0002).

Figure 1. Buruli ulcer on left ankle.
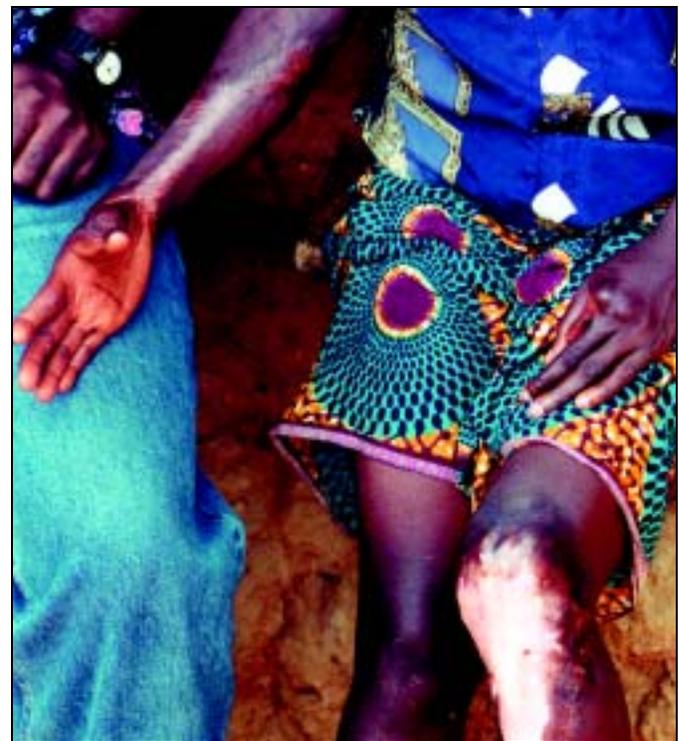
Figure 2. Healed Buruli lesions with scarring, right forearm and left knee.

Of 5,772 lesions for which the information was available, 25.1% were located on the arms and hands, 65.6% on the legs and feet, 5.4% on the trunk, and 3.8% on the head and neck. The distribution of lesions on the limbs (upper or lower limbs) is significantly associated with the age of the patient. Patients >20 years of age have more lesions on the limbs than the younger ones (chi square=16.4; p=0.0001; OR= 1.43; 95% CI 1.20-1.72).

The distribution of lesions on the trunk and lower limbs did not differ by sex. Males, however, had significantly more lesions on the head and neck than females (chi square=6.71; p=0.006; OR=0.67; 95% CI 0.51-0.89), while females had significantly more lesions on the upper limbs than males (chi square=4.56; p=0.03; OR=1.16; 95% CI 1.01-1.33).

Among females, 64.3% of lesions were on the lower limbs, 27% were on the upper limbs, 5.5% on the truck, and 3.2% on head and neck. Among males, 67% of lesions were on the limbs, 23% were on the upper limbs, 5.6% on the trunk and 4.2% on head and neck.

Cases of Buruli ulcer were identified in all 10 regions. Table 1 shows the prevalence rates per region, based on estimated 1999 population figures from the 2000 census. The Central Region has the highest overall prevalence rate of active cases, followed by the Ashanti Region; the Northern and Upper West Regions had the lowest prevalence rates (Figure 3). Cases of the disease were identified in 90 (81.8%) of Ghana's 110 districts. Table 2 shows the prevalence rates of the disease in the 10 districts with the highest caseloads. Amansie West had the highest rate (prevalence 150.8 per 100,000), followed by Asante Akim North (prevalence 131.5 per 100,000) and Upper Denkyira (prevalence 114.7 per 100,000).

With regard to access to health care, 38.0% of patients lived in communities >5 miles from the nearest health facility, 37.1% lived 1 to 5 miles away, and 24.9% lived within a mile of the nearest health facility.

Discussion

All cases were diagnosed on the basis of clinical case definitions without laboratory confirmation; as a result, atypical cases such as early and healed lesions may be confused with other diseases endemic in Ghana (e.g., yaws). Experience, however, shows that in disease-endemic communities Buruli ulcer is readily diagnosed empirically.

The overall crude prevalence rate of 20.7 per 100,000 exceeds that of leprosy (9 per 100,000) in 1999, making Buruli ulcer the second most prevalent mycobacterial disease in Ghana after tuberculosis (prevalence 66 per 100,000). Before
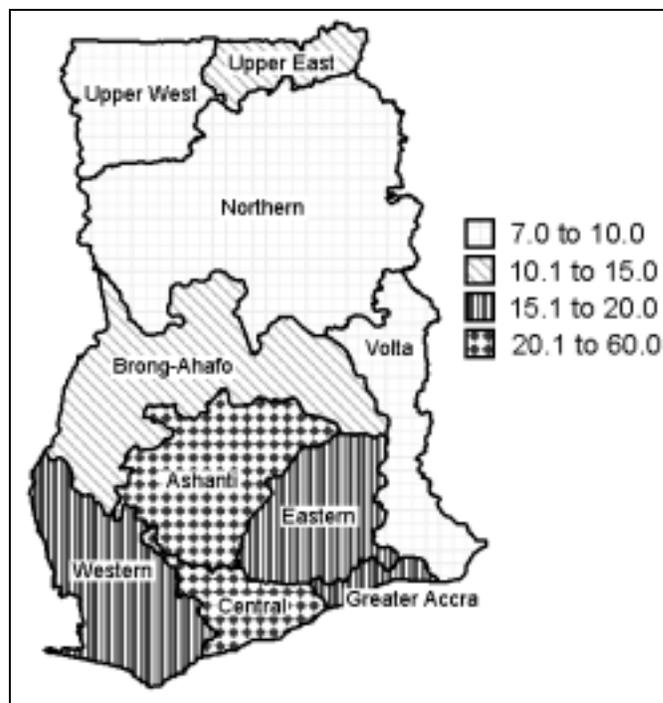
Figure 3. Prevalence of suspected active cases of Buruli ulcer, by region, Ghana, 1999.

the case search, a cumulative total of approximately 1,200 cases of Buruli ulcer had been reported from five regions in Ghana over a 6-year period from 1993 to 1998. The case search has confirmed that the disease is grossly underreported. The distribution of the disease is much more widespread than earlier thought; suspected cases were identified in all 10 regions and at least 90 of 110 districts. Although the infection was thought to be restricted to relatively small patchy isolated foci separated by large disease-free areas (11), our impression is that the more one looks for the disease in known disease-endemic and nearby areas the more likely additional cases will be found.

Our study confirms findings elsewhere that the disease affects children more than adults (7,8). Marston et al. found the highest rate of infection among children 10-14 years of age in a disease-endemic area in the Daloa Region of Côte d'Ivoire (14). This observation has been misinterpreted to mean that the disease affects only children. Our study demonstrates that all age groups can be affected. At least 25% of persons with active lesions were >50 years old.

The preponderance of lesions on the extremities is once again confirmed by our study. About 92% of lesions were located on the extremities in the study by Marston et al. in Côte d'Ivoire (14), compared with 91% in our study. Barker noted that among girls and women there was equal frequency of arm and leg lesions, while among boys leg lesions predominate (9). Our results, however, show that lesions on the leg predominate for all age groups and both sexes. Even for females, leg lesions were 2.4 times more frequent than arm lesions.

Table 1. Prevalence of active Buruli ulcer cases by region in Ghana, 1999

Region	No. of males	No. of females	Total no. of active cases	Prevalence (rate per 100,000)
Ashanti	482	475	957	30.8
Brong Ahafo	113	110	223	12.5
Central	519	395	914	59.2
Eastern	202	150	352	16.9
Greater Accra	259	255	514	18.5
Northern	65	68	133	7.4
Upper East	34	63	97	10.7
Upper West	21	21	42	7.4
Volta	78	74	152	9.6
Western	181	140	321	17.9
Unknown			20	
Ghana	1,751	1,954	3,725	20.7

Table 2. Prevalence of Buruli ulcer in the 10 districts with the highest caseloads, Ghana, 1999

District	Region	No. of active cases	No. of active and healed lesions	Prevalence (rate of active cases per 100,000)
Ga	Greater Accra	467	1,113	87.7
Amansie West	Ashanti	159	474	150.8
Assin	Central	159	173	83.7
Gomoa	Central	158	161	81.9
Asante Akim N	Ashanti	138	265	131.5
Wassa Amenfi	Western	136	167	61.1
Kwawu South	Eastern	122	132	57.0
Upper Denkyira	Central	121	306	114.7
Afigya Sekyere	Ashanti	118	149	107.1
North Tongu	Volta	107	129	85.7

At least 40% of patients with the disease lived in communities ≥5 miles from the nearest health facility capable of providing the minimum service of wound dressing. This distance poses problems for patients who have to travel repeatedly for care for such a chronic ailment.

The study has shown that Buruli ulcer disease in Ghana is much more widespread than previously thought. In all areas where Buruli ulcer cases have been identified, the extent of the disease is likely to be much greater than currently recognized through the routine reporting system. The data set on the disease from the study is among the largest anywhere in the world and can contribute substantially to the epidemiologic description of this relatively new disease. The information generated should contribute greatly to the design, implementation, and evaluation of Buruli ulcer control programs in Ghana.

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Clinical Significance and Epidemiology of NO-1, an Unusual Bacterium Associated with Dog and Cat Bites

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From 1974 to 1998, 22 isolates of an unusual bacterium, designated as CDC nonoxidizer 1 group (NO-1), were sent to the Centers for Disease Control and Prevention for identification. The organism's phenotypic characteristics were similar to asaccharolytic strains of *Acinetobacter*, but differed in their cellular morphology and cellular fatty acid profile. We report here on NO-1's clinical and epidemiologic significance. In all cases, isolates were recovered from an animal bite wound; 17 (77%) were isolated from a dog bite wound, 4 (18%) from a cat bite wound, and one (5%) from an unspecified animal bite. Clinical data were retrieved and reviewed for 12 (55%) of the 22 bite victims. None of the patients had preexisting conditions associated with immunosuppression. Seven (58%) patients were hospitalized for a median stay of 4 days (range 2 to 11 days). The median time between bite to the worsening of symptoms was 17.5 hours (range 3 to 78 hours). All patients recovered following antibiotic treatment.

An estimated 4.4 million animal bites occur each year in the United States (1,2). The annual incidence of dog and cat bites has been reported as 300 bites per 100,000 population (3). The most common organisms isolated from infected dog and cat bite wounds are *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., anaerobes, and *Pasteurella multocida* (4,5).

In December 1974, the Special Bacteriology Reference Laboratory (SBRL) in the Meningitis and Special Pathogens Branch, Centers for Disease Control and Prevention (CDC), received a bacterial isolate recovered from a cutaneous bite wound of an 8-year-old boy in Texas. The isolate was a unique, fastidious, non-oxidative, gram-negative rod and was named nonoxidizer group 1 (NO-1). From 1974 to 1998, the SBRL identified 21 additional isolates received from state and local hospital laboratories across the United States and Canada. The biochemical and phenotypic characteristics of the organism have been described elsewhere (6). However, clinically and epidemiologically relevant data accompanying these specimens were mostly limited to source of specimen, date of collection, and occasionally sex and age of the patient. Clinical course descriptions of patients infected with NO-1 have not been previously described. To evaluate the significance of this recently identified group, we conducted a review of clinical records for patients from whom NO-1 was isolated and submitted to SBRL for identification between 1974 and 1998.

Methods

Inclusion was limited to those patients from whom isolates sent to SBRL were identified as NO-1 between 1974 and

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1998. In collaboration with state and territorial public health epidemiologists and hospital infection control practitioners, we gathered relevant clinical records and exposure data to determine the clinical significance of and risk factors for NO-1 infection. State epidemiologists, hospital infection control practitioners, or both were notified of cases that occurred in their state or hospital and were requested to submit clinical records for each case, including chart notes, discharge summaries, and clinical laboratory results. Using a standardized form, we abstracted clinical data for each patient. Information recorded included demographic characteristics, signs and symptoms of illness, treatment, laboratory results, and outcome. We collected information on symptoms including erythema, swelling, drainage, cellulitis, loss of motion, and any other symptoms that treating physicians noted.

This protocol was determined to be exempt from human subjects review requirements because the gathering of clinical information to accompany strains was a standard part of reference diagnostic submission, and, therefore, considered surveillance activity for a newly emerging pathogen. No specific research question was investigated.

Data were entered and analyzed with Epi Info version 6.04 (CDC, Atlanta GA). Continuous variables were evaluated by using the Kruskal-Wallis test, and categorical variables were compared by using the chi-square test or Fisher's exact methods.

Results

Of the 22 patients from whom NO-1 isolates were recovered, 11 (50%) were male and the a median age of all patients was 22 years (range 20 months to 78 years). The median age of males was significantly different from that of females (9 vs. 25 years, p<0.05). In all 22 cases, the organism was isolated from an animal bite wound site: 17 (77%) from a dog bite

wound, 4 (18%) from a cat bite wound, and 1 (5%) with no recorded bite source. Fourteen (64%) isolates came from bites on the extremities, 6 (27%) came from face or neck wounds, and for 2 (9%) no anatomic site was specified.

We were unable to retrieve 10 (45%) of the 22 medical records for reasons including 1) records were lost, destroyed by accident or natural occurrence, or archived off-site, or 2) a patient was seen in the emergency room and notations were not in the patient record. The 12 patients whose records were available for review are summarized in Table 1. Of these patients, 4 (33%) were male with a median age of 40 years (range 7 to 61 years). Eight (67%) had dog bite wounds, and the remaining 4 (33%) had cat bites. Eleven (92%) received bite wounds on the extremities, including hands, arms, or legs; one bite occurred on the face. The median time interval from bite to onset or worsening of symptoms was 17.5 hours (range 3 to 78 hours). Ten patients were afebrile; in two cases, a temperature >38.9°C was recorded. At treatment, 67% of patients reported increased pain associated with erythema and swelling around the wound site. Purulent drainage was noted in 50% of cases, and a diagnosis of cellulitis was made for 67% of patients. There was no evidence of sepsis, although blood cultures were attempted in only four cases. For those patients for whom records were retrieved, decreased range of motion was documented in 4 (36%) of the 11 with extremity wounds. Other reported symptoms included ecchymosis (8%), increased local temperature (8%), tingling sensations (8%), chills (8%), adenitis/lymphadenopathy (25%), and discoloration (25%) including "streaking." Total leukocyte counts ranged from 8,100 to 12,700 cells/mm^3. Hematocrit (range 36% to 43%) and platelet counts (range 140 to 331 K/mm^3) were normal. Laboratory reports on cultures from wound specimens are summarized in Table 2. Isolates later identified as NO-1 were originally described as fastidious gram-negative rods, polymorphic gram-negative rods, or gram-negative bacilli. Concentrations of NO-1 in these specimens ranged from light to moderate. Most of the specimens contained mixtures of two or more bacterial species, although specimens from Cases 6 and 11 yielded apparently pure cultures of NO-1. Other bacterial organisms isolated from these wounds included *Weeksella zoohelcum*, *Eikenella corrodens*, *Pasteurella multocida*, *Staphylococcus aureus*, and *Corynebacterium* spp., coagulase-negative *Staphylococci*, and unidentified gram-positive cocci and enteric gram-negative rods. None of the NO-1 isolates were identified by the original hospital laboratory.

Table 1. Characteristics of patients for whom records were available and from whom NO-1 isolates were obtained

Case no.	State	Gender/ age (yrs)	Animal	Wound site	No. days ill	Sutured	Major clinical features	Antibiotic treatment/outcome
1	MA	F/22	Cat	Hand	8	Unk.	Cellulitis, loss of motion, purulent drainage, fever	Improved with cephradine + penicillin (OP)
2	IL	M/45	Dog	Hand	5	Unk.	Purulent drainage, redness and swelling extending up to forearm	Cefadroxil (OP); next day admitted to hospital and gradual improvement with ampicillin/sulbactam (IP)
3	IL	F/11	Dog	Hand	6	Yes	Cellulitis, purulent drainage, local increase in temperature	Improved with ampicillin + ceftriaxone, local bacitracin (IP)
4	RI	F/78	Cat	Hand	3	Unk.	Cellulitis, decreased movement	Antibiotic ointment + amoxicillin/clavulanic acid (OP1); penicillin (OP2); admitted to hospital next evening and gradually improved on ampicillin/sulbactam (IP)
5	SC	F/37	Dog	Hand	7	Unk.	Cellulitis, purulent drainage	Cephradine (OP); admitted to hospital and improved on gentamicin + ampicillin (IP)
6	GA	M/7	Dog	Leg	11	Yes	Cellulitis, purulent drainage, fever, red streaking, inguinal adenitis, chills	Amoxicillin (OP); improved on penicillin + cefazolin, changed to cephalexin (IP)
7	MA	M/61	Dog	Arm	Unk.	Unk.	Superficial laceration with streaking	Penicillin[a] (OP)
8	CA	M/35	Dog	Hand	3	Yes	Cellulitis, loss of motion	Iproved with cefazolin + cephradine (OP)
9	CA	F/24	Cat	Hand	2	Unk.	Cellulitis, purulent drainage, lymphadenitis, tingling sensations	Resolved with cefazolin + penicillin (IP)
10	TN	F/51	Dog	Face	5	Yes	Pustule of wound	Improved with cephalexin (OP)
11	NV	F/56	Cat	Arm	Unk.	Unk.	Swelling, lymphadenopathy	Dicloxacillin[a] (OP)
12	MI	F/21	Dog	Hand	3	Yes	Cellulitis, impaired range of motion	Cefaclor (OP) + improved with cefazolin (IP)

Unk. = Unknown; OP = outpatient; OP1 = outpatient visit 1; OP2 = outpatient visit 2; IP = inpatient.
[a]Incomplete information on condition of infection following treatment.

Table 2. Description of all bacterial isolates cultured from infected wounds

Case no.	Bacterial organisms cultured from wound
1	No documentation of culture results in record
2	Light *Weeksella zoohelcum*; light fastidious gram-negative bacilli[a]
3	Few fastidious gram-negative rods;[a] coagulase-negative *Staphylococci* on subculture only
4	No growth of organisms noted in record
5	Enterics, few gram-positive cocci; some polymorphic gram-negative rods[a]
6	Light growth of gram-negative bacilli[a]
7	No documentation of culture results in record
8	Three types of gram-negative rods[a]
9	Few unidentified gram-negative rods;[a] few mixed aerobic skin flora
10	Rare *Eikenella corrodens*; few gram- negative bacilli[a]
11	Moderate growth of gram-negative bacilli[a]
12	Numerous *Pasteurella multocida*; rare *Staphylococcus aureus*; few gram-negative rods;[a] numerous *Corynebacterium* species

[a]Identified as Centers for Disease Control and Prevention group nonoxidizer 1 (NO-1).

No data were available on the depth of the wounds. Standard cleansing, including irrigation and debridement of wounds followed by dressing, was documented for 8 of 12 patients. Five of the 12 patients had wound closure by suture after irrigation. No associations were seen between suture therapy and severity of symptoms or duration of recovery.

Seven (58%) persons were hospitalized for treatment; 5 (42%) were managed as outpatients (Table 1). The 7 hospitalized patients had a median stay of 4 days (range 2 to 11 days). All 12 patients were given one or more antibiotic treatments within 1 to 3 days of receiving bite wounds. Initial therapy for patients included at least a beta-lactam antibiotic (Table 1). Five patients (Cases 2, 4, 5, 6, and 12) were initially treated as outpatients and were subsequently admitted for worsening of symptoms. In-hospital treatment for these five patients included either intravenous beta-lactam or intravenous beta-lactam with beta-lactamase inhibitor. One patient also received an aminoglycoside. Eight (67%) of 12 cases, including all 7 inpatients, received intravenous antibiotic therapy. Four of the five outpatients received oral antibiotics only. However, no documentation of compliance for these patients was found. Though most patients were released before infection completely resolved, all patients for whom follow-up information was available had documented improvement of the infection. Symptoms resolved within 2 to 11 days (median 5.5 days).

A history of asthmatic bronchitis and respiratory allergies, arthritis, and a ventricular septal defect was noted for Cases 6, 4, and 9, respectively. Two patients were elderly (>60 years). No preexisting conditions or illnesses were documented for the rest of the patients.

Discussion

NO-1 is a recently identified bacterium associated with dog and cat bite wounds. Infections in which NO-1 bacteria were isolated appear to be local (i.e., abscess and cellulitis). Following receipt of a bite wound, NO-1 infections, without severe disease, can occur in healthy persons with no underlying illness. The most common clinical features associated with NO-1 infections included purulent drainage, increased pain with erythema and swelling, and cellulitis, which are clinically similar to features of infections caused by other animal bite-related organisms (5). Apparently pure cultures of NO-1 were obtained from wound specimens of Cases 6 and 11. Although Case 11 resolved with outpatient dicloxacillin treatment, Case 6 required hospitalization and multiple antibiotic treatments. This patient also had symptoms consistent with septicemia (fever, chills). Taken together, these facts suggest that NO-1 infections can progress from localized to systemic forms. At this time there is no available information on potential virulence factors for NO-1.

As described by Hollis et al., the phenotypic characteristics of NO-1 are similar to those observed with asaccharolytic *Acinetobacter* species (6). NO-1 bacteria fail to acidify carbohydrates and are oxidase, indole, and urease negative. Cellular fatty acids and ubiquinone analysis are useful in differentiating NO-1 from *Acinetobacter* species (6). Studies are under way at the SBRL to determine the taxonomic classification of NO-1.

The patients from whom NO-1 were isolated were successfully treated with a variety of antibiotics. In general, intravenous antibiotics may have an advantage over oral antibiotics in preventing bacterial infections associated with bite wounds, including those caused by NO-1, because of more rapid delivery of drug to affected tissues (7). Previously reported antimicrobial susceptibility testing of 17 of the 22 strains by a standard broth microdilution method showed all strains to be susceptible to aminoglycosides, beta-lactam antibiotics, tetracyclines, quinolones, and sulfonamides. Fifty percent were resistant to trimethoprim. Some of the isolates were noted to grow poorly in the broth test for the antimicrobial susceptibility testing, but all control wells had sufficient growth for interpretable results (6).

Most dog and cat bite wounds in young children occur on the face, head, and neck; by contrast, the extremities tend to be injured in young adults and adults (2,8,9). In our study, of the 6 patients who received bite wounds to the face, head, or neck, 4 (67%) were ≤8 years, 1 (17%) was a 51-year-old woman, and 1 (17%) was of unknown age. Of the 14 victims who received bite wounds on an extremity, 1 (7%) was <10 years. Across all ages, 14 (64%) of all 22 isolates came from bite wounds on the extremities, consistent with the distribution of animal bite wounds reported in other studies (5,10,11).

In addition to other pathogens that are associated with bite wounds, such as *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., anaerobes, and *Pasteurella multocida*, NO-1 should be considered in the differential diagnosis of infected bite wounds from cats and dogs. Group NO-1 organisms

have been shown to be similar to two isolates from oral and nasal fluids from dogs called "unidentifiable species no. 4" by Bailie et al. (12). Likewise, the association of human NO-1 infections with animal bite wounds received from cats and dogs suggests that these animals are a reservoir for NO-1 bacteria.

At present no surveillance system exists for reporting dog or cat bite wound-associated infections and, therefore, the incidence of NO-1 isolated from bite wound infections is not known; however, these 22 reported cases likely represent only a fraction of the true number of potential NO-1 infections. Several of the case records noted the gram-negative rods as possible exogenous contaminants; thus, many more NO-1 infections may be unrecognized and undocumented. The etiologic role and the pathogenicity of NO-1 are unclear. Enhanced awareness of this organism by physicians will improve our understanding of this new zoonotic infection and clarify its clinical significance. With over 4 million animal bites occurring in the United States each year, bacteria associated with dog and cat bite wounds, including NO-1, are an important public health problem.

Acknowledgments

Robyn Kaiser served as a guest researcher in the Bacterial Zoonosis Unit of the Meningitis and Special Pathogens Branch at the Centers for Disease Control and Prevention from 1998 to 2001. She received her M.P.H. from Emory University Graduate School of Public Health in 2000 and is currently in her first year of medical school at the University of Nebraska.

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Comparative Antibiotic Resistance of Diarrheal Pathogens from Vietnam and Thailand, 1996–1999

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Antimicrobial resistance rates for shigella, campylobacter, nontyphoidal salmonella, and enterotoxigenic *Escherichia coli* were compared for Vietnam and Thailand from 1996 to 1999. Resistance to trimethoprim-sulfamethoxazole, ampicillin, chloramphenicol, and tetracycline was common. Quinolone resistance remains low in both countries, except among campylobacter and salmonella organisms in Thailand. Nalidixic acid resistance among salmonellae has more than doubled since 1995 (to 21%) in Thailand but is not yet documented in Vietnam. Resistance to quinolones correlated with resistance to azithromycin in both campylobacter and salmonella in Thailand. This report describes the first identification of this correlation and its epidemiologic importance among clinical isolates. These data illustrate the growing magnitude of antibiotic resistance and important differences between countries in Southeast Asia.

Antimicrobial resistance among enteric pathogens in developing countries is a critical area of public health concern. The usual causative agents of dysentery—shigella, campylobacter, and nontyphoidal salmonella—are becoming increasingly resistant to most agents commonly in use (1-3). The current recommendation for management of travelers' diarrhea is self-therapy with antibiotics if illness develops (4,5); fluoroquinolones are standard treatment in much of the world. Although travelers' diarrhea has traditionally been associated with enterotoxigenic *Escherichia coli* (ETEC), some studies from Asia have shown that campylobacter is of equal or greater importance (6-8). In the early 1990s, fluoroquinolone-resistant strains of campylobacter rapidly became prevalent in Thailand, increasing from 0% to 84% of isolates from 1990 to 1995 (1).

Azithromycin has been suggested as a replacement for quinolones for empiric treatment of travelers' diarrhea in Thailand (9). Although this drug may be a reasonable choice for travelers, its high cost precludes its use as routine treatment for dysentery in disease-endemic areas. Furthermore, the emergence in Thailand of campylobacter resistant to both quinolones and azithromycin (1) threatens the continued usefulness of both.

The seriousness of the problem has prompted calls for improved surveillance for antimicrobial resistance in developing countries, which could provide early warnings of the emergence of resistant bacterial strains (10). The prevalence of quinolone- and macrolide-resistant campylobacter in areas of Southeast Asia other than Thailand is unknown, and resistance patterns in Thailand may not apply to other countries in the region. Poorer countries such as Vietnam could benefit by avoiding a rush to newer, more expensive agents if sensitivity to older drugs could be demonstrated. Data from Vietnam relative to this issue are scarce or outdated. Surveillance data from the World Health Organization's Western Pacific Region collaboration for 1992-93 reported 0% and 2% prevalence of fluoroquinolone resistance among nontyphoidal salmonella and shigella, respectively, in Vietnam, although the numbers of isolates and shigella groups were not specified (11). Sullivan et al. (12) reported resistance rates in >3,000 isolates of shigella, salmonella, and *Escherichia coli* collected before 1971 from U.S. soldiers and Vietnamese residents with diarrhea; levels of resistance to tetracycline, chloramphenicol, streptomycin, and novobiocem were high, particularly among shigellae. Other than these two studies, no data have been published in English on resistance patterns in enteric diarrheal pathogens in Vietnam, and no campylobacter resistance data from this country have ever been published.

The Armed Forces Research Institute of Medical Sciences in Bangkok has been coordinating studies of the causes, epidemiology, and treatment of diarrheal diseases in Thailand and Vietnam. Antibiotic susceptibility of bacterial isolates, including shigella, ETEC, and nontyphoidal salmonella from patients with diarrhea, have been tested against the most common antibiotics, including ampicillin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole (SXT), nalidixic acid, and fluoroquinolones. In recent years, azithromycin resistance among these pathogens, as well as the emergence of fluoroquinolone and azithromycin resistance in campylobacter species, has been monitored. A review of 15 years of data from 1981 to 1995 has been published (1). Our study further documents increasing antibiotic resistance in Thailand from 1996 to 1999 and presents resistance data from Vietnam, offering

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insight into regional differences between two countries that are proximal geographically, but politically and economically distant.

Methods

Source of Isolates

Bacterial isolates from studies of community-acquired diarrhea conducted in Thailand and Vietnam from 1996 to 1999 were routinely assayed for sensitivity to various antimicrobial agents. Populations under study were predominantly children <5 years of age from both urban and rural environments but also included small numbers of adult U.S. soldiers on military maneuvers in Thailand. Some isolates were obtained from asymptomatic persons. These data archives were searched initially for all isolates of shigella (other than *Shigella dysenteriae* 1), nontyphoidal salmonella, campylobacter, and ETEC. The lists were reviewed for the presence of duplicate isolates from the same person, and duplicates were excluded.

Microbiology

Enteric pathogens were cultured and identified by standard methods (13-15). ETEC isolates were identified with DNA probes. Antibiotic susceptibilities of shigella, salmonella, and ETEC were determined by the disk-diffusion method of Bauer and co-workers (16), with commercially prepared antibiotic disks containing ampicillin, tetracycline, chloramphenicol, sulfisoxazole, SXT, streptomycin, azithromycin, nalidixic acid, and ciprofloxacin. MIC agar-plate dilution methods (17,18) were used to test for nalidixic acid, ciprofloxacin, and azithromycin resistance among campylobacter species. Because of periodic shortages of reagents, not all isolates were tested to all antimicrobial agents.

Results

Overall, 2,218 isolates were studied, 76% from Thailand and 24% from Vietnam. Pathogenic bacteria were obtained from subjects without gastrointestinal symptoms in 4.2%. Adult travelers (U.S. soldiers on maneuvers) accounted for 6.4% of Thai isolates, but none of the Vietnamese isolates. All other isolates were obtained from children <5 years of age. Sixty-one percent of Thai isolates were from an urban setting (Bangkok). All Vietnamese isolates were rural. All isolates were obtained either from cohorts enrolled in epidemiologic studies evaluating the prevalence of bacterial diarrhea pathogens or the incidence of diarrhea or from case-control studies evaluating diagnostic techniques.

Routine Susceptibility Testing for Shigella Species Other than *S. dysenteriae* 1, ETEC, and Salmonella

From Thailand, 175 shigella (65% *S. sonnei*, 32% *S. flexneri*), 696 nontyphoidal salmonella, and 203 ETEC isolates were studied during the 3-year period. From Vietnam, 305 shigella (18% *S. sonnei*, 71% *S. flexneri*), 30 salmonella, and 113 ETEC isolates were studied (Table 1).

Among shigella, high levels of resistance to tetracycline and SXT were noted in both Thailand and Vietnam. Significantly less resistance was noted among Thai isolates to ampicillin (29% vs. 77%, p<0.05) and chloramphenicol (21% vs. 64%, p<0.05). Sorting by shigella group showed that nearly all the difference between Thai and Vietnamese isolates was accounted for by differences in the prevalence and susceptibility of *S. sonnei* compared with the more resistant *S. flexneri* strains. In Thailand, *S. sonnei* was almost all susceptible to ampicillin and chloramphenicol; in Vietnam, *S. sonnei* was more commonly resistant to these agents.

ETEC isolates demonstrated high-level resistance in both countries to ampicillin, SXT, tetracycline, and sulfisoxazole, but chloramphenicol resistance was relatively low in both countries. In contrast, nontyphoidal salmonella from Thailand had consistently and substantially higher levels of resistance to all agents, compared with those from Vietnam. Serogroup B salmonellae in Thailand were generally more resistant than other serogroups, except for serogroup C isolates, which were relatively more resistant to nalidixic acid and azithromycin.

Quinolone and Macrolide Resistance among Shigella, Salmonella, and ETEC

Resistance to nalidixic acid was rare among shigella (<1%) and ETEC (3%) in both Thailand and Vietnam but was more common among Thai salmonella (21%). Resistance to ciprofloxacin was documented in <1% of isolates, including two salmonellae (from Thailand) and four ETEC (three from Thailand, one from Vietnam). No shigellae were resistant to ciprofloxacin in either country (Table 1).

With the exception of *S. sonnei*, azithromycin resistance was found in ≤8% of shigella, salmonella, and ETEC, with no significant difference between Vietnam and Thailand. Azithromycin resistance was present in 28% of *S. sonnei* isolates from Vietnam but only 2% of Thai isolates (p<0.05).

Resistance of Campylobacter to Quinolones and Macrolides

We studied 608 campylobacter isolates from Thailand and 88 from Vietnam. Resistance to nalidixic acid (73% vs. 7% respectively, p<0.05) and ciprofloxacin (77% vs. 7% respectively, p<0.05) differed markedly between Thai and Vietnamese isolates. Among Vietnamese isolates, quinolone resistance was predominantly among *Campylobacter coli* rather than *C. jejuni,* but rates among Thai isolates were similar between species. Resistance to azithromycin was low in Thailand (6%), where it predominated among *C. coli*. No azithromycin resistance was present in Vietnamese isolates (Table 1).

Co-Resistance to Quinolones and Macrolides among Campylobacters and Salmonellae

Vietnamese isolates of campylobacter were uniformly sensitive to azithromycin, so we were unable to examine correlations with quinolone resistance. Among Thai isolates, however, azithromycin resistance correlated with resistance to

Table 1. Antibiotic resistance patterns among selected enteric pathogens in Thailand and Vietnam, 1996–999[a]

Pathogen	Site	AM N	AM %R	CM N	CM %R	SXT N	SXT %R	NA N	NA %R	CIP N	CIP %R	TE N	TE %R	AZM N	AZM %R
ETEC	Thailand	203	54	203	13	203	51	203	3	203	2	203	43	68	4
	Vietnam	113	67	113	17	113	63	112	<1	113	<1	113	65	37	3
Salmonella (all)	Thailand	696	28	696	26	696	37	696	21	696	<1	695	59	284	5
	Vietnam	30	3	30	7	30	10	30	0	30	0	30	13	19	0
Group B	Thailand	258	49	258	45	258	67	258	23	258	<1	258	77	82	1
	Vietnam	6	0	6	0	6	0	6	0	6	0	6	0	3	0
Group C	Thailand	192	5	192	13	192	12	192	33	192	0	191	62	101	12
	Vietnam	1	0	1	0	1	0	1	0	1	0	1	0	1	0
Group D	Thailand	68	31	68	31	68	38	68	18	68	0	68	46	28	4
	Vietnam	7	0	7	14	7	14	7	0	7	0	7	1	4	0
Group E	Thailand	133	24	133	11	133	16	133	4	133	0	133	18	52	5
	Vietnam	14	0	14	0	14	7	14	0	14	0	14	7	10	0
Other	Thailand	45	9	45	11	45	16	45	4	45	0	45	18	21	5
	Vietnam	2	50	2	50	2	50	2	0	2	0	2	100	1	0
Shigella (all)	Thailand	175	29	175	21	175	90	175	<1	175	0	175	91	68	2
	Vietnam	305	77	305	64	305	78	305	<1	305	0	305	80	233	8
Group A	Thailand	3	0	3	0	3	0	3	0	3	0	3	33	0	NA
	Vietnam	6	50	6	17	6	50	6	0	6	0	6	50	6	0
Group B	Thailand	56	82	56	61	56	86	56	0	56	0	56	96	18	0
	Vietnam	216	84	216	74	216	83	216	<1	216	0	216	87	165	5
Group C	Thailand	2	0	2	0	2	0	2	0	2	0	2	0	0	NA
	Vietnam	28	54	28	46	28	68	28	0	28	0	28	75	22	0
Group D	Thailand	114	4	114	3	114	97	114	<1	114	0	114	92	50	2
	Vietnam	55	62	55	36	55	67	55	0	55	0	55	60	40	28
Campylobacter	Thailand	ND		ND		ND		608	73	531	77	ND		520	6
	Vietnam	ND		ND		ND		88	7	85	7	ND		62	0
C. jejuni	Thailand	ND		ND		ND		489	72	426	75	ND		415	2
	Vietnam	ND		ND		ND		71	1	69	1	ND		48	0
C. coli	Thailand	ND		ND		ND		108	75	94	80	ND		94	26
	Vietnam	ND		ND		ND		17	29	16	31	ND		14	0

[a]AM=ampicillin; CM=chloramphenicol; SXT=trimethoprim-sulfamethoxazole; NA=nalidixic acid; CIP=ciprofloxacin; TE=tetracycline; AZM=azithromycin; ND=not done; %R=percent resistant; NA=not applicable.

ciprofloxacin (p=0.007) (Table 2). Among campylobacter tested to both these agents, all isolates (n=29) that were resistant to azithromycin were also resistant to ciprofloxacin. Based on the 23% prevalence of ciprofloxacin sensitivity among azithromycin -susceptible strains, a similar rate of discordance among azithromycin-resistant strains would be expected if these resistance patterns were independent. Coresistant isolates included both *C. coli* (n=22) and *C. jejuni* (n=7), were composed of multiple serogroups and resistance profiles, were present in both travelers and indigenous Thais, and were dispersed geographically around Thailand and tem-

porally over the 4 years of data; therefore, they are not likely to be clonally related. These isolates were significantly more likely to be *C. coli* (76%) than were azithromycin sensitive isolates (*C. coli* 15%; p<0.001).

Too few salmonellae were resistant to ciprofloxacin for analysis of cross-resistance; however, among isolates tested for resistance to both the first-generation quinolone nalidixic acid and azithromycin (Table 3), a highly significant correlation was found (p<0.001). Based on the 31% prevalence of nalidixic acid resistance among azithromycin sensitive strains, a similar proportion would be expected among azithromycin-

Table 2. Co-resistance[a] to azithromycin (AZM) and ciprofloxacin (CIP) among campylobacter isolates in Thailand, 1996–1999

		AZM	
		R[a]	S
CIP	R	29[b]	358
	S	0	109

[a]R = resistant; S = sensitive.
[b]Chi square = 7.4, p=0.007.

resistant strains if the two were independent, rather than the 80% documented.

Multiple-Antibiotic Resistance among Shigellae, Salmonellae, and ETEC

Multiple resistance to ampicillin, chloramphenicol, tetracycline, SXT, and streptomycin was quite common among shigella isolates in both Thailand and Vietnam, but was significantly more so in Vietnam (46% vs. 21%, p<0.001). Lower levels of multiple resistance to these drugs were noted among salmonella and ETEC isolates from both countries (3% to 7%).

Association between Symptoms and Prevalence of Antimicrobial Resistance

No association was found with presence or absence of gastrointestinal symptoms among isolates from Vietnam or among campylobacter or shigella isolates from Thailand. Thai ETEC and salmonellae that were isolated from participants with symptoms were significantly more likely to be resistant across the spectrum of antimicrobial agents studied (Mantel-Haensel summary chi square p=0.01 and p<0.01, respectively).

Discussion

This study points out several new findings relative to antimicrobial resistance among enteric pathogens in Southeast Asia. We have documented the existence of quinolone resistance among campylobacter, shigella, and ETEC in Vietnam. If the pattern of campylobacter in Thailand holds true in Vietnam, one can expect rapidly increasing resistance rates to quinolones over the next few years in this genus. The reasons for the marked difference in resistance rates to this class of drugs between the two countries are unknown but likely reflect differences in human and veterinary use of fluoroquinolones.

Table 3. Co-resistance[a] to azithromycin (AZM) and nalidixic acid (NA) among salmonella isolates in Thailand

		AZM	
		R[a]	S
NA	R	12[b]	55
	S	3	122

[a]R = resistant; S = sensitive.
[b]Chi square = 12.5, p<.001.

Quinolone resistance among nontyphoidal salmonella was not seen in isolates from Vietnam, and ciprofloxacin resistance remains rare among this species in Thailand; however, progressive resistance to nalidixic acid among Thai isolates is cause for concern. Hoge (1) documented steadily increasing nalidixic acid resistance among nontyphoidal salmonellae in Thailand, from <1% in 1991-92 to 4% in 1993-94 and 9% in 1995. We documented 21% resistance in this genus during 1996 to 1999, more than double 1995 rates (chi square for trend, p<0.001). As resistance to nalidixic acid due to first-step resistance mutations is generally thought to precede resistance to fluoroquinolones (19), close continued monitoring of resistance rates to both these drugs among nontyphoidal salmonellae is warranted.

Although cases of infections due to macrolide- and fluoroquinolone-resistant campylobacter have been reported, these have usually been among immunocompromised persons who have received multiple antibiotics (20-22). Our study is the first to document a statistical correlation between these two resistance patterns among a large number of community-acquired enteric isolates. Although the prevalence of azithromycin resistance remains relatively low in Thailand (6%) while ciprofloxacin resistance is high (77%), our study had sufficient power to confirm that the 100% correlation between resistance to these two agents was not likely due to chance. Previous studies of smaller numbers of azithromycin-resistant isolates from Thailand also support this 100% correlation (1,7,9). Resistance to quinolones is generally mediated by mutations in the chromosomal genes for DNA gyrase or topoisomerase IV, which are targets of action by the quinolone class, or less commonly in genes responsible for membrane flux of the drug (19), including plasmid-mediated enhanced efflux mechanisms. Resistance to macrolides most commonly results from mutations in the genes encoding ribosomal proteins but has also been associated with decreased permeability of the cell envelope in enterobacteriaceae, including plasmid-mediated mechanisms (21,23). Cross-resistance due to decreased membrane permeability has been documented between quinolones and chloramphenicol, trimethoprim, tetracycline, and cefoxitin, but not macrolides (19). Possible novel cross-resistance mechanisms in these isolates deserve to be more closely evaluated, as a common plasmid-mediated mechanism would increase the likelihood of horizontal spread.

Although resistance to quinolones among campylobacters from Vietnam was markedly lower than that from Thailand, multidrug resistance to other agents among Vietnamese shigella isolates was significantly higher than among Thai isolates, perhaps reflecting differences in human use patterns. In Vietnam in 1997, ampicillin, SXT, tetracycline, and chloramphenicol were the antimicrobial agents most commonly dispensed over the counter (24). Thai data from 10 years earlier (1987) document common dispensing of the same four drugs by Bangkok pharmacists (25), although fluoroquinolones were more frequently used for children with diarrhea admitted to Thai hospitals during 1990-1992 (26), and quinolones have

likely become increasingly used in the decade since. Our data suggest that ampicillin, SXT, tetracycline, and chloramphenicol have no reasonable role in the empiric treatment of dysentery in Vietnam and should be replaced with quinolones or macrolides for this indication. Shigellae in Thailand are uniformly sensitive to quinolones, but dysentery and travelers' diarrhea in Thailand are predominantly caused by quinolone-resistant campylobacter rather than shigella and are thus better treated empirically with macrolides. Although ampicillin resistance among Thai shigellae was stable during the 4 years of this study, data from the preceding 15 years (1) demonstrate that resistance has decreased linearly (chi square for trend, p<0.001), perhaps reflecting overall reduced human use of this drug in Thailand as other agents have become more popular.

Concern in Thailand over the possibility of increasing rates of resistance to azithromycin developing in campylobacter species is not yet borne out by our study. Hoge (1) demonstrated azithromycin resistance rates of 15% in 1994 and 7% in 1995 among campylobacters. Our data show 7% resistance among isolates from 1996 to 1999, failing to confirm an upward trend.

Limitations of our study include the fact that isolates were obtained from multiple populations, both indigenous and U.S. military, in Thailand. However, the rates documented likely reflect overall resistance among human isolates. Additionally, the populations under study in Vietnam were all from areas around Hanoi and therefore do not necessarily reflect resistance rates seen in other parts of Vietnam.

This study highlights the need to be aware of regionally specific resistance rates to avoid inappropriate antibiotic use. Additionally, given the evidence for progressive resistance to the quinolone class and co-resistance between quinolones and azithromycin among salmonellae and campylobacters, the need to develop newer classes of antibiotics to treat dysentery and travelers' diarrhea is apparent. More efficient use of antimicrobial agents, for example, avoidance of antimicrobial therapy for acute noncholera watery diarrhea and use of effective data-based antimicrobial choices for dysentery, would likely help limit the development of resistance, although the impact of these measures would be offset by the widespread availability of antimicrobial agents over the counter in both these countries. Effective vaccines against the major causes of bacterial diarrhea are being developed but are still years away from commercial production. Parallel drug development efforts are required.

Dr. Isenbarger is a physician and epidemiologist in the Department of Enteric Infections, Walter Reed Army Institute of Research. His professional interests include clinical internal medicine, epidemiology of diarrheal disease in developing countries, and clinical trials of enteric vaccine candidates.

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Erratum Vol. 8, No. 1

In the article, "A Large Outbreak of Legionnaires' Disease at a Flower Show, the Netherlands," by Jeroen W. Den Boer et al., errors appear in two figures and their legends.

In Figure 3, part 3a should be identified as IgM and part 3b as IgG. The correct legends are as follows:

Figure 3a. Smoothed mean geometric immunoglobulin (Ig) M antibody titers to *Legionella pneumophila* of nearest 35 exhibitors in Halls 3 and 4 per 63 cm^2 of exhibition area; confirmed and probable cases among exhibitors in halls 3 and 4.
 ● = confirmed case in exhibitor; ◒= probable case in exhibitor;
 Bu = bubblemat; W = whirlpool spa.

Figure 3b. Smoothed mean geometric IgG antibody titers to *L. pneumophila* of 35 exhibitors nearest to whirlpool in halls 3 and 4 per 63 cm^2 of exhibition area; exhibitors ill with confirmed and probable cases in halls 3 and 4.
 ● = confirmed case in exhibitor; ◒= probable case in exhibitor;
 Bu = bubblemat; W = whirlpool spa.

In Figure 4, parts b and c were inadvertently omitted. The complete figure appears below. The correct legends are as follows:

Figure 4. Exhibition hall, West Frisian Flower Show, Bovenkarspel, the Netherlands, 1999.
4a. Circles indicate locations in water-supply system where water samples were taken. PE = polyethylene.
4b. Assessment of risk for *Legionella pneumophila* infection, by distance from water-using devices.
4c. Water samples taken and culture status, by distance from water-using devices.

We regret any confusion these errors may have caused. The article is corrected online, available at http://www.cdc.gov/ncidod/eid/vol8no1/01-0176.htm
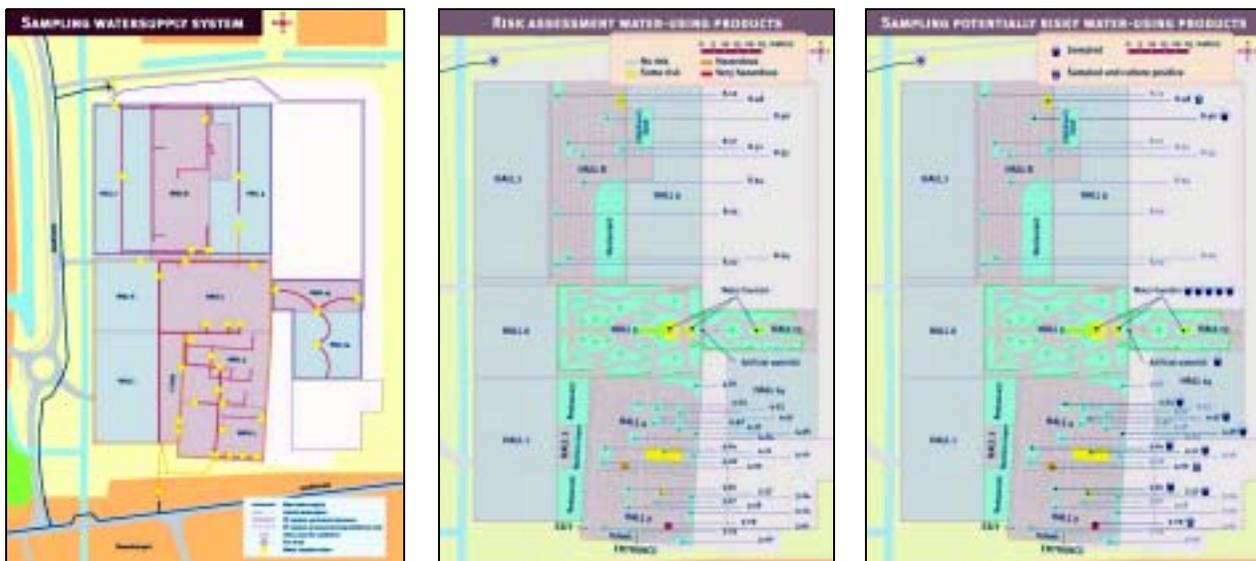
Epidemiology of *Burkholderia cepacia* Complex in Patients with Cystic Fibrosis, Canada

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The *Burkholderia cepacia* complex is an important group of pathogens in patients with cystic fibrosis (CF). Although evidence for patient-to-patient spread is clear, microbial factors facilitating transmission are poorly understood. To identify microbial clones with enhanced transmissibility, we evaluated *B. cepacia* complex isolates from patients with CF from throughout Canada. A total of 905 isolates from the *B. cepacia* complex were recovered from 447 patients in 8 of the 10 provinces; 369 (83%) of these patients had genomovar III and 43 (9.6%) had *B. multivorans* (genomovar II). Infection prevalence differed substantially by region (22% of patients in Ontario vs. 5% in Quebec). Results of typing by random amplified polymorphic DNA analysis or pulsed-field gel electrophoresis indicated that strains of *B. cepacia* complex from genomovar III are the most potentially transmissible and that the *B. cepacia* epidemic strain marker is a robust marker for transmissibility.

*B*urkholderia cepacia complex is an important group of pathogens in immunocompromised hosts, notably those with cystic fibrosis (CF) or chronic granulomatous disease (1,2). Lung infections with *B. cepacia* complex in certain patients with CF result in rapidly progressive, invasive, fatal bacteremic disease (3). Furthermore, the bacteria have a potential for patient-to-patient spread, both within and outside the hospital (4-9), raising questions about optimal measures for infection control.

The disease risk for infection with *B. cepacia* complex in patients with CF is substantially higher than with *Pseudomonas aeruginosa* alone or with bacteria other than *B. cepacia* or *P. aeruginosa* (10). However, there is a dramatic heterogeneity in outcome among CF patients infected with *B. cepacia* complex: some patients have a fulminant decline in pulmonary function, and others harbor *B. cepacia* complex for extended periods of time with no obvious adverse effects. The marked difference in prognosis among infected patients has not been adequately explained but is thought to result in part from differences among infecting strains of *B. cepacia* complex.

B. cepacia is a genetically highly diverse class of bacteria, which is composed of several different species and discrete groups constituting the *B. cepacia* complex (11). Each group differs sufficiently from the others to constitute a species, and those that are phenotypically distinct have been assigned species designation. Those that cannot be differentiated phenotypically but are genetically distinct are defined as genomovars (11). As phenotypic differentiation among the genomovars has improved over the past decade, new species designation has been assigned as follows: genomovar II = *B. multivorans*, genomovar IV = *B. stabilis*, genomovar V = *B. vietnamiensis*,

and genomovar VII = *B. ambifaria*. Genomovars I and III cannot be differentiated phenotypically, nor can *B. multivorans* and genomovar VI; these species must be distinguished by genetic methods. Bacteria from each of the genomovars have been recovered from patients with CF, but the predominant isolates in North America are from genomovar III and *B. multivorans* (12).

Numerous questions about the epidemiology of *B. cepacia* complex in CF are unanswered; for example, it is not known if certain genomovars or strains are more virulent than others. The relative risk for patient-to-patient spread of strains from each of the different genomovars is also unknown. Two genetic elements have been identified in strains having a propensity for epidemic spread. First, *cblA,* which encodes the protein for cable pilus production, is found in a single highly transmissible lineage from genomovar III that clusters among patients in the United Kingdom and Canada (13). Second, the *B. cepacia* epidemic strain marker (BCESM), which encodes a protein of unknown function, is found in many different strains from genomovar III, each of which is clustered in specific CF treatment centers (14).

Infection with bacteria from the *B. cepacia* complex has a profound effect on the lives of patients with CF. Since *B. cepacia* complex infection can be spread from one CF patient to another, provisions have been introduced in hospitals to limit contact among these patients. Infected patients are prohibited in some countries from attending social gatherings where other CF patients may be in attendance. Furthermore, since virulence appears to differ among strains and one strain may replace another, policies have been introduced in some centers to limit contact among patients who are infected with any strain from the *B. cepacia* complex. Lack of a clear understanding about the epidemiology of *B. cepacia* complex and the relative risk of infection with each of the different genomovars has spawned anxiety and confusion among CF patients,

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their caregivers, and families. Infection control policies have been developed in an effort to balance the rights of CF patients with careful consideration of their physical and mental health.

With burgeoning knowledge about the taxonomy, epidemiology, and virulence of the *B. cepacia* complex, many questions about appropriate infection control precautions have been raised. Consensus has been difficult to attain because of incomplete and conflicting data from various regions throughout the world. We undertook this study to provide a database from which infection control questions could begin to be answered.

In 1994 a *B. cepacia* complex research and referral repository for Canadian CF clinics was established in Vancouver. Since that time, *B. cepacia* complex isolates from an estimated 75% of infected Canadian CF patients have been evaluated for genomovar and species identity, random amplified polymorphic DNA (RAPD) strain type, and putative markers of transmissibility. These data have permitted inferences about the potential transmissibility of different strains and facilitated the development of rational infection control guidelines. We report data and conclusions from our observations to date.

Materials and Methods

Patients and Clinics

Canadian CF clinics are linked through the Canadian Cystic Fibrosis Foundation, which collates annual summary data in its patient data registry. Approximately 3,200 patients with CF receive care at 36 clinics in the 10 provinces. Each clinic provides care for 20 to 300 patients (median age 17 years). The number of patients with CF in Canada has increased by approximately 90 each year since 1994, although the median survival age has plateaued at approximately 30 years.

In 1994, the Canadian *B. cepacia* Complex Research and Referral Repository was established at the British Columbia Research Institute for Children's and Women's Health in Vancouver. Each clinic director was notified about the new laboratory and encouraged to send archived and new isolates of *B. cepacia* complex to the Vancouver laboratory for strain typing and confirmation of species identity (7). At least one isolate from each infected patient was solicited, as well as subsequent isolates that were considered phenotypically different.

Species and Genomovar Determination

A polyphasic scheme (15) was used to determine the species or genomovar classification of each isolate.

Phenotypic Identification of *B. cepacia* Complex and Other Organisms

Isolates were identified as described (15,16): purity, morphology, and hemolysis were observed, and oxidase activity (Pathotec cytochrome oxidase, Remel, Lenexa, KA) was tested after growth on Columbia agar with 5% sheep blood

(PML Microbiologicals, Richmond, British Columbia, Canada). Bacteria were incubated for up to 7 days at 35°C in the following sugars: glucose, maltose, lactose, xylose, sucrose, and adonitol. Moeller lysine, ornithine, and negative control were also heavily inoculated and incubated at 35°C for 48 hours. The API 20 NE strip (Biomerieux Vitek Inc., Hazelwood, MO) was set up according to manufacturer's instructions, except that the strip was incubated at 35°C and observed at 24 and 48 hours. Growth on MacConkey agar without crystal violet (Difco Laboratories, Detroit, MI) and on *B cepacia* selective agar (16) at 35°C was observed at 24 and 48 hours. Pigment production and growth on tryptic soy agar at 35°C and 42°C were observed at 24 and 48 hours.

Molecular Methods

Genomovar-Specific PCR for the *recA* Gene

Polymerase chain reaction (PCR) with selected *recA* primers was performed essentially as described (15,17). Tests were done with the six *recA* subgroup genomovar-specific primers (15,17) and a seventh primer pair for genomovar VII as described (15,18). After amplification, 8 µL of each reaction mixture was subjected to electrophoresis in 1.5% agarose gel. PCR products were photographed after ethidium bromide staining. *B. cepacia* complex strains that did not react with the specific primers described above were subjected to nucleotide sequence analysis of the *recA* gene (17). Placement in the complex was then done phylogenetically by analysis of 500 bp of the N-terminal encoding sequence according to the algorithm described (17).

Speciation with the 16S *rRNA* Gene

Restriction fragment length polymorphism (RFLP) analysis of the 16S *rRNA* gene PCR product with the enzyme *Dde*1 was performed as described (15,17).

Genotypic Identification of *B. gladioli*

A PCR reaction with primer pair LP1/LP4, directed toward a species-specific region of the 23S *rRNA* gene, was used (19).

Strain Typing of *B. cepacia* Complex Isolates

Each isolate was evaluated for RAPD strain type by RAPD analysis (20). If typing results were ambiguous, pulsed-field gel electrophoresis (PFGE) analysis was performed (21).

Groups of isolates that were unambiguously identical by RAPD with or without PFGE were each assigned numerical types. Unique isolates were designated X until another identical isolate was identified.

Evaluation for Markers of Transmissibility

Southern dot blot analysis was performed (14) to determine if each isolate encoded either of the genetic markers of transmissibility (*cblA* or BCESM).

Results

Isolates Received from Canadian CF Clinics

A total of 922 isolates considered to be *B. cepacia* complex (or possible *B. cepacia* complex) by the referring laboratory were received through July 2000 (Table 1). These isolates were recovered from 459 different patients. Most specimens were received after a request for strains was made in 1994 through the Canadian Cystic Fibrosis Foundation; however, 95 isolates were archived specimens obtained from 1981 to 1991. Seventeen isolates (from 17 patients) were organisms that had been misidentified as *B. cepacia* complex. Fourteen of these 17 isolates had been received before 1997, when information about the importance of correct identification began to be disseminated by the International *B. cepacia* Working Group (http://allserv.rug.ac.be/~tcoenye/). The 17 isolates included 5 of *Stenotrophomonas maltophilia*; 4 of *Pseudomonas* species; 3 of *Alcaligenes xylosoxidans*; and 1 each of *Enterobacter agglomerans*, *Candida* species, mixed gram-positive bacteria, and 2 undescribed new species. Eighteen isolates belonged to species that are phenotypically similar to *B. cepacia* complex (Table 2). *Pandoraea* species and *B. fungorum* have only recently been described (22,23).

Geographic Distribution of CF Patients Infected with *B. cepacia* Complex

Since this study was conducted by passive ascertainment of bacterial cultures, the representation from regions of Canada differed considerably. Isolates confirmed as members of the *B. cepacia* complex were received from 8 of 10 provinces (Table 1). Only Saskatchewan, Prince Edward Island, and the territories (Yukon, Nunavut, and Northwest) did not submit specimens.

The consistently monitored data in the Patient Data Registry of the Canadian Cystic Fibrosis Foundation provide good estimates of regional patterns of *B. cepacia* complex prevalence and repository representation. To provide more stable estimates and preserve confidentiality in provinces with a small number of centers, the cumulative prevalence of infection from 1992 to 1997 is reported for four regions of Canada (Table 3). The prevalence of *B. cepacia* complex was highest in Ontario and in the eastern provinces. Although Quebec and Ontario are contiguous and have similar populations, the prevalence of infection with *B. cepacia* complex was substantially higher in Ontario. The provinces west of Ontario had a combined prevalence intermediate between those of Ontario and the eastern provinces, but this region covers a very large geographic area, which may include heterogeneous provincial prevalence rates.

Although the relative prevalence of different genomovars varied from province to province, genomovar III was found in every province in which patients were infected with *B. cepacia* complex (Table 1). The only province from which *B. multivorans* was frequently recovered was British Columbia. Each isolate of *B. multivorans* was unique except for those recovered from sibling pairs (24).

Prevalence of Genomovars and Species of Isolates from the *B. cepacia* Complex

All initial isolates and those that appeared phenotypically different from previously received isolates from individual patients were evaluated for genomovar and species identity by a polyphasic scheme involving both biochemical analyses and molecular methods (*recA* PCR and 16s *rRNA* RFLP) (Table 2). Most isolates (80%) were from genomovar III and included all strains that clustered in individual centers and appeared to be transmitted from patient to patient. Approximately 9% of infected patients were infected with *B. multivorans* (genomovar II), but there was little evidence among these isolates of genotypic clustering as determined by RAPD and PFGE.

Table 1. *Burkholderia cepacia* complex isolates and phenotypically similar organisms recovered from patients with cystic fibrosis in Canada

Province	No. of patients from whom isolates were submitted	Total no. of isolates submitted	No. of genomovar/species submitted from different patients		
			B. multivorans (genomovar II)	III	Other
British Columbia	95	394	33	52	20
Alberta	45	55	2	32	13
Manitoba	7	7	3	3	1
Saskatchewan	0	0	0	0	0
Ontario	243	292	2	233	8
Quebec	23	48	3	15	5
Prince Edward Island	0	0	0	0	0
New Brunswick	4	4	0	3	1
Nova Scotia	17	89	0	18	2
Newfoundland	13	16	0	13	0
Territories	0	0	0	0	0
Total (including territories)	447	905	43	369	50

Table 2. Genomovar or species of *Burkholderia cepacia* complex or phenotypically similar isolates from cystic fibrosis patients in Canada

Species or genomovar	No. of patients infected with species or genomovar[a]	Percentage of patients (%)
Genomovar I	1	0.2
Burkholderia multivorans (genomovar II)	43	9.3
Genomovar III	369	80.0
Burkholderia stabilis (genomovar IV)	17	3.8
Burkholderia vietnamiensis (genomovar V)	7	1.6
Burkholderia cepacia complex (not genomovar I-VII)	8	1.8
Burkholderia fungorum	1	0.2
Burkholderia gladioli	5	1.1
Ralstonia pickettii	5	1.1
Pandoraea spp.	5	1.1
Total	461	

[a]Some patients were counted twice if two or more different strains were recovered.

Isolates from the other genomovars and species (genomovar I, *B. stabilis, B. vietnamiensis,* and *B. cepacia* complex bacteria of indeterminate genomovar status) were recovered, but at very low frequency (Table 2). Several patients were infected with more than one strain or genomovar from the *B. cepacia* complex, but in all but one case, one strain replaced another that had been identified previously**.** Replacement of genomovar II (*B. multivorans*) by genomovar III occurred in six patients (24).

Eight patients were infected with *B. cepacia* complex bacteria that did not belong to any of the currently defined genomovars (Table 2). The full-length *recA* gene was amplified from these isolates by using primers BCR1 and BCR2 (17). These strains produced novel *recA* RFLP products, and none reacted with the PCR primers developed to identify the current genomovars (17,18). Their biochemical profile was consistent with that of the *B. cepacia* complex (data not shown). Analysis of the 16S rRNA gene by RFLP demonstrated that these strains were not *B. multivorans, B. vietnamiensis*, or genomovar VI, since they had the single RFLP profile shared by all the remaining current genomovars/species (*B. stabilis,* I, III, and

VII; pattern 2 [17]). The nucleotide sequence of the *recA* gene from five isolates representative of these novel strains was examined phylogenetically (Figure). These strains form two unique, distinct clusters with the current *B. cepacia* complex (Figure), suggesting that they are members of the current complex but may be novel taxonomic groups or subgroups of the existing genomovars not detected by the current molecular tests.

Geographic Distribution of *B. cepacia* Complex from Different Epidemic RAPD Strain Types

We have identified four genetic lineages of *B. cepacia* complex genomovar III that cluster by geographic region in Canada (Table 4). Each of these types was defined by RAPD and confirmed by PFGE. All the isolates from types 01, 04, and 06 harbored the BCESM, but only those from RAPD type 02 encoded both BCESM and *cblA*.

RAPD type 02 was the predominant genomovar III lineage in Canada. This is the same clone that is reported to have spread intercontinentally between Canada and the United Kingdom and is also known as ET12 (5). The *cblA* gene codes for production of a cable pilus thought to enhance adhesion to epithelial cells.

Discussion

This analysis of isolates from the *B. cepacia* complex from a broad geographic distribution may facilitate insights into the epidemiology and virulence of this evolving class of bacteria in patients with CF. Most Canadian CF patients are cared for at centralized clinics in each province, and the data are relayed to a central registry at the national office of the Canadian CF Foundation. Regular audits by the Foundation enhance the quality of care at the individual CF clinics; as a result, microbiologic investigation of CF patient samples is optimized. The rate of misidentification of *B. cepacia* complex organisms in Canada has been very low since standard methods for culture and identification were publicized in 1997. Our recent experience contrasts with other reports of misidentification in Canada and the United States (25,26). An estimated 75% of prevalent *B. cepacia* infection was reported to the Canadian *B. cepacia* Complex Research and Referral Repository, and regional differences were similar to those recorded in the patient data registry. Therefore, the data reported here proba-

Table 3. Regional prevalence of cystic fibrosis (CF) patients infected with *Burkholderia cepacia* complex and representation of their isolates in Canadian *B. cepacia* Research and Referral Repository, 1992–1997

Region of Canada	1996 census, population, thousands	No. of CF patients, 1992-1997	No. (%) of CF patients infected with *B. cepacia* complex	No. (%) of CF patients with samples of *B. cepacia* complex in repository
West	8,816	975	117 (12)	91 (78)[a]
Ontario	11,101	1293	285 (22)	241 (85)
Quebec	7,274	1088	55 (5)	16 (29)
East	2,381	405	103 (25)	27 (26)

[a]Archived samples from the main study center have been excluded here to provide comparative regional estimates.
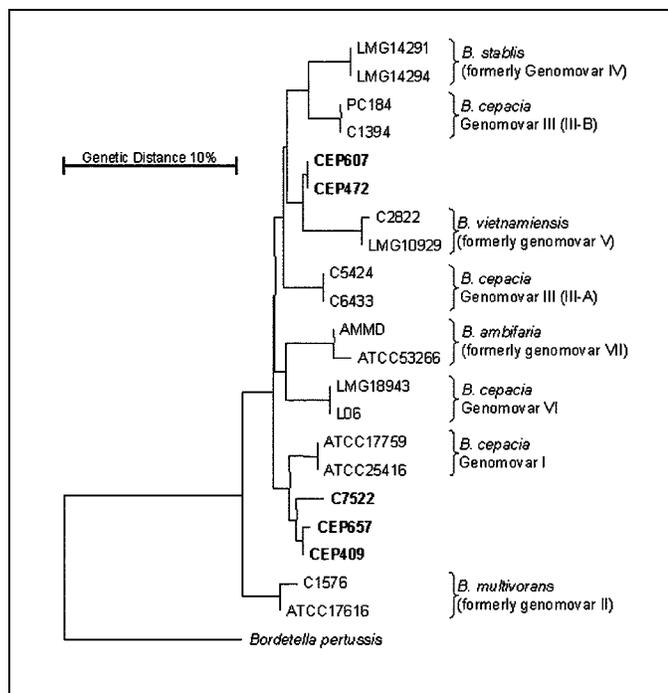
Figure. Phylogenetic analysis of the *recA* gene from the *Burkholderia cepacia* complex. The phylogenetic diversity of the *B. cepacia* complex observed after nucleotide sequence analysis of the *recA* gene is shown. Isolates recovered from Canadian CF patients that are representative of strains of currently indeterminate genomovar status (Table 2) appear in bold and lack species identification; all fall within the current *B. cepacia* complex. The tree was drawn as described (16). The *recA* sequence from *Bordetella pertussis* was used as a root, and the genetic distance is indicated by the bar.

bly reflect true national trends in prevalence and strain distribution of *B. cepacia* complex organisms in CF patients in Canada.

Remarkable differences in prevalence and RAPD strain type clustering were noted among Canadian provinces. This was most striking in Ontario and Quebec, Canada's two most populous provinces. These provinces are contiguous, but the prevalence of *B. cepacia* complex infection was about 10-fold higher in Ontario than in Quebec. Patients in these two provinces probably had very little contact with each other because of the geographically wide separation between clinics in the major population centers (Toronto and Montreal). Furthermore, the difference in primary language between the two provinces may have discouraged social mixing when opportunities arose. The predominant RAPD strain type recovered from patients in Ontario (RAPD type 02) was rarely cultured from patients in Quebec. This strain is prevalent throughout the United Kingdom and appears to have spread to Britain as well as to other parts of Canada as a result of common exposures in summer camps in Ontario (9).

Clustering of RAPD strain types by province suggests patient-to-patient spread. Most isolates of RAPD type 02 in British Columbia and in the Maritime provinces (Nova Scotia and Newfoundland) can be traced to care received in Toronto, where that is the predominant type (E. Tullis, pers. comm.). Since cohorting of patients was instituted in Canadian clinics in 1994, the spread of RAPD type 02 has slowed (24). Most new acquisitions of *B. cepacia* complex organisms in British Columbia since 1994 have been *B. multivorans* (24). Each new isolate has had a unique genetic fingerprint, suggesting that acquisition has not been from other patients, but from the environment. Recent reports from France and Italy describe the rhizosphere as an important environmental reservoir for *B. cepacia* complex isolates (27,28). We are searching for potential environmental reservoirs in Canada.

Clusters of common RAPD strain types in CF clinics have been from genomovar III. The four common RAPD strain types (01, 02, 04, and 06) all encode BCESM, but only type 02 encodes *cblA*. Although the latter appears to enhance adhesion of the bacteria to epithelial cells, the role of BCESM in transmissibility has not been determined.

Table 4. Number of cystic fibrosis patients infected with *Burkholderia cepacia* complex genomovar III RAPD strain type, by Canadian province

Province	RAPD strain type				
	01	02	04	06	Other (BCESM+)[a]
British Columbia	9	11	16	7	10 (5)
Alberta	2	14	16	0	0
Manitoba	0	1	1	0	1 (0)
Saskatchewan	0	0	0	0	0
Ontario	1	223	2	0	7 (5)
Quebec	5	7	1	0	2 (2)
Prince Edward Island	0	0	0	0	0
New Brunswick	0	0	2	0	1 (0)
Nova Scotia	0	2	12	0	4 (0)
Newfoundland	0	13	0	0	0
Total	17	271	50	7	25 (12)

[a]Number of patients whose isolates had *Burkholderia cepacia* epidemic strain marker (BCESM) in the category "other genomovar III RAPD strain types." Strain types 01, 02, 04, and 06 all encoded BCESM. RAPD = random amplified polymorphic DNA.

Evidence of patient-to-patient spread of genomovar III *B. cepacia* complex strains has been documented in studies from different geographic regions (9,24). Our results support the likelihood that spread of these strains occurs throughout Canada. Most patients infected with genomovar III RAPD type 02 may have acquired the strain directly or indirectly from patients from Ontario. The factors that enhance such patient-to-patient spread have not been clearly determined, but segregation appears to have been successful in limiting transmission.

B. multivorans (genomovar II), in contrast to genomovar III, does not appear to have spread from patient to patient in Canada. Each of the isolates was typed by RAPD, and each had a unique genetic fingerprint. The only exceptions were isolates from a sibling pair who transiently shared the same strain. This observation contrasts with those from other parts of the world, where *B. multivorans* has been observed to cluster in CF clinics, suggesting patient-to-patient spread (12,29,30). The differences between our observations and those of others may be explained on the basis of difference in infecting strain types; the Canadian *B. multivorans* isolates may lack the putative factors necessary for patient-to-patient spread. Alternatively, infection control practices in Canada may differ from those elsewhere. The differences between the epidemiology of *B. multivorans* in Canada and the United Kingdom are analogous to that of *Pseudomonas aeruginosa* in CF. No evidence of patient-to-patient spread of *P. aeruginosa* in Canada has been documented (despite intensive investigation), but well-documented outbreaks of epidemic spread among patients in Liverpool and Manchester, United Kingdom (31,32), and Melbourne, Australia (33), have been reported.

The evidence of patient-to-patient spread of bacteria from the *B. cepacia* complex among patients with CF and the adverse prognosis of those who are infected (10,34) demands stringent efforts to prevent new acquisition. Strategies have been introduced in Canada, the United States, the United Kingdom, and elsewhere to limit spread both within and outside hospitals. These strategies appear to have limited the epidemic spread of certain clones of *B. cepacia* complex, but the prevalence of infection has remained largely unchanged. Infection control precautions are based on lessons learned from the control of spread of other respiratory tract pathogens; they may or may not be relevant for CF, with its unique host-pathogen relationship. Furthermore, *B. cepacia* complex is an opportunistic pathogen and is commonly found in the natural environment in such places as soil and plant roots (27,28,35-37). The mode of acquisition of *B. cepacia* complex in CF patients appears to be both from other patients and from the environment. Until more is known about risk factors for acquisition, rational infection control strategies will be difficult to design. We are attempting to identify the factors that may be correlated with acquisition of this problematic pathogen in Canadian patients with CF.

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Broad-Range Bacterial Detection and the Analysis of Unexplained Death and Critical Illness

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Broad-range rDNA polymerase chain reaction (PCR) provides an alternative, cultivation-independent approach for identifying pathogens. In 1995, the Centers for Disease Control and Prevention initiated population-based surveillance for unexplained life-threatening infections (Unexplained Death and Critical Illness Project [UNEX]). To address the causes of UNEX cases, we examined 59 specimens from 46 cases by using broad-range bacterial 16S rDNA PCR and phylogenetic analysis of amplified sequences. Specimens from eight cases yielded sequences from *Neisseria meningitidis* (cerebrospinal fluid from two patients with meningitis), *Streptococcus pneumoniae* (cerebrospinal fluid from one patient with meningitis and pleural fluid from two patients with pneumonia), or *Stenotrophomonas maltophilia* (bone marrow aspirate from one patient with pneumonia). *Streptococcus pneumoniae* rDNA sequence microheterogeneity was found in one pleural fluid specimen, suggesting the presence of multiple strains. In conclusion, known bacterial pathogens cause some critical illnesses and deaths that fail to be explained with traditional diagnostic methods.

I n 1995, the Centers for Disease Control and Prevention (CDC) initiated a large-scale, population-based surveillance study to detect life-threatening infectious diseases in previously healthy persons 1 to 49 years of age; this study is known as the Unexplained Death and Critical Illness Project (UNEX) (1). Surveillance was performed through the CDC Emerging Infections Program sites in California (Alameda, Contra Costa, and San Francisco counties), Connecticut (New Haven County), Minnesota, and Oregon. At the start of the project, the surveillance population base was 7.7 million. The study design was a prospective case series obtained by enhanced passive surveillance. Only cases from which cultures and routine serologic tests failed to provide a microbiologic diagnosis were selected for further study. Work-up of cases was syndrome based and algorithm directed. Case investigation included laboratory analysis by broad-range 16S rDNA polymerase chain reaction (PCR) to identify the presence of bacteria in specimens collected from normally sterile anatomic sites.

Broad-range bacterial PCR is based on the use of primers that recognize conserved sequences of bacterial chromosomal genes encoding ribosomal RNA (rDNA). The resulting amplified rDNA sequences also contain variable regions and provide a reliable basis for the analysis of phylogenetic relationships among different forms of cellular life. Broad-range bacterial PCR has been used to identify previously uncharacterized as well as known bacterial pathogens directly in clinical specimens, including cerebrospinal fluid (CSF), synovial fluid, blood, and heart valve tissue (2-9). The use of broad-range bacterial 16S rDNA PCR for identifying bacterial culture isolates in the clinical laboratory is well established (10), and this technique is useful as a routine supplemental method for direct bacterial detection from clinical specimens (11). However, broad-range bacterial rDNA PCR has not previously been applied in a systematic manner for the diagnosis of unexplained deaths and life-threatening illnesses with features suggesting infection. In our study, we examined this issue.

Materials and Methods

Patients and Specimens

Patients were recruited as part of the CDC Emerging Infections Program's UNEX study and met all project inclusion criteria, including absence of important preexisting medical conditions, development of an illness with defined features suggestive of infectious etiology, severity leading to admission to an intensive care unit or to death, and failure of cultivation and routinely available serologic tests to provide a microbiologic diagnosis (1). Of specimens available from more than 139 cases fulfilling these criteria, only those from anatomic sites that are usually culture negative in healthy persons and those from patients with syndromes of suspected bacterial etiology were selected for broad-range bacterial PCR analysis. These 59 specimens from 46 patients were collected from July

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1995 to January 2000. The median age of the 46 patients (21 female, 25 male) was 18.5 years (range 1 to 47 years). Most had neurologic (n=17) or respiratory manifestations (n=14). Five had cardiac, one had abdominal, and nine had multiorgan manifestations. The patients resided in New Haven County (CT) (n=5), Minnesota (n=6), Oregon (n=20), or three counties in the San Francisco Bay Area (n=15).

Bacterial Strains

Partial 16S rDNA sequences were determined from three *Streptococcus pneumoniae* clinical isolates obtained from patients in the San Francisco Bay Area during 1997 and 1998 (strains SF10014, SF10175, and SF10314; California Department of Health Services, Berkeley, CA). *Escherichia coli* strain B DNA (Sigma, St. Louis, MO) was used as a positive control and as template for measurement of PCR assay sensitivity in reactions with primers fD1mod and 16S1RR-B (see below).

Specimen Processing

Specimens were processed in parallel with interspersed negative control specimens. DNA was extracted from serum (n=4), blood (n=25), blood culture (n=9), and bone marrow (n=2) specimens by using the chaotropic properties of guanidine isothiocyanate, followed by DNA extraction and purification by alcohol precipitation with the IsoQuick Nucleic Acid Extraction Kit (ORCA Research, Bothell, WA). Pleural fluid (n=3) was digested with proteinase K (Sigma) and non-ionic detergent, essentially as described (12). CSF (n=16) was processed by either of these methods.

Broad-Range Bacterial rDNA PCR

All specimens were analyzed with PCR methods using at least one of the following four bacterial broad-range 16S rDNA primer pairs: fD1mod (5'-AGAGTTTGATCYTG-GYTYAG-3', corresponding to positions 8-27 in the *E. coli* 16S rRNA gene) (4) and 16S1RR-B (5'-CTTTACGCCCAR-TRAWTCCG-3', 575-556) (6); 63F (5'-CAGGCCTAACA-CATGCAAGTC-3') (13) and 16S1RR-B; 8F2 (5'-TGGAGAGTTTGATCCTGGCTCAG-3', 5-27) and 806R (5'-GGACTACCAGGGTATCTAAT-3', 806-787) (3); and 515F (5'-GTGCCAGCAGCCGCGGTAA-3', 515-533) (3) and 13R (5'-AGGCCCGGGAACGTATTCAC-3', 1390-1371) (3). The presence of amplifiable DNA and PCR inhibitors was assessed with human beta-globin gene PCRs and primers PCO4 and GH20 (3).

Forty-three specimens from 34 patients were analyzed with primer pair fD1mod/16S1RR-B. Reactions based on these primers contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 200 µM each deoxynucleoside triphosphates, 2.5 U AmpliTaq LD DNA polymerase (PE Biosystems, Foster City, CA), and 5-µL template, as well as 20 pmol of each primer in a total volume of 50 µL. After a denaturation step of 3 min at 94°C, PCR steps at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec were repeated 30 to 36 times, followed by

an elongation step at 72°C for 7 min in a GeneAmp PCR system 2400 thermal cycler (PE Biosystems). Products were detected by agarose gel electrophoresis and DNA staining with ethidium bromide. In most cases, the presence of product was also assessed by attempting to generate recombinant plasmid clones (see below). PCR amplification conditions used with some other bacterial broad-range primer pairs included 100-µL reaction volumes and a 4-min initial denaturation step. Reactions with all reagents necessary for PCR, including water processed in parallel with clinical specimens, were performed as negative controls.

DNA Sequence Determination and Phylogenetic Analysis

PCR products were sequenced directly, as well as after cloning, by using the TA or TOPO cloning systems (Invitrogen, Carlsbad, CA). Automated ABI PRISM 373 or 377 DNA sequencers (PE Biosystems) and BigDye Terminator Cycle sequencing chemistry were used for determining DNA sequences. Both DNA strands were analyzed, and base-editing was performed together with manual review of the electropherograms, using AutoAssembler (PE Biosystems). The 16S rDNA sequences were compared with those in the GenBank database by using the BLAST search tool (14). In addition, the automated 16S rRNA sequence alignment tool and phylogenetic algorithms from the ARB software package (Technical University of Munich, Germany) were used (15). Complete sequences of the four rRNA operons of an *S. pneumoniae* serotype 4 strain were obtained from The Institute for Genomic Research, Rockville, MD (16). Unpublished sequence data from the rRNA operons of a different *S. pneumoniae* strain were obtained from SmithKline Beecham, Philadelphia, PA.

Results

Bacterial 16S rDNA sequences were detected in specimens from 8 of the 46 patients. When the same PCR conditions were used, negative control reactions failed to yield a visible product of the expected size. The absence of large amounts of inhibitors of the PCR reaction in specimens from the remaining 38 patients was demonstrated by the ability to amplify human beta-globin sequences from those specimens. The specimen types with positive bacterial 16S rDNA PCR results were CSF (three patients), pleural fluid (two patients), bone marrow aspirate (one patient), and blood culture bottle material (two patients) (Table 1). The sensitivity of this PCR assay with primers fD1mod/16S1RR-B was 5-50 *E. coli* 16S rDNA gene copies, as assessed by using purified *E. coli* DNA as template.

CSF was studied from 14 project cases with unexplained meningitis. Specimens from five subjects were analyzed with broad-range bacterial rDNA primer pairs 8F2/806R and 515F/13R. PCR products of approximately the anticipated size (804 and 876 bp, respectively) were generated from specimens from cases XOR34 and XOR06. From case XOR34, a 1342-bp 16S rRNA gene consensus sequence was generated from the overlapping gene fragments. This sequence shared 99.3% similarity (1332/1342 nucleotide positions) with the four identical

Table 1. Characteristics of the bacterial 16S rDNA broad-range polymerase chain reaction (PCR)-positive cases

Case ID	Sex	Age (yrs)	Duration of antibiotic therapy before specimen obtained	Clinical syndrome(s)[a]	16S rDNA PCR and sequencing results	Specimen	Outcome
XOR6	M	18	10 min	Neurologic	*Neisseria meningitidis*	CSF	Survived
XOR34	M	13	3 days	Neurologic	*N. meningitidis*	CSF	Survived
XCA73	F	19	1 day[b]	Respiratory & neurologic	*Streptococcus pneumoniae*	CSF	Survived
XEB44	F	10	3 days	Respiratory	*S. pneumoniae*	Pleural fluid	Survived
XMN22	M	43	2 weeks	Respiratory	*S. pneumoniae*	Pleural fluid	Survived
XOR63	M	29	1 month	Respiratory	*Stenotrophomonas maltophilia*	Bone marrow aspirate	Died (no autopsy)
XOR56	M	11	none	Multisystem	*Staphylococcus epidermidis*	Blood culture material	Survived
XCT29	F	10	1 week	Cardiac	*Bacillus* sp., *Halomonas* sp., *Enterococcus* sp.	Blood culture material	Survived

[a]The primary clinical syndrome(s) during hospitalization.
[b]A second cerebrospinal (CSF) sample obtained 5 days later also contained *S. pneumoniae* rDNA.

16S rRNA gene sequences from each of the recently published complete *N. meningitidis* serogroup A and B genomes (GenBank accession numbers AL162758 and AE002551); these GenBank sequences were the closest match to the XOR34 case sequence. Direct sequencing of PCR products from case XOR06 generated 654 bp of sequence homologous to the *N. meningitidis* serogroup A and B 16S rRNA genes. Limited CSF from this case prevented a more complete sequence analysis of the bacterial rDNA in this specimen.

CSF from nine other patients was analyzed by using primers fD1mod/16S1RR-B. Two CSF specimens drawn on different days from case XCA73 each generated PCR products whose directly determined sequences were identical (526/526 bp) to the published 16S rDNA sequence from a *S. pneumoniae* reference strain (NCTC 7465T, AJ001246) and with the 16S rDNA sequences from three *S. pneumoniae* strains isolated from patients in the same region and from the same time period (SF10014, SF10175 and SF10314); these reference sequences were determined as part of this study (Figure). The sequences of two clones from each of the amplified XCA73 products were identical to the sequences obtained directly from the PCR products. The CSF specimens from cases XOR06, XOR34, and XCA73 had been collected after empiric antibiotic therapy had begun.

From two of the three culture-negative pleural fluid specimens (cases XEB44 and XMN22) DNA products of the expected size (~568 bp) were amplified with bacterial broad range primers fD1mod/16S1RR-B. In addition to analysis of cloned molecules from these products, the PCR product from case XEB44 was sequenced directly; insufficient product was obtained from the pleural fluid of case XMN22 for direct sequencing. From the XEB44 pleural fluid specimen, DNA was also amplified with bacterial broad-range primers 63F/16S1RR-B. Three recombinant plasmid clones from this product were characterized by DNA sequencing (Table 2). One

clone sequence from each of the pleural fluid specimens (clones C and G in Table 2) was identical (497/497 and 509/509 bp) to an *S. pneumoniae* rDNA sequence deposited in
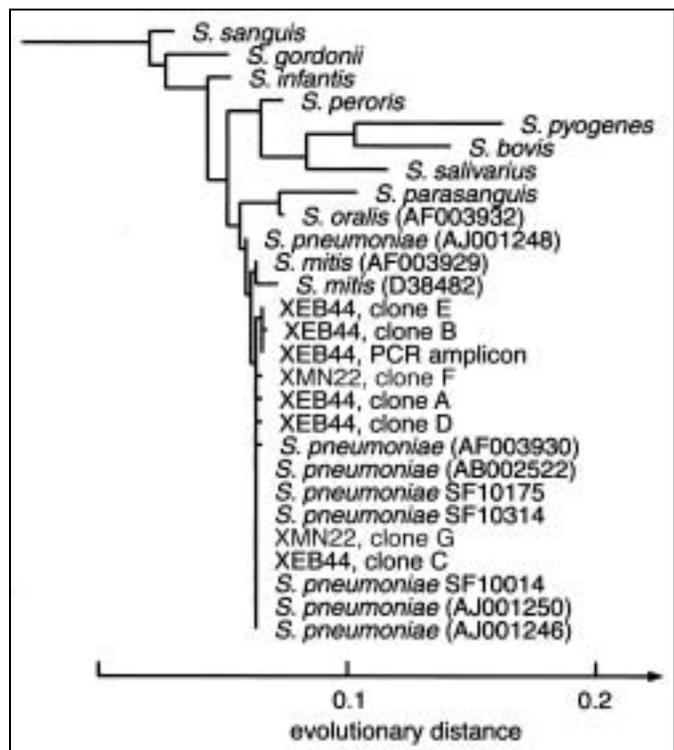
Figure. Phylogenetic analysis of the bacterial 16S rDNA sequences obtained from cases XEB44 and XMN22. The tree was rooted with *Staphylococcus aureus* and *Escherichia coli* as outgroups and constructed with a maximum-likelihood algorithm using 468 homologous sequence positions that were selected from a sequence dataset of 497 total positions. *Streptococcus pneumoniae* clinical isolates sequenced for this study are marked as SF10175, SF10314, and SF10014. GenBank database accession numbers for published sequences are given in parentheses. All six sequences from the case XCA73 cerebrospinal fluid were identical to those of the *S. pneumoniae* reference strain (accession no. AJ001246). PCR = polymerase chain reaction.

Table 2. Variability in the amplified *Streptococcus pneumoniae* 16S rDNA sequences[a]

Corresponding *E. coli* 16S rDNA position	Pleural fluid								Cerebrospinal fluid						Bacterial isolates			
	XEB44[b]						XMN22		XCA73[c]									
	Specimen 1-PCR analysis 1			Specimen 1-PCR analysis 2			Specimen 1-PCR analysis		Specimen 1-PCR analysis			Specimen 2-PCR analysis						
	Original PCR product	Clones from PCR products		Clones from PCR products			Clones from PCR products		Original PCR product	Clones from PCR products		Original PCR product	Clones from PCR products					
		A	B	C	D	E	F	G		H	I		J	K	SF10 014	SF10 175	SF10 314	Reference strain[d]
120	**A/G**	**G**	**G**	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
260	**A/G**	G	**A**	G	G	**A**	G	G	G	G	G	G	G	G	G	G	G	G
273	A	A	A	A	A	A	**G**	A	A	A	A	A	A	A	A	A	A	A
279	A	A	A	A	**G**	A	A	A	A	A	A	A	A	A	A	A	A	A
442	T	T	T	T	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T

[a] Nucleotides in bold differ with the reference strain sequence at the indicated rDNA position.
[b] One pleural fluid sample was analyzed with PCR on two occasions.
[c] Two cerebrospinal fluid specimens were taken 5 days apart from the same patient.
[d] *Streptococcus pneumoniae* NCTC 7465T (GenBank accession no. AJ001246).
E. coli = *Escherichia coli*; PCR = polymerase chain reaction.

GenBank (reference strain NCTC 7465[T], #AJ001246)(Figure). However, four other clones from XEB44 (clones A, B, D, and E) and one from XMN22 (clone F) contained one or two positions with variant nucleotides. This variability was confirmed in case XEB44 by examining the sequence obtained directly from the PCR product (Table 2, original PCR product). In contrast, ambiguities were not seen in directly sequenced PCR products from the three clinical isolates (SF10014, SF10175, SF10314), nor in the sequences obtained from case XCA73 CSF.

To understand better the variation observed in these pneumococcal sequences, a model of the secondary structure of the *S. pneumoniae* 16S rRNA was constructed, based on the published secondary structure of the *Bacillus subtilis* homolog (17) (supplemental data available at http://relman.stanford.edu). Two of the five polymorphic positions are predicted to participate in a "stem" structure and can therefore be assessed for compensatory changes at the paired position. Of the two, only the polymorphism at position 442 creates a noncanonical pairing, thereby suggesting a possible *Taq* polymerase incorporation error. Intrachromosomal allelic variability was another possible explanation for the observed polymorphisms in these pleural fluid *S. pneumoniae* 16S rDNA sequences. However, the complete genome sequence of each of two *S. pneumoniae* strains contains four identical copies of the 16S rRNA operon (16) (unpub. data, Michael A. Loretto and Martin Rosenberg). Therefore, our data are consistent with the hypothesis that patient XEB44 was infected in the pleural space by at least two different *S. pneumoniae* strains. Insufficient amounts of data from the patient XMN22 specimen hamper speculation of a similar nature for this case.

PCR analysis of a bone marrow aspirate from a case of fatal respiratory disease (XOR63) showed a 16S rDNA sequence that was 99.8% (527/528 bp) similar to 16S rDNA sequences from *Stenotrophomonas maltophilia* (strain ATCC 13637, #AB008509). This sequence was obtained directly from the PCR product as well as from two recombinant clones; all were identical. A bone marrow aspirate from another patient in our study did not reveal bacterial 16S rDNA sequences.

Two blood culture bottle specimens (from cases XOR56 and XCT29) of nine that were studied gave positive results for bacterial rDNA using PCR primer pair fD1mod/16S1RR-B. All these specimens had been collected from inoculated culture bottles that had failed to exhibit signs of bacterial growth during routine incubation. From one of the two positive specimens (case XOR56), sequences obtained directly from the PCR product and from two recombinant clones were identical (at 535 positions) to the 16S rDNA sequence of a *Staphylococcus epidermidis* type strain (ATCC 14990T, #D83363). From the other PCR-positive blood culture specimen (case XCT29), direct sequencing of the PCR products indicated the presence of multiple, different DNA molecules. Five recombinant clones were sequenced. Two clones were 99.8% (545/546 bp) similar to 16S rDNA sequences from *Enterococcus faecium* (strain DSM20477, #AJ276355) and *E. durans* (strain DSM20633, #AJ276354). One clone was 99.8% similar to a sequence from *Bacillus stearothermophilus* (strain IF012550, #AB021196), one was 98.6% similar (509/516) to a sequence from a *Halomonas sp.* (strain ML-028, #AF139994), and one clone with a truncated sequence was 96.4% similar (323/335 bp) to several *Bacillus* species. The clinical relevance of the rDNA findings in the etiology of these two cases remains uncertain.

Discussion

In this study, we applied a molecular diagnostic method, broad-range bacterial rDNA PCR, to 59 specimens from 46 UNEX cases. Positive findings were obtained for eight of the cases. In six of these cases, the organism detected in the specimens is a known cause of the type of syndrome the patient had.

The diagnosis of infectious diseases with molecular data, rather than from cultivation or serology, requires a careful examination of criteria for causation (18) and may require new molecular surveys of host microbial ecology. *Streptococcus pneumoniae* and *N. meningitidis* are common causes of bacterial meningitis in the United States (19), and *S. pneumoniae* is a well-known cause of pneumonia and empyema. Despite some unconfirmed reports of *S. pneumoniae* DNA in the serum of healthy persons (20-22), our failure to find other bacterial sequences in the two pleural fluid specimens with broad-range primers leads us to conclude that *S. pneumoniae* was the cause of these two cases of respiratory tract disease. In CSF, DNA from these organisms has been detected only in meningitis cases. The response of project patients to appropriate antibiotics further supports these conclusions. Similarly, *S. maltophilia* was the only bacterial species detected in a privileged anatomic site (bone marrow) in case XOR63. The known association of this organism with lower respiratory tract disease and septicemia strongly suggests that it caused at least some of this patient's respiratory disease. However, additional specimens were not available for confirmation; in addition, *S. maltophilia* is ubiquitous in nature and, despite our negative controls, may in theory contaminate laboratory reagents. We speculate that it may have been acquired after the patients admission to the hospital and contributed to the late stages of the patient's illness.

In the cases of multisystem failure (XOR56) and cardiac disease (myocarditis, XCT29), the role played by the organisms detected in the blood culture specimens in causing disease is much less clear. Although *S. epidermidis* was also cultivated from the blood of case XOR56 by local physicians, it is a well-recognized blood culture contaminant. Determining its clinical relevance requires additional data. *Bacillus* sp. are also occasional blood culture contaminants, and their DNA may contaminate blood culture media (23). An *Enterococcus* sp. is the most likely disease-causing agent among those detected in case XCT29. In general, with reliance on molecular methods, conclusions about causation are most reliably drawn after 1) repeated detection of the same organism in separate specimens from the same patient collected only at clinically relevant time points; 2) direct detection of the organism in situ in areas of pathology (24); 3) documentation of response to appropriate therapy; and 4) assessment of specific immune responses. Many of the same concerns and goals are applicable to cultivation-based diagnosis. In practice, especially when case investigation occurs after resolution of illness or death, as in this project, the preferred type and amount of specimens are not always available.

Our findings from these "unexplained" cases raise several important questions. Why weren't these well-known bacterial pathogens detected during the routine care of these patients? Specimens are not always collected from the appropriate anatomic site, at the appropriate time, in a sufficient amount, nor processed in an optimal fashion. In addition, the sensitivity of cultivation and serology is imperfect, even for organisms amenable to these approaches. Most of the project patients had received broad-range antibiotics before specimens were collected for this study.

In applying broad-range rDNA PCR to clinical specimens, one confronts the problem of 16S rDNA sequence microheterogeneity. Small discrepancies between directly amplified sequences and reference sequences in public databases pose difficulties for the definition of taxon boundaries and complicate clinical interpretation of laboratory data (25,26). In our investigation, the finding of *Streptococcus pneumoniae* 16S rDNA sequence microheterogeneity in the pleural fluid of a patient with culture-negative empyema suggests that some cases of pneumococcal disease, and perhaps other bacterial disease, may be caused by multiple concurrent strains of the same species.

Why have we failed to explain most of these project cases? Are some unexplained cases caused by novel or previously unrecognized pathogens? Our experiments addressed only the possibility of a bacterial cause with bacterial domain-specific broad-range PCR primers; current efforts include broad-range primers for various families of viruses and for fungi and the *Archaea*. Multiple broad-range primer pairs may be necessary for each group (as we describe for the bacteria) to detect unrecognized organisms with polymorphisms in conserved primer recognition sites.

In addition to the specimen problems listed above, PCR inhibitors may cause some false-negative results. The timing of specimen acquisition may also be relevant, since the average delay between onset of illness and specimen collection was 9 days for our PCR-positive cases versus 16 days for all cases studied with broad-range PCR. Although bacterial DNA persists longer than cultivatable organisms after therapy is initiated (27), the former still has a limited half-life. Finally, it must be kept in mind that some of these cases may not have a microbial cause.

Applying broad-range bacterial PCR to this set of difficult clinical cases raised several important points about the future use of this diagnostic approach. First, certain specimen types, such as CSF (3/16 specimens positive), pleural fluid (2/3 positive), and bone marrow (1/2 positive), may provide higher diagnostic yield in bacterial disease than other types, such as serum and blood. Second, the microbial sequence "background" in clinical specimens from healthy persons (e.g., blood) must be better characterized before findings from ill persons can be reliably interpreted. Third, sequence microheterogeneity in anatomically isolated sites of microbial disease may be more common than previously assumed. Our findings from the XEB44 pleural fluid illustrate this point. There are

several possible explanations for the *S. pneumoniae* 16S rDNA heterogeneity in this specimen (see above); however, we believe that at least some of the heterogeneity is best explained by the presence of two (or more) *S. pneumoniae* strains. To our knowledge this is the first case of invasive disease associated with multiple *S. pneumoniae* strains, although dual infections with other pathogens have been described (28,29). In fact, carriage of multiple strains of *Haemophilus influenzae* was recently shown to correlate with an increased risk for otitis media in children (30), and mixed-strain infections with *Mycobacterium tuberculosis* have also been reported (31). Multiple-strain infections with *S. pneumoniae* may have been previously missed with culture-based methods because of the common laboratory practice of subculturing a single representative when there is only one apparent colony morphotype.

The search for microbial causes of unexplained illnesses and deaths must continue to integrate traditional and molecular methods and will inevitably challenge assumptions about the mechanisms of microbial disease causation. Refined techniques, novel approaches, and study of other populations such as immunocompromised or impaired hosts are likely to provide new insights into the spectrum of infectious diseases.

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Dr. Simo Nikkari is a visiting assistant professor at Stanford University School of Medicine. His work focuses on the development of molecular methodologies to study infectious and chronic idiopathic diseases.

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Antibiotic Resistance Patterns of Bacterial Isolates from Blood in San Francisco County, California, 1996–1999

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Countywide antibiotic resistance patterns may provide additional information from that obtained from national sampling or individual hospitals. We reviewed susceptibility patterns of selected bacterial strains isolated from blood in San Francisco County from January 1996 to March 1999. We found substantial hospital-to-hospital variability in proportional resistance to antibiotics in multiple organisms. This variability was not correlated with hospital indices such as number of intensive care unit or total beds, annual admissions, or average length of stay. We also found a significant increase in methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and proportional resistance to multiple antipseudomonal antibiotics. We describe the utility, difficulties, and limitations of countywide surveillance.

Many national sampling and hospital surveillance systems exist to monitor antimicrobial resistance patterns in bacteria (1-4). Previously, organisms resistant to multiple antibiotics were largely confined to hospital settings and were typically described through studies involving single hospitals or intensive care units (ICUs). These single-hospital studies often reported substantially different resistance patterns from one another (5-7). National surveillance systems provided key data on large-scale resistance trends, but, similarly, continued to show marked variability in proportional resistance among participating hospitals (1-3,8,9).

We hypothesized that countywide surveillance data would not only provide information on changes in bacterial antimicrobial resistance but also potentially identify hospital demographic data to account for interhospital variability in resistance patterns. Additionally, countywide surveillance should provide greater insight into the relationship between single hospitals and their neighboring communities as data accumulate to support the spread of resistant organisms from the hospital into the community and vice versa. Morgan et al. (8) reported data from Wales that 66% of patients colonized or infected with methicillin-resistant *Staphylococcus aureus* (MRSA) are being discharged to their homes, leading to risks of intrafamilial transmission (10-12). Goetz et al. (6) showed that health-care workers similarly bring resistant organisms home. Additionally, outpatient dialysis units, rehabilitation centers, and outpatient intravascular devices have been shown to be reservoirs of colonization with MRSA and vancomycin-resistant *Enterococcus* (VRE) in many patients in the community (13-16).

MRSA and VRE colonization and infection in the absence of hospital risk factors are also being increasingly recognized in the community (17-22). Day-care centers and isolated communities may play a notable role (20,23). Patients colonized from these community reservoirs can subsequently cause hospital outbreaks after admission (24).

As hospital and community colonization and infection begin to exert pressure on one another, single-hospital surveillance data may become less useful in isolation. Countywide surveillance may provide more insight into the sources and extent of outbreaks and prompt focused investigations into the spread and containment of resistant organisms.

We conducted an observational study to evaluate the changes in antibiotic resistance in selected bacteria isolated from blood in San Francisco County, California, to determine if these changes were associated with specific hospital demographics and to define the utility, limitations, and potential areas of improvement in a county-based surveillance system.

Methods

All bacterial strains recovered from blood were identified from available microbiology department records of all 13 hospitals in San Francisco County from January 1, 1996, to March 31, 1999. For three hospitals (4,5,9), data from 1996 had been purged and were no longer available. For each isolate, data were collected on organism type and susceptibility pattern. Information was also obtained on the ward, age, and gender of the patient. Only the first positive blood culture of a given species was included for a single patient throughout the study period, regardless of susceptibility pattern. Cultures positive for *S. epidermidis* were considered representative of clinical bacteremia if at least two isolates with identical susceptibility patterns were obtained from a minimum of two separate sets of blood cultures. Each such set was considered a single bacteremic event. All other cultures positive for *S. epidermidis* were

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excluded, as were other common skin contaminants (e.g., *Propionibacterium acnes, Peptostreptococcus*, *Corynebacterium*).

All but one hospital used automated systems (VITEK [bioMerieux Vitek, Hazelwood, MO] or Microscan [Baxter Laboratories, West Sacramento, CA]) for the susceptibility testing of gram-negative bacteria. All hospitals used Kirby-Bauer disk-diffusion techniques for the evaluation of susceptibility profiles for *Streptococcus pneumoniae* and other streptococcal species according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines. Kirby-Bauer results were supplemented by E-testing. One hospital (12) performed all susceptibility testing using Kirby-Bauer disk-diffusion techniques. All microbiology laboratories used MIC breakpoints established by the NCCLS. The annual number of blood culture sets processed by each microbiology laboratory was also obtained.

Data from five hospitals were obtained as text files or Microsoft Excel (Redmond, WA) files and subsequently imported into a Microsoft Access (Redmond, WA) database. Data from four hospitals were obtained in printed form, scanned as image files, converted into text files using Text-Bridge Pro 98 (ScanSoft Inc., Peabody, MA), and imported into the database. All scanned entries were verified for accuracy. Data from the remaining four hospitals were obtained from stored index cards, entered manually into the database, and verified for accurate entry.

We also obtained bed size and census data for all 13 hospitals in San Francisco County. Total hospital admissions were tabulated from quarterly administrative records from 1996 through 1998. For each hospital, average length of hospital stay was obtained from publicly available resources (25).

Data Analyses

The number of isolates for each species was tabulated for each year and for the entire study period. Only species for which the total number of countywide isolates exceeded 100 during the study period were further evaluated. For each organism, proportional annual resistance to an antibiotic was calculated as the yearly number of organisms with intermediate or full resistance divided by the total number of organisms isolated in San Francisco County that year. Because of laboratory variability in susceptibility testing, not all isolates are included in descriptions of proportional resistance.

Means were calculated from the annual countywide percentages in each of the 4 years studied. Percent annual resistance was determined for any antibiotic tested in $\geq$50 isolates and analyzed for increasing or decreasing annual trend from 1996 through 1999. Data for 1999 were based on the first quarter culture results. Strains with full or intermediate resistance to an antibiotic were counted as resistant in all statistical analyses.

Organisms demonstrating increasing or decreasing annual resistance to a given antibiotic (p<0.05) were further described by calculating mean proportional resistance over the study period according to categories of hospital, ward, patient age

(in 10-year intervals), and patient gender. Spearman rank tests were used to determine any correlation between hospital indices (beds, annual admissions, average length of stay) and proportional antibiotic resistance. P values were not adjusted for the effect of multiple comparisons. P values remained unchanged with respect to alpha level (0.05) after removal of the three hospitals missing data from 1996.

Results

A total of 11,573 bacterial strains were recovered from blood cultures by the 13 hospitals. After excluding duplicate cultures, we had 8,072 remaining clinical isolates.

Information on hospital size, census, and blood culture volume is provided in Table 1. Despite being distinct and nonadjoining, hospitals 4 and 5 are reported together because of unified microbiology and administrative centers. Hospital 13 is a skilled nursing facility. On average, 74,600 sets of blood cultures were processed each year in San Francisco County; 9.9% of these were positive for bacterial species.

Of the 8,072 isolates, *Staphylococcus aureus* (1,858), *Escherichia coli* (1,634), and *S. pneumoniae* (725) were the most common organisms. The numbers of *S. aureus, Enterococcus faecalis, Bacteroides fragilis, E. coli,* and *Serratia marcescens* increased annually during the 4-year period. Fourteen species had >100 isolates and were considered for further analysis (Figure 1). These 14 organisms accounted for 85% to 86% of all yearly totals.

Gram-Positive Organisms

The proportion of MRSA and *E. faecium* resistant to vancomycin (VRE *faecium*) increased annually (Figure 2). Coun-

Table 1. Hospital size and census, San Francisco County, 1996 to 1999

Hospital	No. of beds	No. of ICU[a] beds	Yearly admissions	Average length of stay (days)	Blood culture sets/year	% + blood cultures
1	482	47	20,340	6.6	16,325	11
2	371	32	14,305	3.8	9,108	9
3	304	30	18,843	7.5	12,929	13
4,5[b]	302	31	10,893	5.8	10,800	6
6	284	12	5,939	8.3	3,580	12
7	253	15	7,982	7.3	4,865	9
8	240	15	9,887	7.6	5,475	6
9	221	18	6,139	6.6	2,582	11
10	209	19	6,655	6.7	4,974	14
11	209	8	3,293	9.3	2,515	10
12	59	7	2,260	5.4	1,464	13
13[c]	1,280	0	1,211	351.2	N/A	—

[a]ICU beds includes medical, surgical, cardiac, and neurologic adult critical care.
[b]Administration, microbiology laboratories merged. Average number per hospital given.
[c]Skilled nursing facility.

Figure 1. All bacterial species that were isolated from blood in >100 persons, January 1996 through March 1999, all hospitals in San Francisco County, California. Each bar is divided by yearly totals. Total number of isolates obtained during the study period is given above each bar. MRSA = methicillin-resistant *Staphylococcus aureus*; VRE = vancomycin-resistant *Enterococcus*.


Figure 2. Increase in percentage of *Staphylococcus aureus* isolates resistant to methicillin and increase in percent of *Enterococcus faecium* resistant to vancomycin on a yearly basis from 1996 through the first quarter of 1999. Number of isolates per year, regardless of susceptibility, appears above each bar. Tests of trend showed significant increases in percent resistance for both organisms.

tywide, the proportion of MRSA isolates rose from 18.1% (1996) to 26.1% (1999) (p<0.001). In total, MRSA constituted 22.4% of all *S. aureus* isolates, including 19.6% of emergency department isolates and 15.9% of isolates in the outpatient setting (Table 2). Methicillin resistance in *S. aureus* isolates was <15% in patients <20 years of age and >20% in all other age groups, with the exception of 30- to 39-year-olds (17.6%) (Table 3). Proportional resistance did not vary by patient gender.

During the study period, 124 VRE isolates and 157 vancomycin-sensitive *Enterococcus* isolates were unspeciated. Among the speciated *E. faecium* isolates, the percentage resis-

tant to vancomycin rose from 0% to 66.7% in the 4-year period (p<0.001). VRE *faecium* was most frequently isolated from inpatient adult wards, but six isolates were cultured from emergency department and outpatient settings. Over 66% of *E. faecium* isolates from skilled nursing facilities were resistant to vancomycin (Table 2). VRE isolates exceeded 40% in all age groups with the exception of ≤10-year-olds (22.2%) (Table 3). Of note, VRE *faecium* isolates showed increasing annual resistance to doxycycline (from 30% to 68%; p = 0.02).

Even after the skilled nursing facility (hospital 13) was excluded, the percentage of MRSA and VRE *faecium* isolates varied substantially among individual hospitals (MRSA 12.5% to 37.5%, VRE *faecium* 12.5% to 80.0%). There was no correlation between proportional resistance and number of ICU or total hospital beds, number of annual admissions, or average length of ICU or total hospital stay. However, for both organisms, there was increasing proportional resistance among adult wards in the following order: outpatient wards, emergency department, medical and surgical floors, medical and surgical ICUs, and skilled nursing facility wards. Notably, a substantial number of VRE and MRSA isolates were cultured within the first 24 to 48 hours of hospital admission (Figure 3).

Of the 678 isolates of *S. pneumoniae*, 13.6% were resistant to penicillin. Proportional resistance increased from 10.8% (21 isolates) in 1996 to 14.6% (27 isolates) in 1998, but this increase was not statistically significant. Proportional resistance was highest at the extremes of age (16.4% in patients >70 years of age and 19.3% in patients <10 years of age (Table 3).

Gram-Negative Organisms

Countywide, *E. coli* (1,634 isolates), *Klebsiella pneumoniae* (428 isolates), and *Pseudomonas aeruginosa* (260 isolates) were the most frequently isolated gram-negative bacilli. This was true in all inpatient and outpatient wards with the

Table 2. Proportional[a] resistance of highly resistant organisms by ward,[b] San Francisco County, 1996 to 1999

Ward	Methicillin-resistant *Staphylococcus aureus*	Vancomycin-resistant *Enterococcus faecium*	Penicillin-resistant *Streptococcus pneumoniae*
Skilled nursing facility	38.0% (100)	66.7% (6)	8.3% (12)
Med/surg ICU	27.2% (298)	50.0% (58)	13.3% (75)
Pediatric ICU	30.3% (33)	25.0% (4)	0% (2)
Med/surg floors	21.8% (709)	47.8% (92)	12.0% (166)
Pediatric floors	9.1% (33)	60.0% (5)	40.0% (10)
Emergency department	19.6% (424)	22.7% (22)	9.5% (305)
Outpatient	15.9% (171)	12.5% (8)	23.6% (72)
Other/unknown	28.6% (7)	100% (1)	32.4% (34)
Total	22.4% (1,782)	44.9% (196)	13.6 (678)

[a]Proportional resistance refers to the proportion of isolates of that species that is resistant to the indicated antibiotic.
[b]Total number of species isolates given in parentheses. Percentages for small numbers of total isolates should be cautiously interpreted.
ICU = intensive care unit.

Table 3. Proportional[a] resistance of highly resistant organisms by age,[b] San Francisco County, 1996 to 1999

Age group (years)	Methicillin-resistant (%) *Staphylococcus aureus*	Vancomycin-resistant (%) *Enterococcus faecium*	Penicillin-resistant (%) *Streptococcus pneumoniae*
≤10	14.3 (98)	22.2 (9)	19.3 (83)
10-19	8.1 (37)	50.0 (4)	20.0 (5)
20-29	21.7 (83)	50.0 (8)	0 (26)
30-39	17.6 (250)	42.1 (19)	13.4 (142)
40-49	21.8 (353)	41.4 (29)	8.7 (138)
50-59	24.8 (234)	43.8 (32)	11.5 (78)
60-69	29.1 (227)	45.5 (44)	10.2 (49)
70-79	24.8 (258)	55.9 (34)	15.6 (64)
≥80	22.8 (237)	41.2 (17)	17.2 (64)

[a]Proportional resistance refers to the proportion of isolates of that species that is resistant to the indicated antibiotic.
[b]Total number of species isolates given in parentheses. Percentages for small numbers of total isolates should be cautiously interpreted.

exception of skilled nursing facilities, where *Proteus mirabilis* was the most common gram-negative isolate after *E. coli*. Proportional resistance by ward for selective gram-negative organisms is shown in Table 4.

Among *E. coli* isolates, resistance to trimethoprim-sulfamethoxazole averaged 28% and resistance to ciprofloxacin averaged 3%. There was no resistance to fluoroquinolones among *E. coli* isolates from pediatric wards. Increasing annual resistance to ticarcillin-clavulanate was seen in both *E. coli* (6% to 16%, p=0.03) and *K. pneumoniae* (0% to 18%, p= 0.007) isolates.

P. aeruginosa isolates showed increasing annual countywide resistance to ciprofloxacin (7% to 21%, p=0.005), ceftazidime (6% to 16%, p=0.02), and imipenem (2% to 18%, p=0.004) (Figure 4). Resistance to each of these three antibiotics exceeded 10% in adult ICU and adult medical and surgical wards. In fact, in these settings, ciprofloxacin resistance approached 20% countywide. No isolates resistant to ciprofloxacin were cultured from pediatric wards (Table 4). Resistance to gentamicin (15%) and piperacillin-tazobactam (12%) also increased but was not statistically significant.

There were 182 *E. cloacae* and 116 *S. marcescens* isolates from January 1996 through March 1999. Ciprofloxacin resistance averaged 4% among *E. cloacae* isolates and 6% among *S. marcescens* isolates. *S. marcescens* isolates also showed increasing annual proportional resistance to gentamicin (0% to 14%, p=0.02) and piperacillin (4% to 29%, p=0.01).

Resistance to ceftazidime, which can be predictive of inducible and extended-spectrum-beta-lactamases, was found in the following overall mean proportions in the study period: *E. coli* (1%), *P. mirabilis* (1%), *K. pneumoniae* (1%), *S. marcescens* (8%), *P. aeruginosa* (13%), and *E. cloacae* (39%).

Only *P. aeruginosa* isolates demonstrated an increasing linear annual trend (p = 0.02).

Discussion

San Francisco County has a population of approximately 735,000 and covers 46.7 square miles (26). It comprises multiple racial and ethnic groups (black 10.9%, Hispanic 13.9%, Asian 29.1%, and Native American 0.5%) and is served by 13 hospitals. We have shown that county surveillance of bacterial resistance is a useful addition to local hospital surveillance, particularly as antibiotic-resistant bacteria increasingly spread from hospital to hospital and into the community at large.

Across the county, annual proportions of MRSA and VRE isolates significantly increased over the 4-year period. Pseudomonal strains resistant to fluoroquinolones, ceftazidime, or imipenem also increased annually. These data allow us to distinguish countywide outbreaks and trends from single-hospital changes in resistance patterns, and enable infection control efforts to expand or narrow to the appropriate scale. With awareness programs, county surveillance can broaden physicians' knowledge of their hospital's effects on the community, as well as the effects of neighboring hospitals on resistance patterns in their particular hospital.

Additionally, county surveillance that includes subcategorization of isolates by ward is invaluable in identifying patients at high risk and locations for transmission of resistant bacteria. Not surprisingly, we report our highest proportion of MRSA and VRE isolates from ICU and nursing home units. Nevertheless, ward variability across hospitals was substantial. Large interhospital differences can lead to further study of ward practices that foster or abate transmission. Awareness can prompt hospital infection control personnel to ensure well-described preventive measures such as swabbing and isolation precautions for VRE and MRSA in ICU settings (27-30) and nasopharyngeal swabbing and eradication of MRSA in hemodialysis wards (1,31). We also identify several MRSA and VRE isolates from outpatient and emergency department
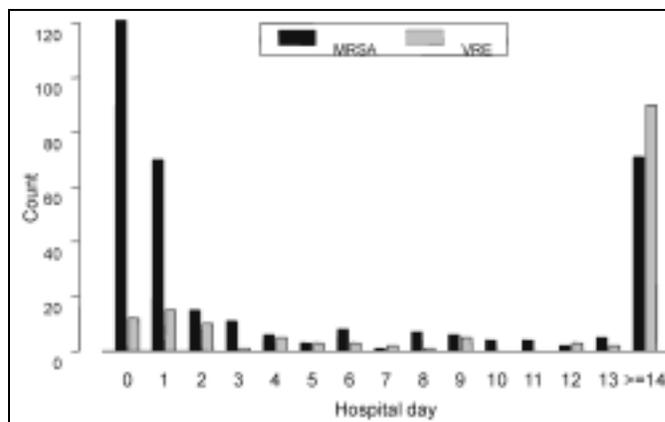

Figure 3. Plot of the number of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) isolates by hospital day of admission. An early peak is noted, corresponding to patients entering the hospital with MRSA or VRE bacteremia. Subsequent cases likely represent nosocomial acquisition.

Table 4. Proportional resistance of selected gram-negative organisms by ward[,a] San Francisco County, 1996 to 1999

	Escherichia coli-cefazolin	*E. coli*-ciprofloxacin	*Enterococcus cloacae*-ceftazidime	*Pseudomonas aeruginosa*-ciprofloxacin	*P. aeruginosa*-ceftazidime	*P. aeruginosa*-imipenem	*Serratia marcescens*-ceftazidime
Skilled nursing facility	9.1% (88)	2.2% (92)	50% (6)	8.3% (12)	25% (12)	20.0% (10)	40.0% (5)
Med/surg ICU	8.4% (166)	1.9% (160)	53.4% (43)	21.5% (65)	15.9% (63)	11.9% (59)	12.1 (33)
Pediatric ICU	18.5% (27)	0% (28)	18.2% (11)	0% (13)	7.7% (13)	9.1% (11)	0% (11)
Med/surg floors	8.4% (586)	4.3% (564)	47.2% (53)	19.1% (89)	12.5% (88)	12.2% (74)	9.5% (21)
Pediatric floors	25.0% (16)	0% (16)	27.3% (11)	0% (10)	0% (10)	0% (8)	0% (6)
Emergency department	8.4% (536)	2.6% (549)	32.3% (31)	23.5% (34)	5.9% (34)	6.7% (30)	0% (17)
Outpatient	8.6% (117)	1.6% (123)	4.8% (21)	4% (25)	4.0% (25)	0% (22)	0% (15)
Other/unknown	11.1% (18)	0% (18)	0% (0)	0% (1)	0% (1)	0% (1)	0% (0)
Total	8.8% (1,554)	2.9% (1,550)	38.1% (176)	16.5% (249)	11.4% (246)	9.8% (215)	7.3% (109)

[a]Total number of species isolates given in parentheses. Percentages for small numbers of total isolates should be cautiously.
ICU = intensive care unit.

settings. Whether or not these represent true community-acquired strains or strains from patients recently released from hospital settings, they suggest that highly resistant bacterial outpatient infections and infectivity are increasing, a result consistent with recent studies (19,21,22).

We also evaluated whether the wide variability in the proportions of resistant bacteria among San Francisco hospitals was linked to hospital indices. In contrast to previous nationwide sampling studies, none of this variability was correlated with the number of hospital ICU beds (3), total beds (1,2,31), annual admissions (32), or annual mean length of stay. This may be due to our small number of hospitals, leading to limited power to detect such correlations. Alternatively, local community and hospital factors (e.g., increasing care of moderately ill patients at home [8], increasing home intravenous antibiotics [15], active transfer of patients between hospitals [33,34], and community-acquired resistant organisms) may now be diminishing the effect of hospital size, census and length of stay on proportional resistance.
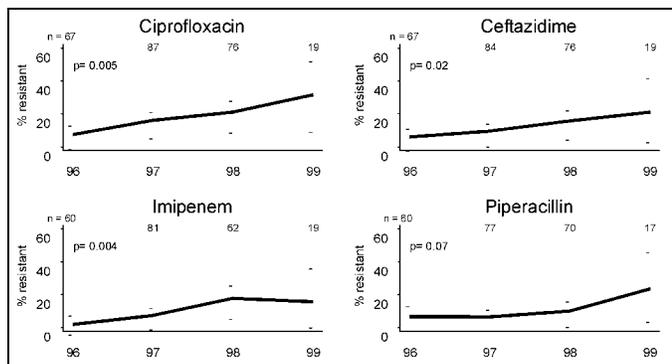

Figure 4. Yearly percent resistance to ciprofloxacin, ceftazidime, imipenem, and piperacillin in *Pseudomonas aeruginosa* isolates from blood. Increasing proportional resistance occurred in three of the four antibiotics commonly used to treat this organism. Annual number of isolates tested to each antibiotic is given at the top of each graph.

There are many additional advantages to a countywide surveillance system for antimicrobial resistance. First, it can help reassess therapy. The 28% trimethoprim-sulfamethoxazole resistance in *E. coli* raises questions about the optimal empiric treatment of urinary tract infections in patients at high risk for bacteremia or urosepsis. Likewise, distinguishing resistance in the outpatient versus inpatient setting can guide empiric therapy in the appropriate setting.

Second, in studying a larger populace, we can obtain sufficient numbers to study uncommon organisms. Similarly, countywide surveillance provides a means to identify and confirm novel resistant pathogens. In our study, one MRSA isolate with intermediate resistance to vancomycin and three vancomycin-resistant *S. epidermidis* isolates were noted in microbiology laboratory reports. As with the organisms recently reported in the United States (35-37), these were reported from nontertiary hospitals. Nevertheless, our reports are unconfirmed and likely represent laboratory error. However, if surveillance could be expedited to real-time use, such reports could be investigated and confirmed rather than dismissed. At its worst, this suggests that highly resistant organisms may be escaping deserved attention and reaction.

Third, countywide surveillance engenders further hypotheses and research regarding interhospital and community transmission of resistant organisms. For example, our finding that 20% of emergency department *S. aureus* isolates are methicillin-resistant provides a flag to further study which county areas have the highest percentages of resistant *S. aureus* and which risk factors are involved (e.g., recent hospital admission [21,22], associated hemodialysis centers [8,38] or nursing homes [21,22], or intravenous drug use [39]). The finding that *P. mirabilis* bacteremia is more common in nursing home wards raises questions about preventing urinary tract infections in that setting. The relative lack of fluoroquinolone resistance in pediatric gram-negative isolates is likely due to the avoidance of fluoroquinolones in children because of potential

detrimental cartilage effects. The study of fluoroquinolone-resistant organisms during the transition years from pediatric to adult medicine may provide insight into the quantity and duration of antibiotic needed to produce selection pressure, as well as the speed and durability of emerging resistance.

Our surveillance method had several limitations. Chart review would have been an invaluable addition in distinguishing between community-acquired and recent hospital or nursing home acquisition of resistant organisms. Second, we did not collect or confirm isolates; thus, although our results reflect microbiologic data actually presented to ordering physicians, they are subject to laboratory differences in speciation and susceptibility determination. Notably, not all organisms were fully speciated or tested against the antibiotics of interest. Fortunately, this occurred in a small proportion of bacteria. Third, we do not provide information on antibiotic use, which is known to be a major determinant of bacterial antibiotic resistance. Fourth, countywide trends can be driven by trends seen in the largest hospitals, particularly since smaller hospitals often lack sufficient numbers of isolates to make statistical analyses meaningful. This was notable for our data on proportional increases in VRE, which were largely driven by three hospitals. On the contrary, MRSA trends were not limited to a few hospitals, nor were they limited to the largest hospitals in the county. No hospital showed a significant decreasing trend in the proportion of MRSA isolates.

Areas for improvement include methods to computerize microbiologic data storage in a universal format. This would expedite surveillance and allow real-time collection and identification of unusually resistant organisms, as well as provide sentinel data regarding countywide outbreaks. In addition, linking of patient information to microbiologic data would have expedited acquisition of sex, gender, and ward information. Furthermore, despite NCCLS guidelines, a fair amount of variability exists in laboratory practices and susceptibility panels. Further standardization of these practices would help ensure the reliability of merging data among hospitals.

Without a doubt, the greatest utility of countywide surveillance lies in its ability to ask screening questions that prompt a more thorough investigation of specific hospitals, wards, or age groups at particular risk for acquiring or transmitting highly resistant organisms.

We have shown how several such questions were raised by our surveillance of bacteremias in San Francisco County and described its many advantages. We have further defined our limitations and difficulties in performing such surveillance, in the hope that this will be helpful to further similar surveillance efforts.

At the time of this study, Dr. Huang was a categorical resident in Internal Medicine at the University of California San Francisco Medical Center. She has since completed a Master of Public Health at the Harvard School of Public Health and is pursuing a fellowship in infectious diseases at Brigham & Women's Hospital and Massachusetts General Hospital, Boston, Massachusetts. Her current research interests are in the field of bacterial antibiotic resistance.

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Prosthetic Valve Endocarditis Caused by *Bartonella quintana*

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We describe the first case of *Bartonella quintana* endocarditis affecting a prosthetic valve in a person with no known risk factors for this infection. *Bartonella* should be considered as a cause of endocarditis in any clinical setting.

Four species of *Bartonella* have been described as a cause of endocarditis in humans: *Bartonella quintana, B. henselae, B. elizabethae*, and *B. vinsonii* subsp. *berkhoffii*. Although infection with the latter two species has been reported only as single cases, endocarditis caused by *B. quintana* and *B. henselae* has been increasingly recognized in recent years. Most cases in which *B. quintana* has been implicated as the infecting species (usually through culture or molecular techniques) have had known risk factors such as homelessness, alcoholism, or human HIV infection (1-3). We report a case of *B. quintana* endocarditis affecting a prosthetic valve in a person with no known risk factors.

Case Report

A 46-year-old Indian woman, who had lived in the United Kingdom for 10 years, was admitted to our hospital in June 2000 with a 3-month history of fever, sweats, and rigors associated with anorexia and 5-kg weight loss. Medical history included prosthetic aortic valve insertion in 1992 for aortic stenosis. In 1998, the patient had a hemorrhagic cerebrovascular event, a presumed consequence of anticoagulation therapy with warfarin. In October 1999, she was admitted to another hospital with fever, anemia, renal impairment, hypergammaglobulinemia, and microscopic hematuria. Several days later, she had sudden loss of vision due to a large right-sided occipital hemorrhage that required surgical evacuation. A transesophageal echocardiogram at that stage revealed no evidence of endocarditis, and three blood cultures were sterile. The patient was unemployed and lived with her father. She did not smoke or drink alcohol and actively disliked and had no contact with animals.

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The patient was clinically anemic, had no fever, and had several subconjunctival hemorrhages. There was no evidence of ectoparasite infestation. Cardiovascular examination showed a water-hammer pulse (Corrigan's sign), prosthetic heart sounds, an ejection systolic murmur, and an early diastolic murmur consistent with aortic regurgitation. Respiratory examination was unremarkable, and splenomegaly (1-cm enlargement) was detected in the abdomen. Residual left hemiparesis and hemianopia, resulting from her previous cerebrovascular accidents, were present.

Urinalysis showed proteinuria and hematuria; urinary protein excretion was measured at 2.54 g/L. The patient was anemic, with a hemoglobin of 7.2 g/dL with normal leukocyte and platelet counts. The serum creatinine was elevated at 168 µmol/L, and serum globulins were increased with low serum albumin (27 g/dL). The C-reactive protein was elevated at 66 g/dL. Six blood cultures were sterile, and an HIV antibody test result was negative. A transthoracic echocardiogram was unremarkable, but a transesophageal study showed two 1.5-cm vegetations attached to the prosthetic aortic valve, with moderate paravalvular regurgitation. A diagnosis of culture-negative endocarditis was made, antibiotic treatment with vancomycin and gentamicin was commenced, and the patient was referred for surgical assessment.

Despite antibiotic therapy, fever, progressive renal impairment (serum creatinine 300 µmol/L), and leukopenia developed. In view of the valvular pathology, the aortic valve prosthesis was replaced with a homograft root into which the coronary arteries were reimplanted. Microbiologic examination of the excised valve showed no organisms on Gram stain and no bacteriologic growth. There was insufficient material for histologic examination.

During screening for rarer causes of endocarditis, *Chlamydia* serology was found to be positive, with *Chlamydia trachomatis* and *C. pneumoniae* immunoglobulin (Ig) G titers >512 by microimmunofluorescence (MRL Diagnostics, Binding Site Ltd, UK). *Bartonella* serology was positive by immunofluorescence, with IgG titers >8,192 for both *B. henselae* and *B. quintana* and a positive IgM for both species (titer =80). Genomic DNA was extracted from the vegetation removed at surgery by using the QIAamp Tissue Kit (QIAGEN Ltd, Crawley, UK). Two pairs of oligonucleotide primers were used to amplify overlapping fragments of the 16S ribosomal DNA (rDNA) gene. The first primer pair amplified a 296-bp segment of the *Bartonella* gene, as described (4). The second primer pair (5'-GAAGGGGGGCTAGCGTTGT-3' and 5'-AACTGAGATGGCTTTTGGAG-3') was designed to amplify a 768-bp fragment of the same gene in alpha-Proteobacteria (5). DNA sequencing of both amplicons allowed analysis of a 720-bp fragment of the 16S rDNA gene. This sequence was most closely related to the four *B. quintana* sequences deposited in GenBank (0 to 3 nucleotide differences, corresponding to 99.7% to 100% similarity). In contrast, the sequence had nine nucleotide differences (98.8% similarity) from that of *B.*

henselae, the next closest match, establishing *B. quintana* as the infecting species in this case.

A serum sample drawn in October 1999 was retrospectively tested and also found to be positive for *Bartonella* IgG and IgM antibodies. Initial postoperative therapy with teicoplanin and ceftriaxone (given for 1 week) was changed to ciprofloxacin for a total of 1 month. Oral clarithromycin was then given for another month. Six weeks after surgery, the patient was afebrile, the valve was functioning satisfactorily, and splenomegaly had resolved. Both the C-reactive protein and serum creatinine had returned to normal.

Conclusions

This case report documents the first description to our knowledge of *B. quintana* endocarditis affecting a prosthetic valve; after surgical and medical therapy the outcome was favorable. The first descriptions of human disease caused by *B. quintana* emerged during World War I (1914-1918), when approximately 1 million cases of trench fever occurred (6). Subsequently, the organism has been shown to be a cause of bacillary angiomatosis in HIV-infected persons. More recently, endocarditis and chronic bacteremic illness resembling trench fever have been described, with affected persons usually being homeless or alcoholic (6). The body louse was shown to be the vector of trench fever and has been postulated as a vector of contemporary *B. quintana* infection, although direct evidence for this is lacking. Valve replacement has been the rule in the few reported cases of *Bartonella* endocarditis. This surgical intervention may reflect either a poor clinical response to medical therapy or the fact that diagnostic delay, as in our case, may lead to valve destruction to a degree that necessitates valve replacement.

An interesting aspect of our case is that none of the previously known risk factors for infection with *B. quintana* were present. *B. quintana* native-valve endocarditis in persons without recognized risk factors appears rare (7). Since clinicians are only likely to investigate the possibility of this infection in patients with known risk factors, reported cases may not accurately reflect levels of *Bartonella* infection. Thus, as with any emerging disease, the clinical and epidemiologic features of contemporary *B. quintana* infection remain to be fully described. Using a large bank of control sera, Raoult et al. (1) estimated that a *Bartonella* IgG titer >1,600 has a positive predictive value for endocarditis of 0.884. Detection of high-titer *Bartonella* antibodies will therefore be a powerful diagnostic tool in cases of suspected endocarditis; such results could have established the diagnosis 8 months earlier for our patient.

In the recent study from Canada and France, *Bartonella* was estimated to cause 3% of all cases of endocarditis (1). The true incidence of *Bartonella* endocarditis in countries such as the United States or the United Kingdom is unknown. Of 66 sera taken from cases of culture-negative endocarditis and sent to the Public Health Laboratory Service in London, United Kingdom, 18% were positive for *Bartonella* antibodies (8). Although ascertainment bias may have increased this figure, this organism is clearly an important cause of culture-negative endocarditis in the United Kingdom (9). As in our case, cross-reacting anti-chlamydial antibodies are frequently detected in cases of confirmed *Bartonella* endocarditis (1), and identifying such antibodies in the context of endocarditis should prompt a search for *Bartonella* infection. We recommend that in the diagnostic work-up of patients with suspected endocarditis, *Bartonella* infection should be sought by serologic testing at an early stage, regardless of the presence or absence of recognized risk factors.

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Cryptosporidium muris Infection in an HIV-Infected Adult, Kenya

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We describe a case of *Cryptosporidium muris* infection in an HIV-infected adult with diarrhea in Kenya. Sequence analysis of an 840-bp region of the 18S rRNA gene locus demonstrated the isolate had 100% nucleotide identity with *C. muris* recovered from a rock hyrax, 98.8% with a *C. muris* "calf" isolate, 95.5% with *C. serpentis*, but only 87.8% with *C. parvum* "human" type.

T yzzer identified the first *Cryptosporidium* species, *C. muris*, in the gastric glands of mice (1). Thereafter, he identified *C. parvum*, which infects the small intestines of many mammals, and described the complete coccidian life cycle. Over the next 70 years, more than 23 different species of the genus were described on the basis of their morphology and natural hosts. However, when animals were experimentally infected, many of the described *Cryptosporidium* species were found to be identical. In the 1970s the classification was revised, and today only six to eight species are recognized as valid, with most human, zoonotic, and mammalian infections being attributed to the different *C. parvum* genotypes (2,3). *C. muris*, which is naturally a murine parasite, appears to have a more limited host range than *C. parvum*. Experimental transmission studies of *C. muris* have shown that the isolate from laboratory mice can infect other animals, including dogs, guinea pigs, rabbits, lambs, and gerbils, although it did not produce patent infections (4). The parasite has also been isolated from a rock hyrax (*Procavia* sp.) from a zoo and a Bactrian camel with chronic cryptosporidiosis (3,5). For many years, the parasite was thought to infect cattle; however, recent studies have shown that the *C. muris* that infects cattle is genetically distinct, and a new species name, *C. andersoni*, has been suggested (6).

Conventional diagnostic methods for *Cryptosporidium* do not differentiate the various species and genotypes, and most infections are diagnosed as *C. parvum*. *C. parvum* "human" and "bovine" genotypes remain the main causes of human cryptosporidiosis, but lately identification of infections with other genotypes and also *Cryptosporidium* species other than

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C. parvum has increased in both immunosuppressed and immunocompetent persons (7-10). Possible asymptomatic human infection with *C. muris* was reported in two healthy girls in Indonesia (11). Morphologic studies on the oocysts showed they were most likely to be *C. muris,* although there was no genotypic or experimental animal confirmation. Phylogenetic analysis has enabled more conclusive assignment to species and genotypes infecting humans and other animals (12,13). We report a case of *C. muris* infection, confirmed by morphology and genotyping, in an adult HIV-infected man from Kenya, hospitalized with diarrhea.

The Study

Fecal samples were collected from diarrheal patients from a hospital in Nairobi, Kenya, as part of a larger study. The patient described was an HIV-infected man who had clinical tuberculosis and diarrhea. *Isospora belli* was also detected in a fecal sample from the patient.

The fecal samples were preserved in both sodium acetate formalin and 2.5% potassium dichromate and kept at 4°C. They were stained with Kinyoun's carbol fuchsin modified acid-fast stain and examined by oil immersion microscopy. An aliquot of 400 µL of the sample suspension in 2.5% potassium dichromate was processed for genotypic analysis. Potassium dichromate was washed 5 times with cold, distilled water until the yellow color cleared. Oocysts were ruptured by freeze-thaw, and DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen, West Sussex, UK) for stool DNA purification as per protocol.

A section of the SSU (18S) rRNA gene was amplified by nested polymerase chain reaction (PCR) as described (14), using the forward primers 5'-TTCTAGAGCTAATACATGCG-3' and the reverse primer 5'-CCCTAATCCTTCGAAACAGGA-3' for primary PCR. Secondary PCR used primers 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and reverse primer 5'-AAGGAGTAAGGAACAACCTCCA-3', employing the Techne (FTGENE2D Techne, Cambridge Ltd., UK) thermal cycler. Restriction fragment analysis of the secondary PCR product was done by digesting 15 µL of product in a 40-mL total reaction volume consisting of 15 U of *Ssp*1 and 3 µL of restriction buffer (Boehringer Mannheim Biochemicals, Indianapolis, IN) for species identification and *Asn*1 (Boehringer Mannheim) for genotyping in the same concentration at 37°C for 1 hour. Digestion products were separated on 2% agarose gel and visualized by ethidium bromide staining. The internal (secondary) fragment was purified by using the Prep-A-Gene DNA purification kit and cloned into PGEM-T Easy plasmid vector (Promega Corporation, Madison, WI) as described by the manufacturer. The cloned product was sequenced and aligned with previously published sequences of the 18S rRNA gene of *Cryptosporidium* species by using the CLUSTALX (EMBL, Heidelberg, Germany) program and manual adjustments. Multiple alignment was done with the Phylogeny Inference Package (PHYLIP version 3.5c, J. Felsenstein and the University of Washington, Seattle, WA). Sequences were analyzed by using

DNADIST followed by neighbor joining (NEIGHBOR, PHYLIP package). One hundred replica samplings were analyzed for percentage bootstrap values. Accession numbers for *Cryptosporidium* 18S rRNA genes used were AF093498, AF093497, AF093496, AF108866 and AF093489, AF093499, AF112569 AF115377.

Results

Microscopic examination of the acid-fast stained fecal smear revealed ovoid oocysts that were an average size of 7.5-9.8 x 5.5-7.0 µm (Figure 1). Cysts of *I. belli* were also identified in the stained smear.

Restriction endonuclease digestion with *Ssp*1 of the secondary PCR 18S rRNA product yielded two fragments of 385 bp and 448 bp in size, while *Asn*1 digestion yielded two visible bands that were 102 bp and 731 bp. The results match restriction fragment patterns observed following similar digestions of *C. muris* amplicons from rock hyrax and Bactrian camel isolates (14).

The resulting sequence 18S r RNA gene fragment of the *C. muris* human isolate was deposited in the EMBL Nucleotide Sequence Database (Accession no. AJ307669). Sequence analysis with ClustalX showed this human *C. muris* isolate had a 100% nucleotide identity to that of a *C. muris* isolate from a rock hyrax and a Bactrian camel (EMBL Accession no. AF093498, AF093497), 98.8% identity to a *C. muris* "calf" isolate (AF093496), 96.5% with *C. serpentis* (AF108866), and only 87.8% identity to *C. parvum* human type (AF093489). *C. muris* calf isolate (AF093496) has since been shown to be a different species from *C. muris* ("mouse" type, Accession no. AF093498) and has been given a new name, *C. andersoni*. The phylogenetic tree showed topology similar to that already published for *Cryptosporidium,* with *C. parvum* clustering in one clade, and our patient's sample and published sequences of *C. muris* (rock hyrax isolate)*, C. andersoni* (calf isolate), and *C. serpentis* clustered in another group (Figure 2).
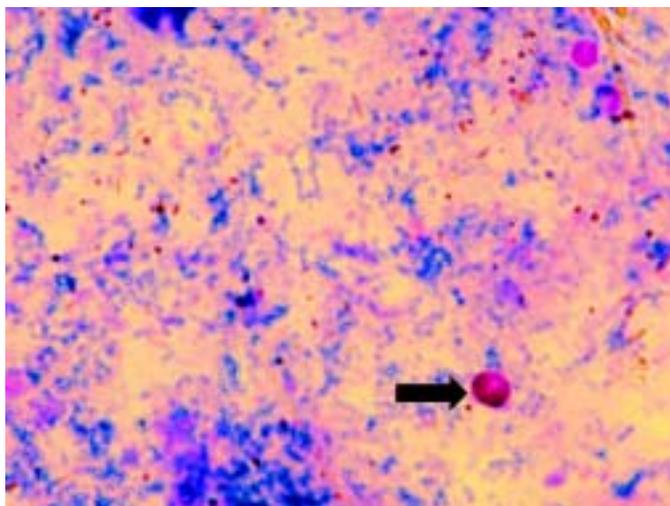

Figure 1. *Cryptosporidium muris* oocysts (under oil X 1,000), stained by Kinyoun's acid-fast staining.

Conclusions

Our study used genotypic analysis to confirm microscopic detection of *Cryptosporidium* oocysts in fecal samples and indicated that *C. muris* can indeed infect humans. Although immunosuppression has been observed to produce an increased susceptibility to cryptosporidiosis, the range of *Cryptosporidia* that can cause human cryptosporidiosis is still being elucidated (8,13,15). Lately, novel genotypes and *non-C. parvum* species such as *C. meleagridis*, *C. felis,* and *C. parvum* "dog" type have been identified not only in HIV-infected persons but also in HIV-uninfected patients (7,9,10). Genotypic analysis of *Cryptosporidium* organisms in fecal samples in the United Kingdom showed the occurrence of *C. meleagridis, C. felis,* and *C. parvum* "dog" type in immunocompetent and immunosuppressed persons (10,16). In another study in Peru, *C. felis, C. parvum* "dog" type, and *C. meleagridis* were identified in children not infected with HIV. In that study, *C. meleagridis* was as common as *C. parvum* "bovine" type; it appeared to be a stable part of the enteric pathogen mix causing cryptosporidiosis, perhaps only being identified with current definitive molecular methods (9).
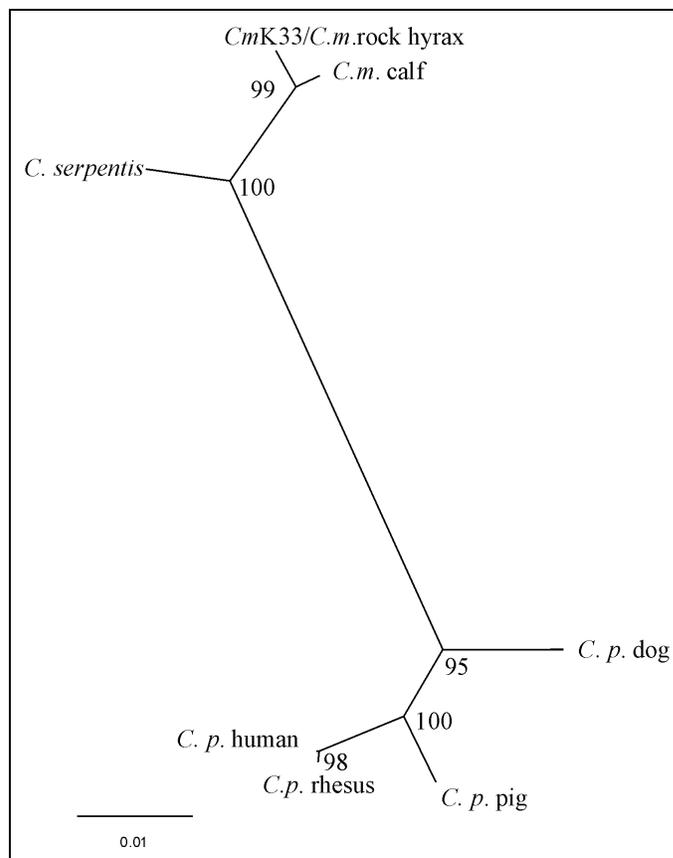
Figure 2. Comparison of 18S rRNA gene sequences of *Cryptosporidium* species. K33-*C.muris* from human patient (current paper Accession no. AJ307669); *C. m (muris)* "rock hyrax" (Accession no. AF093498); *C.m. (muris)* "calf" isolate (AF093496), now renamed *C. andersoni; C. serpentis* (AF093499); *C.p (parvum)* "dog" (AF112576); *C.p.* "rhesus monkey" (AF112569); *C.p.* "human" (AF093489); and *C.p.* "pig" (AF115377). Numbers refer to the percentage of repeated analyses that gave the same tree topology (bootstrap values).

C. muris infects the gastric glands of immunocompetent or immunocompromised (nude and SCID) mice (17); however, since our patient was co-infected with *I. belli*, the role of *C. muris* in our patient's gastroenteritis and its possible site of infection in this patient are unclear.

A report of possible asymptomatic *C. muris* infection in healthy persons (11) and our finding of it in an immunosuppressed patient suggest that this may be yet another *Cryptosporidium* species with a zoonotic potential. The range of animal reservoir hosts in which *C. muris* has been identified or experimentally transmitted adds to the importance of *Cryptosporidium* species as a public health concern (3,4,15). The current genotypic analyses are making it possible to make more conclusive diagnoses and to speculate on possible sources of infection (14-16). These techniques will need to be applied more widely to identify and characterize isolates of *Cryptosporidium* for more definitive epidemiologic mapping.

Acknowledgments

Wangeci Gatei is a Commonwealth Universities Scholar, working toward a doctorate in parasitology in the Tropical and Medical Schools, University of Liverpool. She qualified in veterinary medicine in 1987 and since then has worked at the Kenya Medical Research Institute in Nairobi as a veterinary parasitologist.

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Rickettsia felis Infection Acquired in Europe and Documented by Polymerase Chain Reaction

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and Didier Raoult†**

We report the first case of *Rickettsia felis* infection in Europe to be documented by polymerase chain reaction (PCR) and serologic testing.

Rickettsia felis (1) was first detected in 1990 as the ELB agent from the midgut epithelial cells of cat fleas (*Ctenocephalides felis*) (2). The pathogenic role of *R. felis* for humans has been demonstrated by its detection by PCR in five patients from Texas, Mexico, and Brazil (3-5). Following isolation of the bacterium and the first establishment of a strain in 2000, a new serologic test allowed the identification of three additional human cases (5).

Case Reports

In August 2000, a 42-year-old woman and her 42-year-old husband were hospitalized in Düsseldorf, Germany, with high fever and rash of 4 and 2 days' duration, respectively. The fever was associated with marked fatigue and headache. Four to 5 days before the onset of fever, both patients had noted a single black, crusted, cutaneous lesion surrounded by a livid halo (on the woman's right thigh and the man's abdomen). On admission, both patients had fever of 39°C and generalized maculopapular rash. The man had enlarged, painful lymph nodes in the inguinal region. Clinical examination was otherwise normal.

Laboratory investigation showed slightly elevated liver enzymes. The woman's values were aspartate amino transferase (ASAT) 48 IU/L (normal <26); alanine amino transferase (ALAT) 29 IU/L (normal <27); gamma glutamyl transferase (g-GT) 32 IU/L (normal <200); and lactate dehydrogenase (LDH) 517 IU/L (normal <250). The man's values were ASAT 38 IU/L, ALAT 32 IU/L, g-GT 79 IU/L, and LDH 498 IU/L. Other notable findings were elevated C reactive pro-tein (12.8 mg/L for the woman and 11.4 mg/L for the man [normal <5]) and thrombocytopenia (93.000 x 10^9/L) for the man. Other clinical laboratory investigations were normal. An abdominal ultrasonography showed splenomegaly in both patients.

Serologic testing for leptospirosis, as well as for other infections endemic in Germany, such as cat-scratch disease, Lyme borreliosis, ehrlichiosis, and Q fever, was negative.

The patients received doxycycline (200 mg/day) for 7 days, recovered within 3 days, and have remained well. Because symptoms resembled those of Mediterranean spotted fever, serum samples were tested for antibodies to *R. conorii;* when titers were found to be elevated, further clinical history was obtained. The patients had traveled to Costa Rica 7 months before the onset of symptoms but had not left Germany since that date. They owned two dogs, one of which had recently been adopted from an animal shelter. Neither of the dogs nor their littermates had traveled outside Germany. Both dogs, which were asymptomatic, had repeatedly had ticks and fleas, but the patients did not recall any recent arthropod bite.

Several serum samples were taken from the woman on days 4 (#1), 24 (#2), 35 (#3), and 43 (#4) and from the man on days 2 (#1) , 22 (#2), 33 (#3), and 43 (#4) after the onset of fever. A serum specimen was taken from each dog on day 35. All sera were analyzed in Marseille. Antibodies to *R. conorii*, *R. slovaca*, "*R. mongolotimonae*," *R. helvetica*, *R. felis*, *R. typhi*, *Coxiella burnetii*, *Bartonella henselae*, and *Francisella tularensis* were determined by microimmunofluorescence (6).

Results of serologic tests were negative for *C. burnetii*, *B. henselae*, and *F. tularensis*. The woman had antibody titers to *R. felis* of 0/0 (immunoglobulin [Ig] G/IgM), 128/64, 128/64, and 128/64 for serum samples #1, 2, 3, and 4, respectively. The man had titers of 0/0, 32/16, 32/0, and 0/0 for serum samples #1, 2, 3, and 4, respectively. Cross-reactions were observed between the rickettsiae tested except for *R. typhi,* preventing the identification of the species infecting the man. A twofold difference in immunoglobulin (Ig) M titer in favor of *R. felis* compared with other antigens was noted for the woman. Both dogs had an IgG titer to *R. felis* of 128, but antibody cross-reactions did not allow the specific etiologic agent to be identified. A Western blot with the same antigens was performed on patient specimen #2 and the dog samples (6). Antibodies specifically directed at *R. felis* were observed for the woman and one of the dogs. Additionally, *R. felis* infection was confirmed by nested polymerase chain reaction (PCR) (7). DNA was extracted from serum #1 from both patients, taken before antibiotic therapy, and from both dog specimens with QIAGEN columns (QIAamp Tissue Kit, QIAGEN, Hilden, Germany). To avoid contamination, no positive control was used. The assay amplified from the woman's serum a fragment of the gene encoding the PS120 protein (Figure), an intracytoplasmic protein with sequence signatures specific for most rickettsiae, including *R. felis* (8). The amplicons were sequenced by an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Foster City,

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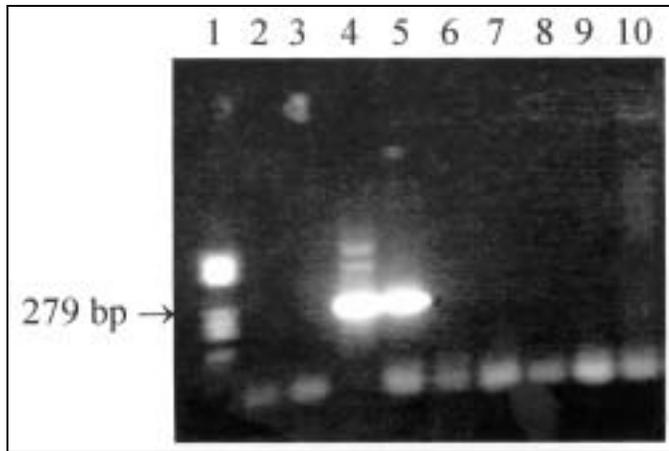
Figure. Results of the nested polymerase chain reaction (PCR) assay performed on the serum specimens from both patients and their dogs. Lane 1: standard DNA size marker V (Boehringer, Mannheim, Germany); lanes 2 and 3: serum #1 from man; lanes 4 and 5: serum #1 from woman; lanes 6 and 7: serum from dog #1; lanes 8 and 9: serum from dog #2; lane #10: negative control; lanes 2, 4, 6, and 8: pure DNA; and lanes 3, 5, 7, and 9: DNA diluted 1:10 in deionized water.

CA). Comparison of resulting sequences to GenBank showed 100% homology with *R. felis*.

Conclusions

Because our patients were in contact with dog ticks, a tick-borne rickettsiosis was suspected. However, no endemic tick-borne rickettsiosis has been identified in Germany to date. The most frequent rickettsiosis in Europe, Mediterranean spotted fever due to *R. conorii*, is contracted in the Mediterranean area; clustered cases, as observed for our patients, are exceptional. In contrast, African tick-bite fever, a rickettsiosis due to *R. africae,* is frequently encountered in travelers to southern Africa (7). Murine typhus, caused by *R. typhi*, which has long been considered the only flea-transmitted rickettsiosis, has not been reported in Germany but is present in southern Europe, including Spain, Portugal, Cyprus, and Greece (9-12). Until 1997, *R. felis* had only been detected in the United States. Since then, it has been detected by PCR in humans in Mexico (4) and Brazil (5) and in cat fleas from Ethiopia (5) and Spain (Marquez FJ, pers. comm.), thus demonstrating its presence in various areas, including the Old World, and supporting our preliminary serologic findings in French patients (5). In this study, serologic techniques discriminated among several rickettsiae for the woman but not her husband. Neither patient had antibodies to *R. typhi*, which suggests that antibodies to *R. felis* should be evaluated systematically in patients with typhuslike illnesses. Although no direct or indirect evidence of *R. felis* infection was obtained for the man, the simultaneous occurrence of symptoms similar to those observed in his wife strongly suggests infection with the same microorganism. Contact with fleas carried by their dogs would account for the simultaneous infection, as *R. felis* has been identified in *C. felis* fleas collected from a dog (13). However, neither fleas nor ticks from of the two dogs were available at the time of examination.

Our report describes the first PCR-confirmed case of human *R. felis* infection in Europe and supports the concept that *R. felis* may be widely distributed in the Old World and should be considered in the diagnosis of typhuslike illnesses, especially following a flea bite. Further studies should be conducted to identify the vectors of this rickettsia in Europe.

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Hemorrhagic Fever with Renal Syndrome Presenting with Hemophagocytic Lymphohistiocytosis

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Hemophagocytic lymphohistiocytosis—which is associated with a variety of infections, malignant neoplasms, autoimmune diseases, and immunodeficiencies—-is an uncommon syndrome with a rapidly fatal outcome. We describe the first case of hemorrhagic fever with renal syndrome due to *Hantaan virus* presenting with reactive hemophagocytosis.

Hemophagocytic lymphohistiocytosis (HLH) is an uncommon syndrome characterized by a reactive, systemic proliferation of benign histiocytes throughout the reticuloendothelial system (1). It is associated with a variety of infections, malignant neoplasms, drugs, autoimmune diseases, and various immunodeficiencies. Infection-induced HLH is often associated with systemic viral infections, particularly Epstein-Barr virus, and occasionally with bacterial, fungal, or parasitic infections. For most patients with HLH, the outcome is rapid and fatal unless the diagnosis is made early and followed by prompt therapeutic intervention (1,2).

Hemorrhagic fever with renal syndrome (HFRS), which is caused by *Hantaan, Puumala, Seoul,* and *Dobrava-Belgrade viruses* (HTNV, PUUV, SEOV, and DOBV, respectively) is acquired primarily through aerosols of infectious rodent urine (3). Recently, Baty et al. (4) reported a case of hemophagocytic syndrome associated with PUUV, the most common cause of HFRS in Europe. Our report describes an unusual case of HFRS caused by HTNV presenting with secondary hemop-hagocytosis.

Case Report

A 57-year-old woman was admitted to Chonnam National University Hospital, South Korea, with fatigue, generalized myalgia, and nausea of 2 weeks' duration. Three weeks before admission, she had worked in a field in a rural area. Her vital signs were blood pressure 140/90 mm Hg, heart rate 80/min, temperature 36.6°C, and respiratory rate 20/min. On examina-

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tion, she appeared acutely ill and had conjunctival suffusion, petechiae in the throat, an erythematous rash on the chest, tender hepatosplenomegaly, and mild tenderness in both flanks. There were no palpable lymph nodes.

Her leukocyte count was 3,200/μL, hemoglobin 9.9 g/dL, platelet count 25,000/μL, and reticulocyte count 0.3%. Blood chemistry revealed total serum protein 6.3 g/dL, albumin 3.0 g/dL, alkaline phosphatase 174 U/L, aspartate aminotransferase 369 U/L, alanine aminotransferase 175 U/L, total bilirubin 0.6 mg/dL (direct, 0.3 mg/dL), blood urea nitrogen 8.6 mg/dL, creatinine 0.5 mg/dL, lactic dehydrogenase 2,066 U/L, total cholesterol 100 mg/dL, HDL-cholesterol 22 mg/dL, triglyceride 285 mg/dL, and ferritin 20,000 μg/L. The coagulation profile included a prothrombin time of 13.6 seconds (control 12.5 seconds), a partial thromboplastin time of 45.2 seconds (control from 28 to 40 seconds), and a fibrinogen assay of 120 mg/dL. Serologic tests for viral infections—including antibodies against Epstein-Barr virus, cytomegalovirus, herpes, *Hepatitis A, B,* and *C viruses,* and HIV—were negative. Serologic tests for Leptospira and *Rickettsia tsutsugamushi* and connective tissue diseases were also negative. HTNV titers using a particle agglutination kit (HANTADIA, Greencross, Korea) were 1:160 (normal <1:80). Cultures of blood, urine, and sputum were sterile. A computed tomographic scan of the abdomen showed moderate hepatosplenomegaly without lymphadenopathy. Bone marrow aspirate revealed proliferation of histiocytes with prominent hemophagocytosis (Figure). On day 8 of hospitalization, the second serologic titer for HTNV was elevated at 1:5,120. Fortunately, the patient recovered completely with only supportive care, including aggressive replacement of blood components, over 14 days (Table).

Conclusions

Patients with infection-associated HLH usually have persistent unexplained fever, cytopenia, lymphadenopathy, and, frequently, hepatosplenomegaly and coagulopathy, causing diagnostic difficulties with malignant histiocytosis or T-cell
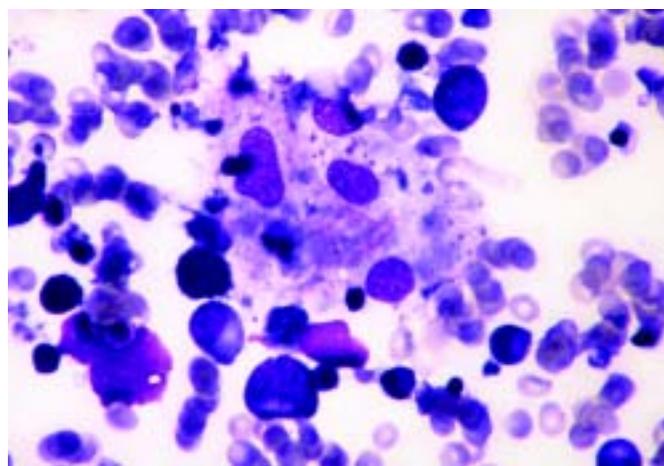

Figure. Bone marrow aspirate showing phagocytosis of neutrophil, nucleated erythrocyte, and platelets by benign histiocytes (Wright stain, x400).

Table. Serial laboratory findings in patient with hemorrhagic fever with renal syndrome from hospitalization until recovery, Korea

Laboratory test	On admission	Day 5	Day 8	Day 14
Leukocyte count (/μL)	3,200	3,300	4,100	4,400
Hemoglobin (g/dL)	9.9	10.2	11.4	11.2
Platelet count (/μL)	25,000	31,000	76,000	147,000
AST (U/L)	369	682	108	21
ALT (U/L)	175	433	205	21
BUN (mg/dL)	8.6	8.8	8.6	13.6
Cr (mg/dL)	0.5	0.8	0.7	0.9
Lactic dehydrogenase (U/L)	2,066	3,206	1,645	472
Ferritin (μg/L)	20,000	-	860	-
Hantaan virus titer	1:160	-	1:5,120	-

AST = aspartate aminotransferase, ALT = alanine aminotransferase; BUN = blood urea nitrogen.

lymphoma (2). The possible immunopathologic mechanism of HLH might be excessive production of Th1 cytokines, such as gamma-interferon, tumor necrosis factor-alpha, interleukin-1, or interleukin-6, from activated lymphocytes or monocytes (1,2). Patients with Epstein-Barr virus-associated HLH (which if not treated is usually fatal because of hemorrhage, infection, or multiorgan failure) should initially be treated with a combination of corticosteroids and chemotherapy (1,2). As with our case, a patient with reactive HLH associated with organisms other than Epstein-Barr virus requires supportive care and treatment of the underlying disease (1,2).

We believe that this is the first case of HFRS caused by HTNV presenting with HLH. Thus, HFRS caused by HTNV or PUUV should also be considered as one of the underlying infectious diseases resulting in hemophagocytosis, requiring early diagnosis followed by prompt therapeutic intervention.

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Distemper Outbreak and Its Effect on African Wild Dog Conservation

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In December 2000, an infectious disease spread through a captive breeding group of African wild dogs (*Lycaon pictus*) in Tanzania, killing 49 of 52 animals within 2 months. The causative agent was identified as *Canine distemper virus* (CDV) by means of histologic examination, virus isolation, reverse transcriptase-polymerase chain reaction analysis, and nucleotide sequencing. This report emphasizes the importance of adequate protection against infectious diseases for the successful outcome of captive breeding programs of endangered species.

The African wild dog (*Lycaon pictus*) is a highly endangered carnivore found in Africa south of the Sahara. Its population, estimated at <5,500, has declined dramatically in recent decades. Suggested causes for this decline include habitat loss, killing by humans, reduced prey availability, competition with other carnivores, and infectious diseases, including rabies and canine distemper (1).

As part of a conservation plan for the African wild dog, a captive breeding program was established in 1995 at Mkomazi Game Reserve, Tanzania, under the auspices of the Government of Tanzania. A founder group of 25 animals was divided into four breeding packs, each housed in a separate fenced enclosure. The founder members and captive-born pups were vaccinated against canine distemper with a vaccine successfully used in seals (2), rabies (Rabdomun, Schering-Plough Animal Health, Brussels, Belgium), and parvoviral disease and leptospirosis (combination vaccine: Dohyvac I-LP, Solvay Duphar, Weesp, the Netherlands). The vaccination schedule consisted of three consecutive vaccinations at 2- to 4-week intervals and annual revaccination, most recently in November 1999. Blood samples from a proportion of the vaccinated animals were collected at the time of vaccination to monitor immune response.

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The Outbreak

On December 20, 2000, two of the African wild dogs in one of the breeding packs became ill from an apparently infectious disease. The disease spread rapidly and was first noted in the other breeding packs on January 16, 18, and 22, 2001, respectively. The first deaths occurred on December 21, 2000; deaths peaked from January 30 to February 6, 2001, when 15 of the wild dogs died. The last death was recorded on February 13, 2001. Forty-nine of the 52 animals died during this outbreak.

Neutralizing antibody levels to *Canine distemper virus* (CDV), determined by methods similar to those used in a study of large felids (3), were measured in serum samples collected from nine African wild dogs on November 8, 2000. One of the three animals that survived the outbreak had a neutralizing antibody titer of 20; the other two were not tested. The sera from the remaining eight animals had a titer of <20, which is considered to be below the level of protection against canine distemper (4).

Tissue samples from nine animals that had died were used for histologic examination (n=6), virus isolation (n=2), and reverse transcriptase-polymerase chain reaction (RT-PCR) with *Morbillivirus*-specific primers P1: 5'ATGTTTATGATCA-CAGCGGT 3' and P2: 5'ATTGGGTTGCACCACTTGTC 3', which have been used before for phylogenetic analysis of morbilliviruses (5) (n=3). The results of analysis were consistent for all animals tested. The main histologic lesion was broncho-interstitial pneumonia with epithelial necrosis and multinucleated syncytial cells. Eosinophilic intracytoplasmic inclusion bodies, characteristic of canine distemper, were found in the epithelium of lung, kidney, intestine, and urinary bladder (Figure 1). Lung samples scored positive by RT-PCR for a *Morbillivirus* P-gene fragment. Phylogenetic analysis of the nucleotide sequences from the resulting PCR fragments demonstrated that the causative virus was most closely related to CDV (Figure 2A) and clustered with the sequences of CDV strains from domestic dogs (*Canis familiaris*), lions (*Panthera leo*), and bat-eared foxes (*Otocyon megalotis*) from East Africa in the 1990s (Figure 2B). P-gene fragments of the virus isolates from lung samples were identical to the sequences of the PCR products obtained directly from the tissue samples.

Conclusions

These results show that the primary cause of death of these African wild dogs was CDV infection. Canine distemper is highly infectious for many species of carnivores and causes high death rates in immunologically naïve populations (7). It is a known cause of death in free-living African wild dogs (8), as well as other wild carnivores, both free-living and captive (9,10). Based on phylogenetic analysis, the causative virus was a CDV strain circulating in the region in the past decade (11,12) (Figure 2B).

Potential routes of transmission of this virus to the captive breeding groups are by direct contact with infected domestic dogs or wild carnivores or indirectly by contact with humans
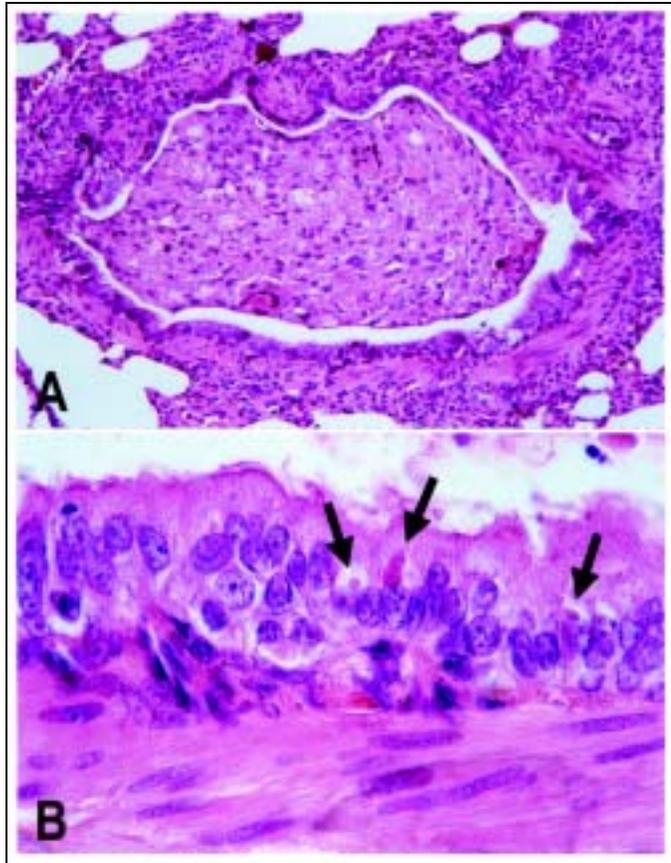
Figure 1. Lung lesions in an African wild dog with canine distemper. Hematoxylin and eosin staining. A. Bronchiole occluded by inflammatory cells and cell debris. B. Detail of A, showing multiple eosinophilic intracytoplasmic viral inclusions (arrows) in bronchiolar epithelium.

Figure 2. Phylogenetic trees based on a 388-bp *Morbillivirus* P-gene fragment. Maximum likelihood trees were generated by using the SEQ-BOOT and DNAML program of PHYLIP (Phylogeny Inference Package [6]) with 1,000 bootstrap replications. When possible, GenBank numbers of the sequences are given in parentheses. A. Virus from African wild dogs and representative *Morbillivirus* members. MV = *Measles virus* (Edmonston): strain (M89920); RPV = *Rinderpest virus*: RBOK strain (X68311); DMV = dolphin morbillivirus (Z47758); PMV = porpoise morbillivirus (5); PDV = *Phocine distemper virus*-1 (X75960); CDV-O = *Canine distemper virus* (CDV): Onderstepoort strain (AF305419); and LP/Tan/01 = CDV African wild dog, Tanzania (this study). B. Virus from African wild dogs and several CDV strains. CDV-O = CDV: Onderstepoort strain (AF305419); Dog/Ger/90 = CDV from dog, Germany (AF259549); Rac/USA/92 = CDV from raccoon, USA (3); Dog/Jap/99 = CDV from dog, Hamamatsu strain, Japan (AB028915); Dog/Tan/94 = CDV from dog, Tanzania (U53715); Fox/Tan/94 = CDV from bat-eared fox, Tanzania (U53714); Lion/Tan/94 = CDV from lion, Tanzania (U53712); and LP/Tan/01 = CDV from African wild dog, Tanzania (this study).

or their equipment. Domestic dog populations in some parts of Tanzania are endemically infected with CDV and were considered to be the source of infection for canine distemper in Serengeti lions in 1994 (13). Domestic dogs were not present at Mkomazi Game Reserve; however, transmission of CDV from domestic dogs in neighboring villages cannot be ruled out.

Because vaccination with live attenuated virus had been suspected of causing past deaths of African wild dogs (14,15) the animals in this breeding program were vaccinated with a CDV-ISCOM vaccine, which does not contain live virus. This vaccine protects harbor seals (*Phoca vitulina*) and dogs against phocine distemper virus infection (2), which is closely related to CDV, and resulted in protective antibody levels to CDV in African wild dogs monitored at the beginning of this captive breeding program (data not shown). However, the lack of neutralizing antibody titers to CDV in sera of these African wild dogs from November 2000 and the high death rate from canine distemper despite recent vaccination indicate vaccination failure. We are investigating possible reasons for this failure, including problems with application, maintenance of the cold chain, efficacy of the vaccine, and antiviral immune response of the African wild dogs.

Conservation of endangered species, both free-living and captive, has been jeopardized by infectious disease outbreaks in the past (10). This outbreak of canine distemper illustrates the disastrous effects that such a disease can have on inadequately protected animals. We therefore conclude that any further attempts to breed African wild dogs in captivity will need to ensure a vaccination regime against canine distemper and other infectious diseases that is both effective in this species and practical to implement under field conditions.

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Postexposure Treatment and Animal Rabies, Ontario, 1958–2000

Christopher P. Nunan,* Rowland R. Tinline,† Janet M. Honig,† David G. A. Ball,† Peggy Hauschildt,† and Charles A. LeBer‡

This paper investigates the relationship between animal rabies and postexposure treatment (PET) in Ontario by examining the introduction of human diploid cell vaccine (HDCV) in 1980 and the initiation of an oral rabies vaccination program for wildlife in 1989. Introducing HDCV led to an immediate doubling of treatments. Both animal rabies and human treatments declined rapidly after the vaccination program was introduced, but human treatments have leveled off at approximately 1,000 per year.

Jurisdictions across North America have identified animal rabies as a serious public health concern (1,2) because the epidemiology of human rabies closely follows the epizoology of animal rabies (3). Recent studies have examined the relationship between animal rabies and postexposure treatment (PET) for reasons of surveillance, economic impact, epidemiology, and appropriate treatment (2,4,5). We investigated the nature of this relationship in Ontario from 1958 to 2000, focusing on the impact of two important advances in rabies prevention: a) the introduction of human diploid cell rabies vaccine (HDCV) and b) the initiation of an oral rabies vaccination program (ORVP).

Animal rabies has had a long and varied history in Ontario. Before the 1950s, sporadic outbreaks of rabies occurred, usually associated with dogs. In the early 1950s, a rabies epizootic swept southward from the Arctic, entered northern Ontario in 1954, and by 1958 became enzootic in southern Ontario in red foxes (*Vulpes vulpes*) and striped skunks (*Mephitis mephitis*) (6). Sylvatic rabies has, in turn, infected companion animals and livestock, the two groups responsible for most subsequent human exposures (Honig JM, unpub. data, 1985). In 1999, the strain of raccoon rabies that has moved north along the eastern seaboard of the United States entered eastern Ontario from northern New York; by the end of 2000, there were 48 reported cases in raccoons (8 during 1999; 40 during 2000) (7). Since 1958, Ontario has averaged 1,200 to 1,300 animal cases per year, for a total of >56,000 cases by 2000. The burden on the public health system has been substantial; for example,

>63,000 PETs were reported in the same period. In addition, public health officials have had to investigate all contacts between humans and animals in which rabies may have been transmitted. In the 1980s, for example, at the height of the rabies enzootic, 15,000 to 25,000 such investigations were carried out annually (8).

In Canada, all animal rabies collection and laboratory diagnoses are handled by the Canadian Food Inspection Agency (CFIA), the federal ministry responsible for establishing the collection protocols and the laboratory diagnosis of submitted specimens that were suspected of carrying *Rabies virus* (RABV). District veterinary officers throughout the country are responsible for specimen collection and the decision to send specimens to federally operated laboratories for testing.

During the study period, Ontario's Ministry of Health and Long-Term Care (MOHLTC) distributed vaccine to physicians, free of charge, for the prevention of human rabies. In the fiscal year 1980-81, the ministry began distributing the newly licensed HDCV to replace earlier Semple and duck embryo vaccines. By the fiscal year 1983-84, all distributed vaccines were HDCV (9). HDCV was an important advance in rabies prevention because "it is a better immunogen with fewer side effects and requires far fewer doses than the previously recommended duck embryo vaccine" (10).

A second important advance in rabies prevention was ORVP. In 1989, the Ontario Ministry of Natural Resources initiated an ORVP in eastern Ontario, targeting the principal wildlife vectors (11). By 1994, the ministry had extended ORVP to cover the epizootic area in southern Ontario, and over 1 million vaccine baits were dropped annually. The program resulted in a dramatic drop in rabies incidence in southern Ontario (11).

Methods

We gathered the PET and animal rabies data (Table) from two government agencies. The MOHLTC annual reports from 1958 to 1978 list the number of courses of rabies vaccine distributed in each calendar year; for this paper, we considered each such course as a PET. For 1979 to 1988 and 1998 to 2000, we obtained similar records directly from internal reports in the MOHLTC. For 1989 to 1997, we obtained vaccine distribution data from the Public Health and Epidemiology Reports for Ontario (12). We obtained the annual number of laboratory-confirmed cases of animal rabies in Ontario directly from CFIA. Data were compiled on all terrestrial animals and bats that tested positive for RABV. Data on the number of negative test results were not available.

Our PET and rabies data were maintained for the entire study period by two central government agencies with a consistent mandate for collecting and reporting. Unfortunately, because these two agencies operate independently, we could not match the individual human treatments to the specific specimens that tested positive for rabies.

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Table. Rabies postexposure treatment (PET) and laboratory-confirmed animal rabies, Ontario, 1958 to 2000

Year	PET	Animal rabies	Ratio PET to animals rabies	PET rate per 100,000[a]
1958	1,647	2,426	0.7	28.3
1959	479	1,210	0.4	8.0
1960	566	241	2.3	9.3
1961	790	636	1.2	12.7
1962	991	879	1.1	15.6
1963	965	907	1.1	14.9
1964	852	1,006	0.8	12.9
1965	1,367	1,352	1.0	20.2
1966	1,168	1,004	1.2	16.8
1967	1,461	1,232	1.2	20.5
1968	1,539	1,924	0.8	21.2
1969	1,187	2,154	0.6	16.1
1970	1,164	1,477	0.8	15.4
1971	960	1,428	0.7	12.4
1972	1,252	2,161	0.6	15.8
1973	1,020	1,503	0.7	12.7
1974	974	1,425	0.7	11.9
1975	1,050	1,954	0.5	12.6
1976	935	1,395	0.7	11.1
1977	957	1,267	0.8	11.3
1978	816	1,422	0.6	9.5
1979	1,002	1,480	0.7	12.0
1980	1,096	1,412	0.8	11.6
Average	1,054	1,387	0.8	14.3
1981	1,833	1,333	1.4	20.8
1982	2,402	2,095	1.1	27.0
1983	2,481	1,834	1.4	27.5
1984	2,027	1,366	1.5	22.1
1985	2,150	1,975	1.1	23.2
1986	4,212	3,274	1.3	44.7
1987	2,621	2,001	1.3	27.2
1988	2,266	1,830	1.2	23.1
1989	2,640	1,870	1.4	26.2
1990	1,991	1,611	1.2	19.4
1991	1,739	1,234	1.4	16.7
1992	2,186	1,371	1.6	20.7
1993	2,581	1,241	2.1	24.2
1994	1,437	613	2.3	13.3
1995	1,182	328	3.6	10.8
1996	937	149	6.3	8.5
1997	1,079	95	11.4	9.6
1998	1,048	80	13.1	9.2
1999	890	100	8.9	7.7
2000	1,073	183	5.9	9.2
Average	1,939	1,229	1.6	19.0

[a]Population figures are from Statistics Canada Quarterly Estimates of Population for Canada, Provinces and Territories, 1951–2000.

Human population data were obtained from Statistics Canada Quarterly Estimates of Population for Canada, Provinces and Territories, 1951-2000.

We used regression analysis to examine the relationship between PET and the number of laboratory-confirmed cases of rabies in terrestrial animals and bats in Ontario. Analyses were done for the periods 1958 to 1980 and 1981 to 2000. As previously noted, HDCV was used during the second period. We used SPSS (release 10.0.5, SPSS Inc., Chicago, IL) to perform the regressions.

Results

From 1958 to 1980, the ratio of human treatments to animal cases was <1 in most years (Table, Figure). After HDCV was introduced in 1980, the yearly ratios of human treatments to animal cases were >1. Furthermore, from 1980 to 1981, the rate of PET per 100,000 persons almost doubled. The annual number of PETs increased from an average of approximately 1,000 in the 1970s to an average of more than 2,000 per year during the 1980s. During the 1980s and early 1990s, the annual number of PETs closely paralleled the annual number of animal cases.

The regression for the 1958 to 1980 period showed a weak but significant relationship between PET and animal rabies (R^2=0.42, p<0.001, n = 23, intercept = 557 [standard error, SE, 135.4], slope = 0.358 [SE 0.092]). After 1980 the relationship was much stronger (R^2=0.91, p<0.001, n = 20, intercept = 861 [SE 100.5], slope = 0.877 [SE 0.067]). The slopes of these regressions indicate that before 1980, there were approximately three reports of rabid animals for every PET, whereas after 1980, the ratio was approximately 1:1. Finally, the regression demonstrates that the base level of treatments after 1980 was 861, approximately 55% higher than the base level (557) before 1980.

Following the initiation of ORVP, the regular cycle of animal rabies was broken in the early 1990s (Figure) and the number of laboratory-confirmed rabid animals declined. The number of human treatments also declined by 50%, from more than 2,000 per year throughout most of the 1980s to approximately 1,000 per year in the late 1990s.
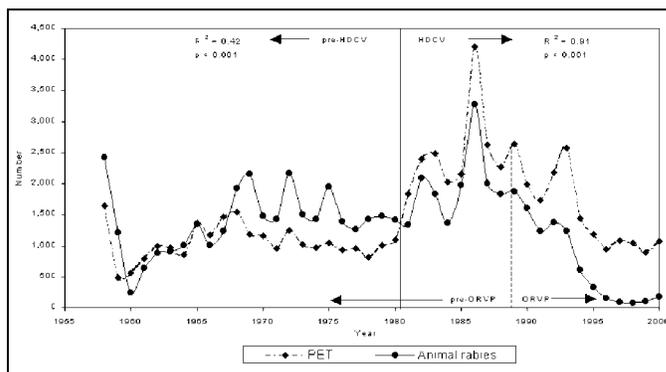

Figure. Postexposure treatment (PET) and laboratory-confirmed animal rabies. HDCV, human diploid cell vaccine; ORVP, oral rabies vaccination program; R^2, coefficient of determination.

During the period 1968 to 1980, there was an apparent change in the relationship between PET and animal rabies compared with the initial 1958 to 1967 period of the enzootic (Figure, Table). The ratios of PET per rabid animal and PET rate per 100,000 persons for the 1958 to 1967 period were 0.9 and 16.0, respectively, and declined to mean values of 0.7 and 13.2, respectively, for the 1968 to 1980 period.

Discussion

There was a dramatic change in the relationship between PET and animal rabies coincident with the introduction of HDCV in 1980. Furthermore, after 1980, the positive correlation between human treatments and animal rabies strengthened. We could not find any directives from the MOHLTC or published studies indicating a change in treatment policy when HDCV was introduced. Since HDCV had fewer side effects and the vaccination regime was simpler and less traumatic than previous treatments, there was, perhaps, less reluctance to administer PET after 1980 and, therefore, the use of PET increased and paralleled the incidence of animal rabies more closely.

When Ontario began its ORVP, one of the arguments for it was that animal rabies would be reduced and human treatments would follow suit. Our findings support this argument and are consistent with reports from other jurisdictions (3,13,14). The decline in human treatments in Ontario, however, was not as rapid as the decline in rabies cases. In fact, in recent years, while animal rabies incidence dropped to approximately 100 to 200 reported cases per year, PET leveled off at approximately 1,000 per year, about the same level as immediately before HDCV was introduced. We suspect the reasons for the continued high ratio of PET to animal rabies are varied and complex. For instance, with the continued presence of rabies, if a suspect animal is not available for testing, PET is administered as a precautionary measure. Furthermore, rabies in bats has been unaffected by the ORVP. Bats have been implicated in more than half (15 of 28 cases) of the human rabies cases diagnosed in the United States since 1980 (15) and in a recent death in Quebec (16). The recent spread of raccoon rabies from New York State into southeastern Ontario has increased the media coverage of rabies and will contribute to uncertainty about the presence of the RABV in the province. Under these circumstances, with the relatively safe HDCV, a continuing high number of treatments should be expected. Even in the absence of further rabies cases, the regression results suggest, at the 95% confidence interval, annual treatments would range from 650 to 1,072 annually.

The decline in PET per rabid animal and PET rate per 100,000 persons in the 1968 to 1980 period hints at other factors affecting the relationship between animal rabies and the administration of PET. We were unable to find any evidence in published studies detailing a change of government policy about the administration of PET or some traumatic event that could initiate a de facto policy change. Indeed, studies during that period recommended a treatment approach similar to today's guidelines (17). Furthermore, compulsory vaccination of companion animals was not an explanation. Under the Health Protection and Promotion Act, a regulation governing rabies immunization was not introduced until 1984 and it has taken until 2000 for all district health units in southern Ontario to be included in it (18). All we know is that, early in this period, rabies incidence across southern Ontario had stabilized and developed regular cycles in various regions (19). We can only speculate that, as animal rabies incidence became more predictable, health professionals and the public learned to manage the risk and there was less pressure to give PET, especially with older vaccines and their lengthy regime of injections. Experience in the United States indicated that consultation with state health departments during management of potential rabies exposure reduced PETs (10).

Conclusions

Our data suggest that human interventions have played a major role in the relationship between PET and animal rabies. The introduction of a new, safer vaccine was associated with a sudden increase in the number of PETs per rabid animal. Furthermore, while the introduction of an ORVP reduced animal rabies, PET did not drop at a similar rate and has appeared to stabilize at approximately 1,000 persons per year. This stabilization, despite the diminishing number of rabies cases, is important in estimating the economic impact of rabies control and public education. However, as our data for the 1968 to 1980 period show, there are other, as-yet-unknown factors that affect the animal rabies/PET relationship.

We believe that two general approaches are needed for the future study of this complex relationship. First, we need details of the circumstances of rabies incidents involving human exposures, such as those assembled by Moran et al. (5), Honig (unpub. data), and the Public Health Branch, Ministry of Health (12). For Ontario, assembling these data will require follow-up interviews on a case-by-case basis. Second, if we can obtain data on the distribution of PETs by the 32 health units in southern Ontario (we have distribution data for animal rabies), we may gain further insight by (a) examining the distribution and interaction of human and animal populations; (b) investigating the influence of the geographic scale at which the relationships are examined; and (c) making regional comparisons of the administration of PET and the relative surveillance efforts in an area over time, given the history of rabies incidence and public awareness campaigns in the area.

Dr. Nunan is a research biologist with the Rabies Research Unit of the Ontario Ministry of Natural Resources. His major research interests lie in the assessment and management of risk in situations involving human response to animal disease.

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It is difficult
to get the news from poems
yet men die miserably every day
for lack
of what is found there.

"Asphodel, That Greeny Flower" by William Carlos Williams, a physician and poet

Dread-Bound News

We cower under stars and stripes
While horrors unfold and threaten the future

Armies, governments, news hounds
Chase their tails and the tales of violence,
But the crucial culprit, collective fear,
Pervades our lives.

Fear that infectious disease of humans,
With strains varied as the common cold,
Has no vaccine or cure and lurks disguised,
Evades diagnosis.

In fear we cannot, must not hide
From the public info-network
That slams us with a headline epidemic
Prompting us to wade in the bay of panic.
The enemy's friend and foe,
It gives voice to the great unknown,
Paints faces on the monster
Who hides under our beds.

United, we wait for disaster
Fearing to suck our thumbs,
Lest mama's apron strings be dusted with anthrax.
A germ becomes a giant!

Zoe Haugo
Atlanta, Georgia

ANOTHER DIMENSION. Submit thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to invoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Medicine to Kill, Medicine to Heal

Neil Shulman and Zoe Haugo

Recent bioterrorism events invite speculation on the nature and extent of human capacity for destructive as well as constructive behavior. Drawing a line between those who create suffering to further their cause and those who strive to alleviate suffering regardless of the cause is fair, though the dichotomy may foster intolerance and further polarize the opposition.

On the one hand, a shortage of open-mindedness and understanding compromises humans' ability to exist in a secure state of peace. A single variable or difference becomes a huge point of contention. Religion has polarized Jews and Muslims in the Middle East, Protestants and Catholics in Ireland, Hindus and Muslims in India; tribal differences decimated Hutus and Tutsis in Rwanda; ideological feuds pounded Cambodia; and skin-color prejudice continues to inflame many countries. A leader can invoke a group of white humans or yellow humans to kill a group of black humans or red humans, but a single white cat could never convince a group of white cats to go out and kill a group of yellow cats. The brains of other animals are generally not wired in this way.

On the other hand, an enormous capacity for compassion drives countless organizations to reduce suffering, eradicate disease, and promote the common good. Departments of public health, for example, join together throughout the world to fight epidemics. Workers from all countries, groups, and belief systems unite to stamp out the diseases that plague us. The road is long, and the breakthroughs are few. Many have risked and lost their lives in the dangerous pursuit of infectious-disease elimination. Disease prevention and control activities deemphasize ethnic differences and acknowledge the value of all human life. Although many public health workers are involved in laboratory research, disease surveillance, and the technical aspects of human health, they remain focused on the goal of their endeavors, the common good.

To prevent humans from harming each other and encourage them to work for the common good, perhaps we can "infect" the world with the value system of public health workers and nurture the circumstances of their psychological make-up. What is it that drives people to choose eliminating disease, improving public health, and extending or saving lives over the deliberate introduction of disease and destruction? Public health research should expand to address the psychodynamics of violent temper, corrosive anger, the use of disease as a weapon of war, and the circumstances and environments that promote them.

If only, in temporary suspension of disbelief, life could imitate science fiction, humans would set aside their differences to fight a common enemy: disease not aliens.

Dr. Shulman, associate professor of medicine and researcher of cardiovascular disease, Emory University School of Medicine, is an author and humanist involved deeply in activities that promote harmony and cooperation among people. He has written a number of books, including children's books, and one of his works, Doc Hollywood, was made into a movie of which he was associate producer.

Ms. Haugo studied Humanistic Studies at McGill University. Her interests are language studies and the performing arts. Currently, Dr. Shulman and Ms. Haugo are developing a documentary celebrating the harmonious atmosphere of Atlanta's diverse Clarkston High School.

Multidrug-Resistant *Pseudomonas Aeruginosa* Bloodstream Infections: Analysis of Trends in Prevalence and Epidemiology

To the Editor: Multidrug-resistant (MDR) *Pseudomonas aeruginosa* bloodstream infection has been described only in patients with cystic fibrosis (1) and in isolated outbreaks in intensive-care unit (ICU) or neoplastic patients (2-4). We investigated the percentage and clinical findings of patients with *P. aeruginosa* bacteremia having MDR strains in a 1,700-bed university hospital in Rome, Italy, over a 10-year period (1990-1999).

All consecutive patients with the first episode of community- or hospital-acquired *P. aeruginosa* bacteremia, according to the definition of the Centers for Disease Control and Prevention (5), were included in the analysis. The term MDR *P. aeruginosa* covered resistance to ciprofloxacin, ceftazidime, imipenem, gentamicin, and piperacillin. In patients with *P. aeruginosa* bacteremia, we evaluated age, gender, type of infection (hospital or community acquired), duration of hospitalization, risk factors, clinical findings, and outcome. Prognosis immediately before bacteremia developed was determined with the revised Acute Physiology and Chronic Health Evaluation (APACHE) III system (6).

Bacteria were identified by using API 20NE (Biomerieux, Marcyl'Etoile, France). MICs were determined by broth microdilution in accordance with the methods of the National Committee for Clinical Laboratory Standards. Contingency data were analyzed by the two-tailed chi-square test or Fisher's exact test, and continuous data were analyzed by Student *t* test. Logistic regression analysis was used to determine which risk factors were independently significant. All statistical analysis was performed with the software program Statistics (Windows Systat Inc., Evanston, IL).

In the study period, *P. aeruginosa* was isolated from 358 of 379,190 hospitalized patients. Among 358 patients with *P. aeruginosa* bacteremia, 133 (37%) were hospitalized in medical wards, 103 (29%) in ICUs, 97 (27%) in surgical wards, and 25 (7%) in neonatology; 45 (12%) had HIV infection and 28 (8%) had hematologic malignancies.

For the study period, the overall hospital incidence of both nosocomial and community-acquired *P. aeruginosa* bacteremia was 0.94 per 1,000 hospital admissions. In particular, the incidence increased from 9.7 to 24.7 per 1,000 hospital admissions (p <0.01; chi square for trend) in ICUs. In HIV-infected patients, the incidence increased from 1.5 to 12.4 per 1,000 hospital admissions until 1996 when, after highly active antiretroviral therapy was introduced, it decreased to 0.7 (p = 0.01, chi square for trend).

The first case of MDR *P. aeruginosa* strain was isolated in the hematologic unit in 1992. After that, the hospital prevalence of MDR strains increased significantly (p=0.03) from 8% (3/37) in 1993 to 17% (9/54) in 1999. Overall, we observed 51 (14% of 358) cases of MDR *P. aeruginosa* bloodstream infections; 49 (96%) were nosocomial. The prevalence of MDR among the total *P. aeruginosa* bacteremia cases per ward was as follows: medical wards 1 (2%) of 60 (95% confidence intervals [CI] = 0.05-10); surgical wards 7 (7%) of 97 (95% CI = 3-15); hematologic ward 3 (11%) of 28 (95% CI = 2-28); ICUs 22 (21%) of 103 (95% CI = 13-31); and infectious diseases ward (in HIV-infected patients only) 18 (40%) of 45 (95% CI = 26-54).

The mean age ± standard deviation of patients with MDR *P. aeruginosa* infections was 52±12 years (range 29 to 77); 35 patients (69%) were men, and 9 (18%) were active intravenous drug abusers. The mean Apache III score at diagnosis of bacteremia was 41±17 (95% CI = 39-56). The mean concentration of circulating polymorphonuclear cells was 2,974±2,790/

mm^3 (95% CI = 2,181-3,796). In HIV-infected patients, the mean number of peripheral CD4+ cells was 71±104 / mm^3 (95% CI = 35-106). Advanced age (odds ratio [OR] = 1.07; 95% CI = 1.04-1.10, p<0.01), HIV infection (OR = 3.94; 95% CI = 1.10-14.11, p=0.03), intravenous drug abuse (OR=13.15; 95% CI=1.65-104.5; p=0.01), and previous therapy with quinolones (OR=3.21; 95% CI=2.14-23.33; p = 0.001) were independent risk factors on logistic regression analysis.

The overall mortality rate of patients with *P. aeruginosa* bacteremia was 31%; death rates were higher among patients with higher APACHE III score (mean 39 versus 27; p=0.01) and MDR *P. aeruginosa* infections (67% versus 23%; OR=15.13; 95% CI=1.90-323.13; p=0.001).

This prospective surveillance of *P. aeruginosa* bloodstream infections clearly indicates, for the first time, that multidrug resistance is statistically associated with HIV infection, as already observed for cystic fibrosis (1). We also identified a significant correlation between MDR *P. aeruginosa* bacteremia and intravenous drug abuse, advanced age, and previous quinolone use.

The association between isolation of MDR strains, HIV infection, and intravenous drug abuse (the most important HIV risk factor in Italy) is not an unexpected result. We have already demonstrated that hospitalized HIV-infected patients are at increased risk of acquiring nosocomial bloodstream infections compared with other immunocompromised hosts (7). Age is a well-known predisposing factor for bacterial infections. In particular, older HIV-infected patients progress more rapidly to AIDS (8).

Resistance following treatment with a single antimicrobial agent may be due in some circumstances to synergy between enhanced production of beta-lactamases and diminished outer membrane permeability (9). More emphasis is now given, however, to the energy-dependent efflux of antibiotics by *P. aeruginosa*. A single opening of a

pump facilitates resistance to quinolones, beta-lactams, tetracycline, and chloramphenicol among the drug efflux (9). The recent characterization of a carbapenem-hydrolyzing metallo-beta-lactamase from *P. aeruginosa* opens new possibilities for reducing the spread of resistant strains (10).

One limitation of our study is the absence of genotypic analysis of MDR strains. However, we are confident that a general outbreak of MDR *P. aeruginosa* did not occur in our hospital. Nevertheless, limited outbreaks involving few patients in different wards remain a possibility. In summary, the observation that 14% of *P. aeruginosa* bloodstream infections are multidrug resistant is worrisome and reflects the growing worldwide problem of antimicrobial resistance. In particular, the fact that HIV-infected patients are at increased risk, as are persons with cystic fibrosis, suggests the need for ongoing worldwide surveillance of *P. aeruginosa* in immunocompromised patients.

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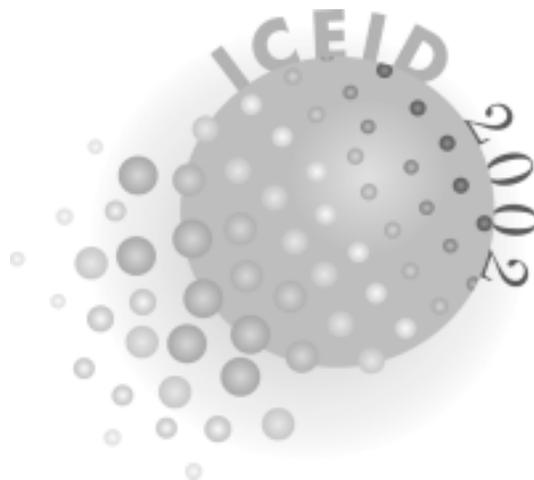
The National Center for Infectious Diseases, Centers for Disease Control and Prevention, has scheduled the Third International Conference on Emerging Infectious Diseases for March 24-27, 2002, at the Hyatt Regency Hotel, Atlanta, Georgia, USA. More than 2,500 participants are expected, representing many nations and disciplines. They will discuss the latest information on many aspects of new and reemerging pathogens, such as *West Nile virus* and issues concerning bioterrorism.

Conference information is available
at http://www.cdc.gov/iceid

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Conference Summary

Clinical Issues in the Prophylaxis, Diagnosis, and Treatment of Anthrax

On November 18, 2001, a meeting was held at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, to discuss the prophylaxis, diagnosis, and treatment of anthrax. Participants included clinicians and health department personnel from areas where anthrax cases were identified, infectious disease experts, representatives of professional societies, and experts from federal agencies. A patient recovering from inhalational anthrax also described her illness. The following is a summary of the presentations and discussion.

Prophylaxis[1]

Ciprofloxacin, doxycycline, and penicillin G procaine have been approved by the Food and Drug Administration (FDA) for prophylaxis of inhalational *Bacillus anthracis* infection, on the basis of efficacy data in monkeys and pharmacokinetic, pharmacodynamic, and safety considerations (1-3). During the recent bioterrorist attacks, interim CDC recommendations for anthrax prophylaxis include ciprofloxacin or doxycycline; amoxicillin (in three daily doses) is an option for children and pregnant or lactating women exposed to strains susceptible to penicillin (4-6), to avoid potential toxicity of quinolones and tetracyclines. Amoxicillin is not widely recommended as a first-line prophylactic agent, however, because of lack of FDA approval, lack of data regarding efficacy, and uncertainty about the drug's ability to achieve adequate therapeutic levels at standard doses.

The optimal duration of prophylaxis is uncertain; however, 60 days was recommended, primarily on the basis of animal studies of anthrax deaths and spore clearance after exposure. The possible need for longer prophylaxis and vaccine use was discussed. In monkeys after aerosol challenge, an estimated 0.5%-1% of spores remained at 75 days and traces were present at 100 days; delaying prophylaxis up to 20 days after exposure prolonged the incubation period without reducing disease risk (7). In one human case during the Sverdlovsk outbreak (former Soviet Union, 1979), anthrax developed 43 days after spores were released into the atmosphere (time of exposure unknown) (2,8). When prophylaxis is delayed or intermittent, several experts recommended a total of 60 days of therapy. (On December 18, the Department of Health and Human Services announced additional options for prophylaxis of inhalational anthrax for persons who wish to take extra precautions, especially those whose exposure may have been high. Three options are now offered: 1) 60 days of antibiotic prophylaxis; 2) 100 days of antibiotic prophylaxis, and 3) 100 days of antibiotic prophylaxis, plus anthrax vaccine as investigational postexposure treatment [3 doses over a 4-week period] [9].)

The need for prophylaxis is determined by public health officials on the basis of an epidemiologic investigation. Prophylaxis is indicated for persons exposed to an airspace contaminated with aerosolized *B. anthracis*. Prophylaxis is not indicated for health-care and mortuary workers who care for patients or attend to corpses using standard precautions, for persons who handle or open mail in the absence of a credible threat, or for prevention of cutaneous anthrax (10).

Successful implementation of mass prophylaxis requires clarity of public health intent and communication, as well as coordination and collaboration. A well-communicated policy on who receives prophylaxis and with which drugs is essential. Agency spokespersons, local health-care providers, employers, and employee organizations (e.g., labor unions) should be familiar with the policy. Local or regional task forces may be helpful in planning and communicating public health policy, and resolving jurisdictional issues. Prophylaxis teams should be predesignated to function around the clock. Team members should have contingency plans for personal needs (e.g., child care). Issues for the point of prophylaxis distribution include layout and managing of traffic flow; security; availability of medical and office supplies, antibiotic and disease fact sheets, multilingual staff, and mental health counselors; legal needs (e.g., for a physician to write orders); and plans for follow-up, including assessment of adherence, illness, and possible drug adverse effects. Collaboration among health departments, health-care delivery organizations, and clinicians is important. In the 2001 outbreak, some patients with possible drug side effects were refused appointments by their private physicians and were referred back to the health department.

Anthrax prophylaxis issues needing further consideration or research include efficacy of additional drugs, optimal duration of prophylaxis, usefulness of a loading dose, safety of prolonged drug use (especially in children and pregnant women), concomitant use of vaccine or antitoxin, level of infectious dose, and definition of high-risk exposure (e.g., according to particle size or degree of environmental contamination).

Clinical Recognition and Diagnosis[2]

Twenty-two confirmed or suspected cases (11 confirmed inhala-

tional; 7 confirmed and 4 suspected cutaneous) were identified in the 2001 outbreak of bioterrorism-related anthrax. Cases were reported from Florida, New York, New Jersey, the District of Columbia, and Connecticut.

Inhalational Anthrax

Of the 11 patients with inhalational anthrax, 9 (and possibly all 11) are believed to have been exposed to mail containing or contaminated with *B. anthracis* spores. Median age was 56 years (range 43-94 years). Average incubation from known exposure to symptoms was 4 days (range 4-6 days). Fever, chills, drenching sweats, profound fatigue, minimally productive cough, nausea or vomiting, and chest discomfort were symptoms reported by most patients. Rhinorrhea and productive cough were uncommon. Chest X-ray at initial examination showed mediastinal widening, paratracheal fullness, hilar fullness, and pleural effusions or infiltrates or both, but in some patients these initial findings were subtle. Pleural effusions were a complication in all 11 patients; among all 8 patients who had not received antibiotics, *B. anthracis* grew in blood cultures drawn at initial examination. Six (55%) of 11 patients have survived with aggressive supportive care and multidrug antibiotic regimens including a fluoroquinolone (11).

The differential diagnosis of inhalational anthrax versus influenzalike illness is challenging. Respiratory viruses, including influenza, are common causes of influenzalike illness and tend to circulate in winter. These viruses are readily communicable, in contrast to anthrax, which is not spread from person to person. A history of influenza vaccination is not helpful in evaluating the likelihood of anthrax. Influenzalike illnesses have many causes besides influenza viruses, and influenza vaccine is not 100% effective. Unlike patients with inhalational anthrax, adults with influenza or other viral respiratory illnesses do not usually have shortness of breath and vomiting but often have sore throat or rhinorrhea. Rapid identification tests for influenza are available but vary widely in sensitivity.

In the current climate, emergency department and primary-care physicians should maintain a high index of suspicion for inhalational anthrax. Complicating diagnosis is the fact that patients initially may not appear very ill (11). A careful history with assessment of epidemiologic risk factors for anthrax (e.g., working for the postal service) should be obtained. Communication between clinicians and health authorities is critical for obtaining up-to-date assistance with diagnosis and management.

The classic chest X-ray findings—widened mediastinum or pleural effusions—may be subtle or absent on initial medical evaluation. In addition, these radiographic findings are not unique to anthrax: histoplasmosis, sarcoidosis, tuberculosis, and lymphoma, for example, are included in the differential diagnosis. A chest computed tomography scan is helpful in detecting hemorrhagic mediastinal lymph nodes and edema, peribronchial thickening, and pleural effusions, findings seen in patients with inhalational anthrax. Hyperdense mediastinal and hilar adenopathy plus mediastinal edema suggest anthrax. The hemorrhagic pleural effusions of inhalational anthrax typically increase during hospitalization.

Blood cultures and *B. anthracis*-specific polymerase chain reaction (PCR) of sterile fluids (e.g., blood and pleural fluid) are important in the diagnosis of inhalational anthrax. Serologic testing has also been valuable. An enzyme-linked immunosorbent assay (ELISA) to detect immunoglobulin (Ig) G response to *B. anthracis* protective antigen (PA) is highly sensitive (detects 98.6% of true positives) but is only approximately 80% specific. To improve specificity, a PA-competitive inhibition ELISA is used as a second, confirmatory step. Preliminary studies indicate that spe-cific IgG anti-PA antibody can be detected as early as 10 days, but peak IgG may not be seen until 40 days after onset of symptoms.

Immunohistochemical examination of pleural fluid or transbronchial biopsy specimens, using antibodies to *B. anthracis* cell wall and capsule, also has an important role in the diagnosis of inhalational anthrax, especially in patients who have received prior antibiotics. Immunohistochemical examination can detect intact bacilli or *B. anthracis* antigens. PCR, serologic tests, and immunohistochemical tests are currently available at CDC or at certain laboratories in the Laboratory Response Network (LRN).

Cutaneous Anthrax

Seven confirmed and four suspected cases of cutaneous anthrax were identified during the 2001 outbreak. Skin trauma was not associated with these cases of cutaneous anthrax. Exposure to contaminated mail was the apparent source of infection in all patients. The incubation period after exposure ranged from 1 to 10 days. The initial symptom was often a pruritic papule resembling an insect bite. The papules vesiculated, with some becoming hemorrhagic. The vesicles ruptured to form depressed ulcers, often with local edema, ultimately forming dry eschars. These stages occur regardless of antibiotic therapy. The differential diagnosis of cutaneous anthrax includes brown recluse spider bite, ecthyma, ulceroglandular tularemia, accidental vaccinia, and necrotic herpes simplex. Cutaneous anthrax is painless, does not include rash, and results in a black eschar. Patients with cutaneous anthrax may have fever, extensive edema, and other systemic signs.

Gram stain and culture of the lesion are recommended; however, prior antibiotic treatment rapidly renders the infected site culture-negative for *B. anthracis*. Serologic testing and punch biopsy at the edge of the lesion,

examined by silver staining and immunohistochemical testing, are useful in diagnosing cutaneous anthrax in patients who have received antibiotic therapy.

Clinical recognition and diagnosis issues needing further consideration and research include rapid, reliable, and readily available detection methods (e.g., PCR and antigen detection); education and ready access to information for clinicians regarding anthrax clinical features and risk stratification; recognition of anthrax in children; and the role of serologic testing in the diagnosis and management of both inhalational and cutaneous anthrax.

Treatment[3]

Treatment recommendations for anthrax infections have been based on historical information and limited data from animals (nonhuman primates), as well as in vitro findings. Susceptibility testing of 65 historical isolates was performed at CDC. In the absence of published guidelines for testing for *B. anthracis*, the standard National Committee for Clinical Laboratory Standards broth microdilution method was used with staphylococcal breakpoints. These 65 isolates and all those associated with the 2001 outbreak were sensitive to the quinolones, rifampin, tetracycline, vancomycin, imipenem, meropenem, chloramphenicol, clindamycin, and the aminoglycosides. The isolates have intermediate-range susceptibility to the macrolides but are resistant to extended-spectrum cephalosporins, including third-generation agents (e.g., ceftriaxone), and to trimethoprim-sulfamethoxazole (12).

The decision regarding the use of penicillins, the drugs historically used for treatment and prophylaxis of anthrax, is complicated. An inhibition assay shows beta-lactamase activity at low levels in the isolates. Genomic sequence data show two beta-lactamases: a potential penicillinase (class

A) and a cephalosporinase (class B), which is expressed. Concern about the use of penicillin arises because an inducible penicillinase could be activated in the face of treatment with beta-lactams, particularly if the number of organisms present is high, as appears typical with inhalational disease. Concerns have also been raised about the poor penetration of beta-lactams into macrophages, the site where *B. anthracis* spores germinate.

Ciprofloxacin has been recommended on the basis of in vivo (animal) findings; other quinolones have not been studied in the primate model. Doxycycline, another first-line agent, should not be used if meningitis is suspected because of its lack of adequate central nervous system penetration. Bacteremic patients are often initially treated with a multidrug regimen to which an offending organism is presumed sensitive; this treatment allows empiric coverage for other pathogens. Thus, the recommendation for initial treatment of inhalational anthrax is a multidrug regimen of either ciprofloxacin or doxycycline along with one or more agents to which the organism is typically sensitive. After susceptibility testing and clinical improvement, the regimen may be altered. The drugs of choice for treatment of cutaneous disease are also ciprofloxacin or doxycycline. A penicillin such as amoxicillin or amoxacillin/clavulanic acid may be used to complete the course if susceptibility testing is supportive.

On the basis of risk for the inhalational form of the disease, cases of both inhalational and cutaneous anthrax associated with the 2001 outbreak are being treated with 60 days of antibiotics. Although zoonotic cutaneous anthrax is treated with a 7- to 10-day regimen, inhaled spores can remain latent for extended periods.

Two months after the 2001 outbreak, 6 of 11 patients with inhalational anthrax had survived. Keys to successful management appear to be early institution of antibiotics and aggressive supportive care. Chest tube drainage of the recurrent pleural effu-

sions, which are typically hemorrhagic, often leads to dramatic clinical improvement. Because these effusions tend to reaccumulate rapidly, insertion of a chest tube or tubes has been beneficial.

Anthrax treatment issues meriting further consideration relate to adjunctive therapies. Clindamycin has been suggested to have antitoxin properties (as in the treatment of toxic shock associated with group A streptococci, *Staphylococcus aureus,* and *Clostridium* infections). Steroids have been used to control the edema of cutaneous disease and have been suggested for the treatment of meningitis or substantial mediastinal edema (13). Other antitoxin agents investigated in vitro include angiotensin-converting enzyme inhibitors, calcium channel blockers, and tumor necrosis factor inhibitors. Specific anthrax IgG antisera, collected from military or other vaccinees, may be an adjunct, as well as administration of the vaccine itself.

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Report Summary

Public Health Assessment of Potential Biological Terrorism Agents

As part of a Congressional initiative begun in 1999 to upgrade national public health capabilities for response to acts of biological terrorism, the Centers for Disease Control and Prevention (CDC) was designated the lead agency for overall public health planning. A Bioterrorism Preparedness and Response Office has been formed to help target several areas for initial preparedness activities, including planning, improved surveillance and epidemiologic capabilities, rapid laboratory diagnostics, enhanced communications, and medical therapeutics stockpiling (1). To focus these preparedness efforts, however, the biological agents towards which the efforts should be targeted had to first be formally identified and placed in priority order. Many biological agents can cause illness in humans, but not all are capable of affecting public health and medical infrastructures on a large scale.

The military has formally assessed multiple agents for their strategic usefulness on the battlefield (2). In addition, the Working Group on Civilian Biodefense, using an expert panel consensus-based process, has identified several biological agents as potential high-impact agents against civilian populations (3-7). To guide national public health bioterrorism preparedness and response efforts, a method was sought for assessing potential biological threat agents that would provide a reviewable, reproducible means for standardized evaluations of these threats.

In June 1999, a meeting of national experts was convened to 1) review potential general criteria for selecting the biological agents that pose the greatest threats to civilians and 2) review lists of previously identified biological threat agents and apply these criteria to identify which should be evaluated further and prioritized for public health preparedness efforts. This report outlines the overall selection and prioritization process used to determine the biological agents for public health preparedness activities. Identifying these priority agents will help facilitate coordinated planning efforts among federal agencies, state and local emergency response and public health agencies, and the medical community.

Overview of Agent Selection and Prioritization Process

On June 3-4, 1999, academic infectious disease experts, national public health experts, Department of Health and Human Services agency representatives, civilian and military intelligence experts, and law enforcement officials[1] met to review and comment on the threat potential of various agents to civilian populations. The following general areas were used as criteria: 1) public health impact based on illness and death; 2) delivery potential to large populations based on stability of the agent, ability to mass produce and distribute a virulent agent, and potential for person-to-person transmission of the agent; 3) public perception as related to public fear and potential civil disruption; and 4) special public health preparedness needs based on stockpile requirements, enhanced surveillance, or diagnostic needs. Participants reviewed lists of biological warfare or potential biological threat agents and selected those they felt posed the greatest threat to civilian populations.

The following unclassified documents containing potential biological threat agents were reviewed: 1) the Select Agent Rule list, 2) the Australian Group List for Biological Agents for Export Control, 3) the unclassified military list of biological warfare agents, 4) the Biological Weapons Convention list, and 5) the World Health Organization Biological Weapons list (8-12). Participants with appropriate clearance levels reviewed intelligence information regarding classified suspected biological agent threats to civilian populations. Genetically engineered or recombinant biological agents were considered but not included for final prioritization because of the inability to predict the nature of these agents and thus identify specific preparedness activities for public health and medical response to them. In addition, no information was available about the likelihood for use of one biological agent over another. This aspect, therefore, could not be considered in the final evaluation of the potential biological threat agents.

Participants discussed and identified agents they felt had the potential

for high impact based on subjective assessments in the four general categories. After the meeting, CDC personnel then attempted to identify objective indicators in each category that could be used to further define and prioritize the identified high-impact agents and provide a framework for an objective risk-matrix analysis process for any potential agent. The agents were evaluated in each of the general areas according to the objective parameters and were characterized by the rating schemes outlined in the Appendix. Final category assignments (A, B, or C) of agents for public health preparedness efforts were then based on an overall evaluation of the ratings the agents received in each of the four areas.

Results

Based on the overall criteria and weighting, agents were placed in one of three priority categories for initial public health preparedness efforts: A, B, or C (Table 1). Agents in Category A have the greatest potential for adverse public health impact with mass casualties, and most require broad-based public health preparedness efforts (e.g., improved surveillance and laboratory diagnosis and stockpiling of specific medications). Category A agents also have a moderate to high potential for large-scale dissemination or a heightened general public awareness that could cause mass public fear and civil disruption.

Most Category B agents also have some potential for large-scale dissemination with resultant illness, but generally cause less illness and death and therefore would be expected to have lower medical and public health impact. These agents also have lower general public awareness than Category A agents and require fewer special public health preparedness efforts. Agents in this category require some improvement in public health and medical awareness, surveillance, or laboratory diagnostic capabilities, but presented limited additional requirements for stockpiled therapeutics

Table 1. Critical biological agent categories for public health preparedness

Biological agent(s)	Disease
Category A	
Variola major	Smallpox
Bacillus anthracis	Anthrax
Yersinia pestis	Plague
Clostridium botulinum (botulinum toxins)	Botulism
Francisella tularensis	Tularemia
Filoviruses and Arenaviruses (e.g., *Ebola virus, Lassa virus*)	Viral hemorrhagic fevers
Category B	
Coxiella burnetii	Q fever
Brucella spp.	Brucellosis
Burkholderia mallei	Glanders
Burkholderia pseudomallei	Melioidosis
Alphaviruses (VEE, EEE, WEE[a])	Encephalitis
Rickettsia prowazekii	Typhus fever
Toxins (e.g., Ricin, Staphylococcal enterotoxin B)	Toxic syndromes
Chlamydia psittaci	Psittacosis
Food safety threats (e.g., *Salmonella spp.*, *Escherichia coli* O157:H7)	
Water safety threats (e.g., *Vibrio cholerae, Cryptosporidium parvum*)	
Category C	
Emerging threat agents (e.g., *Nipah virus*, hantavirus)	

[a]Venezuelan equine (VEE), eastern equine (EEE), and western equine encephalomyelitis (WEE) viruses

beyond those identified for Category A agents. Biological agents that have undergone some development for widespread dissemination but do not otherwise meet the criteria for Category A, as well as several biological agents of concern for food and water safety, are included in this category.

Biological agents that are currently not believed to present a high bioterrorism risk to public health but which could emerge as future threats (as scientific understanding of these agents improves) were placed in Category C. These agents will be addressed nonspecifically through overall bioterrorism preparedness efforts to improve the detection of unexplained illnesses and ongoing public health infrastructure development for detecting and addressing emerging infectious diseases (13).

Agents were categorized based on the overall evaluation of the different

areas considered. Table 2 shows the evaluation schemes as applied to agents in Categories A and B. For example, smallpox would rank higher than brucellosis in the public health impact criterion because of its higher untreated mortality (approximately 30% for smallpox and ≤2% for brucellosis); smallpox has a higher dissemination potential because of its capability for person-to-person transmission. Smallpox also ranks higher for special public health preparedness needs, as additional vaccine must be manufactured and enhanced surveillance, educational, and diagnostic efforts must be undertaken. Inhalational anthrax and plague also have higher public health impact ratings than brucellosis because of their higher morbidity and mortality. Although mass production of *Vibrio cholera* (the biological cause of cholera) and *Shigella* spp. (the cause of

Table 2. Criteria and weighting[a] used to evaluate potential biological threat agents

Disease	Public health impact		Dissemination potential		Public perception	Special preparation	Category
	Disease	Death	P-D[b]	P - P[c]			
Smallpox	+	++	+	+++	+++	+++	A
Anthrax	++	+++	+++	0	+++	+++	A
Plague[d]	++	+++	++	++	++	+++	A
Botulism	++	+++	++	0	++	+++	A
Tularemia	++	++	++	0	+	+++	A
VHF[e]	++	+++	+	+	+++	++	A
VE[f]	++	+	+	0	++	++	B
Q Fever	+	+	++	0	+	++	B
Brucellosis	+	+	++	0	+	++	B
Glanders	++	+++	++	0	0	++	B
Melioidosis	+	+	++	0	0	++	B
Psittacosis	+	+	++	0	0	+	B
Ricin toxin	++	++	++	0	0	++	B
Typhus	+	+	++	0	0	+	B
Cholera[g]	+	+	++	+/-	+++	+	B
Shigellosis[g]	+	+	++	+	+	+	B

[a]Agents were ranked from highest threat (+++) to lowest (0).
[b]Potential for production and dissemination in quantities that would affect a large population, based on availability, BSL requirements, most effective route of infection, and environmental stability.
[c]Person-to-person transmissibility.
[d]Pneumonic plague.
[e]Viral hemorrhagic fevers due to Filoviruses (*Ebola, Marburg*) or Arenaviruses (e.g., *Lassa, Machupo*).
[f]Viral encephalitis.
[g]Examples of food- and waterborne diseases.

shigellosis) would be easier than the mass production of anthrax spores, the public health impact of widespread dissemination would be less because of the lower morbidity and mortality associated with these agents. Although the infectious doses of these bacteria are generally low, the total amount of bacteria that would be required and current water purification and food-processing methods would limit the effectiveness of intentional large-scale water or food contamination with these agents.

Discussion

Although use of conventional weapons such as explosives or firearms is still considered the most likely means by which terrorists could harm civilians (14), multiple recent reports cite an increasing risk and probability for the use of biological or chemical weapons (15-18). Indeed, the use of

biological and chemical agents as small- and large-scale weapons has been actively explored by many nations and terrorist groups (19-20). Although small-scale bioterrorism events may actually be more likely in light of the lesser degrees of complexity to be overcome, public health agencies must prepare for the still-possible large-scale incident that would undoubtedly lead to catastrophic public health consequences. The selection and prioritization of the potential biological terrorism agents described in this report were not based on the likelihood of their use, but on the probability that their use would result in an overwhelming adverse impact on public health.

Most evaluations of potential risk agents for biological warfare or terrorism have historically been based on military concerns and criteria for troop protection. However, several charac-

teristics of civilian populations differ from those of military populations, including a wider range of age groups and health conditions, so that lists of military biological threats cannot simply be adopted for civilian use. These differences and others may greatly increase the consequences of a biological attack on a civilian population. Civilians may also be more vulnerable to food- or waterborne terrorism, as was seen in the intentional *Salmonella* contamination of salad bars in The Dalles, Oregon, in 1984 (21). Although food and water systems in the United States are among the safest in the world, the occurrence of nationwide outbreaks due to unintentional food or water contamination demonstrates the ongoing need for vigilance in protecting food and water supplies (22-23). Overall, many other factors must be considered in defining and focusing multiagency efforts to protect civilian populations against bioterrorism.

Category A agents are being given the highest priority for preparedness. For Category B, public health preparedness efforts will focus on identified deficiencies, such as improving awareness and enhancing surveillance or laboratory diagnostic capabilities. Category C agents will be further assessed for their potential to threaten large populations as additional information becomes available on the epidemiology and pathogenicity of these agents. In addition, special epidemiologic and laboratory surge capacity will be maintained to assist in the investigation of naturally occurring outbreaks due to Category C "emerging" agents. Linkages established with established programs for food safety, emerging infections diseases, and unexplained illnesses will augment the overall bioterrorism preparedness efforts for many Category B and C agents.

The above categories of agents should not be considered definitive. The prioritization of biological agents for preparedness efforts should continue. Agents in each category may

change as new information is obtained or new assessment methods are established. Disease elimination and eradication efforts may result in new agents being added to the list as populations lose their natural or vaccine-induced immunity to these agents. Conversely, the priority status of certain agents may be reduced as the identified public health and medical deficiencies related to these agents are addressed (e.g., once adequate stores of smallpox vaccine and improved diagnostic capabilities are established, its rating within the special preparedness needs category would be reduced, as would its overall rating within the risk-matrix evaluation process). To meet the ever-changing response and preparedness challenges presented by bioterrorism, a standardized and reproducible evaluation process similar to the one outlined above will continue to be used to evaluate and prioritize currently identified biological critical agents, as well as new agents that may emerge as threats to civilian populations or national security.

Appendix

Risk-Matrix Analysis Process Used to Evaluate Potential Biological Threat Agents

In the area of public health impact, disease threat presented by an agent was assessed by evaluating whether the illness resulting from exposure could be treated without hospitalization. In addition, mortality rates for exposed, untreated persons were considered (24-26). Biological agents were given a higher rating for morbidity (++) if illness would most likely require hospitalization and a lower rating (+) if outpatient treatment might be possible for a large part of the affected population. Agents were also rated highest (+++) for expected untreated mortality $\geq$50%, medium (++) for mortality of 21% to 49%, and lowest (+) for an expected mortality $\leq$20%.

Agents were rated according to their overall potential for initial dissemination to a large population (+ to +++) and their potential for continued propagation by person-to-person transmission (0 to ++). Overall dissemination potential of an agent

was based on an assessment of 1) the capability for mass production of the agent (assessment based on availability of agent and Biosafety Level (BSL) requirements for quantity production of an agent), and 2) their potential for rapid, large-scale dissemination (assessment based on the most effective route of infection and the general environmental stability of the agent). Agents were rated (++) if they were readily obtainable from soil, animal/insect, or plant sources (most available; e.g., *B. anthracis*), (+) if mainly available only from clinical specimens, clinical laboratories, or regulated commercial culture suppliers (e.g., *Shigella* spp.), and (0) if available only from nonenvironmental, noncommercial, or nonclinical sources such as high-level security research laboratories (least readily available; e.g., *Variola* or *Ebola* viruses).

BSL requirements for an agent were based on recommended levels for working with large quantities of an agent (27). BSL ratings were used to estimate the level of technical expertise and containment facilities that would be required to work with and mass produce an agent safely. Agents that required higher BSL levels were given lower ratings, as they would require greater technical capabilities and containment facilities to be produced in large quantities. Agents were given (+) for BSL 4 production safety requirements, (++) for BSL 3 requirements, and (+++) for BSL 2 or lower requirements.

Agents were also assessed with regard to their main routes of infection, with the assumption that those causing infection via the respiratory route could be more readily disseminated to affect large populations. Agents were assigned (++) if most effective at causing illness via an aerosol exposure route (air release potential) and (+) if most effective when given by the oral route (food/water release potential). Dissemination potential should also take into account the stability of an agent following its release. Information regarding the expected general environmental stability of agents was obtained from multiple sources (24,28-31). Agents that may remain viable in the environment for $\geq$1 year were given (+++), while agents considered less environmentally stable were given (++) (potentially viable for days to months) or (+) (generally viable for minutes to hours). The ratings system for environmental stability was assigned to reflect

the wide range of stability of the agents, while maintaining a simple overall scheme that contained only a few categories (minutes to hours, days to months, >1 year). The ratings for all the subcategories evaluated for production and dissemination potential were then totaled and agents were assigned a final rating for production and dissemination capability. If the total rating in the subcategories was $\geq$9, the agent was given (+++); for a total of 7-8, the agent was given a (++); and for a total of $\leq$6, the agent was given a final rating of (+) for the overall production and dissemination capability.

As potential outbreak propagation through continued person-to-person transmission would also increase the overall dissemination capabilities of an agent, they were evaluated separately for this characteristic. Agents were rated highest if they had potential for both person-to-person respiratory and contact spread (+++) and lower for mainly respiratory (++) or contact spread potential alone (+). Agents were rated (0) if they presented low or no transmission risk.

Agents were also assessed (0 to +++) according to preexisting heightened public awareness and interest, which may contribute to mass public fear or panic in biological terrorism events. The number of times an agent or disease appeared in a selected form of media was used as a surrogate to determine the current level of public awareness and interest for the agent or disease. Titles of newspaper articles and radio and television transcripts from June 1, 1998, to June 1, 1999, in an Internet database (32) were retrospectively searched by agent name and disease. This database contained articles and transcripts from approximately 233 newspapers and 70 radio or television sources. If a disease was caused by multiple agents (e.g., viral hemorrhagic fever), the database was searched for each of the agents in addition to the name of the disease. Articles or transcripts were only counted if the name of the agent, disease, or other general terms such as bioterrorism, biological terrorism, terrorism, and weapons of mass destruction appeared in the title. Multiple hits for the same title were counted only once unless they appeared in different newspapers or transcripts. Agents were rated based on the number of times they appeared in these forms of media within the 1-year period. Agents were given (0)

rating for <5 titles, (+) for 5-20 titles, (++) for 21-45 titles, and (+++) for >45 titles identified within the search period.

Requirements for special public health preparedness were also considered. Higher ratings were given to agents with different requirements for special preparedness. An agent was given a (+) for each special preparedness activity that would be required to enhance the public health response to that agent. These distinct preparedness requirements included 1) stockpiling of therapeutics to assure treatment of large numbers of people (+), 2) need for enhanced public health surveillance and education (+), and 3) augmentation of rapid laboratory diagnostic capabilities (+). Therefore, if all three special preparedness efforts would be required to provide a strong public health response for that agent, it was given (+++) for this category. Agents that did not require all special preparedness efforts were given lower ratings (++ or +).

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About the Cover

Experimentation du virus charbonneux: "Le Pelerin," 1922. Homage à Louis Pasteur. Dessin de Damblans.

In 1881, Louis Pasteur announced his theory that vaccinating livestock, in effect giving animals a disease, would protect them from that disease. Many contemporaries of Pasteur were skeptical about this radical idea as they were skeptical about the germ theory of disease. One of these contemporaries, the famous veterinarian Monsieur H. Rossignol, challenged Pasteur to test his theory in public by vaccinating animals on his farm at Pouilly-Le-Fort, a small village outside Paris.

Rossignol's challenge came early in Pasteur's efforts to prove the germ theory of disease and develop vaccines. No vaccine had been tested yet outside the laboratory. The risk of having something go wrong with the experiment was very high. Pasteur, however, took the challenge, confident that what had worked with 14 sheep in the laboratory would work with 50 in the field.

In early May, 25 animals at Rossignol's farm were inoculated for "charbon," a disease now known as anthrax. Another 25 received no vaccine. On May 31, all 50 animals were injected with a culture of very virulent anthrax. Within 2 days, a group of farmers, veterinarians, pharmacists, and agriculture officials gathered at Rossignol's farm to observe the results of the experiment. The results were as Pasteur had anticipated: all 25 sheep that had not been inoculated had died; all 25 inoculated sheep were in perfect health. Those gathered at Pouilly-Le-Fort that day witnessed the first successful vaccine and the introduction of effective protection against anthrax.

Anthrax, an ancient disease reported by Homer and Hippocrates as extremely deadly, killed thousands of animals each year. Pasteur's vaccine provided not only protection against anthrax but also proof for the germ theory of disease. Protection against anthrax opened the door for vaccines against smallpox and other diseases. From Pasteur's work emerged the disciplines of immunology and bacteriology, which eventually led to vaccination of millions of people and prevention of many diseases.

Abstracted from Hero for Our Time by Paul Trachtman, Smithsonian, January 2002.

In the next issue of

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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.

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