EMERGING INFECTIOUS DISEASES

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News and Notes

Porcine Reproductive and Respiratory Syndrome Virus: Origin Hypothesis

Peter G.W. Plagemann*

Porcine reproductive and respiratory syndrome is a serious swine disease that appeared suddenly in the midwestern United States and central Europe approximately 14 years ago; the disease has now spread worldwide. In North America and Europe, the syndrome is caused by two genotypes of porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus whose genomes diverge by approximately 40%. My hypothesis, which explains the origin and evolution of the two distinct PRRSV genotypes, is that a mutant of a closely related arterivirus of mice (lactate dehydrogenase-elevating virus) infected wild boars in central Europe. These wild boars functioned as intermediate hosts and spread the virus to North Carolina in imported, infected European wild boars in 1912; the virus then evolved independently on the two continents in the prevalent wild hog populations for approximately 70 years until independently entering the domestic pig population.

Several human and animal virus diseases, generally caused by RNA viruses, have emerged in the last 40 years (1,2). Some of these diseases are caused by preexisting viruses that have the capacity to infect alternate hosts under certain conditions (e.g., Ebola virus, hantavirus, and Nipah virus). Other diseases are caused by viruses that seem to have adapted to new hosts after accidental transspecies transmission. In addition to AIDS, which is caused by HIV, porcine reproductive and respiratory syndrome (PRRS) is a prime example of the latter class of diseases. PRRS, which affects domestic pigs, was first recognized approximately 14 years ago in North America (3) and in central Europe (4); this disease is now found worldwide and causes considerable economic losses in the swine industry (5). Initially, the disease was referred to as "mystery swine disease" until its cause was determined to be a positive-stranded RNA virus, designated porcine reproductive and respiratory syndrome virus (PRRSV), that together with murine lactate dehydrogenase-elevating virus (LDV), equine arteritis virus, and simian hemorrhagic fever virus, belongs to the family *Arteriviridae (*6). However, the origin of PRRSV is still a mystery, especially since the European and North American PRRSV isolates cause similar clinical symptoms but represent two distinct viral genotypes whose genomes diverge by approximately 40% (7). The European and North American PRRSV prototypes are Lelystad virus (4) and VR-2332 (3), respectively. Retrospective serologic tests did not detect antibodies (Abs) to PRRSV in domestic pigs in Iowa and in Germany before the mid-1980s (8,9). The first seropositive pigs were discovered in herds in Iowa in 1985, Minnesota in 1986, and the former East Germany in 1988–1989.

Researchers have postulated that LDV and PRRSV, which are closely related, are derived from a common ancestor (10,11). I suggest that PRRSV is derived from LDV and that wild boars have functioned as intermediate hosts, based on the following observations.

The primary structural proteins of arteriviruses are the nucleocapsid (N) protein, the integral membrane/matrix (M) protein, and the primary envelope glycoprotein, GP5 (10–12). Both the M protein and GP5 of LDV and PRRSV seem to be triple membrane–spanning proteins whose short ectodomains of approximately 11 and 30 amino acids, respectively, are disulfide linked (11,13,14). The ectodomain heterodimer seems critical for the infection of macrophages, the primary host cell of all arteriviruses, perhaps playing a role in receptor interaction (14), but neither the GP5 ectodomain nor the M protein ectodomain appears to determine host cell tropism (15,16).

LDV was first isolated from tumor-bearing laboratory mice but later found to be endogenous in wild house mouse populations (*Mus musculus domesticu*s; 10,17). LDV invariably causes a lifelong asymptomatic infection in mice that is recognized only by an elevation of plasma lactate dehydrogenase activity. The virus replicates cytocidally in a subpopulation of permissive tissue macrophages that clears excess lactate dehydrogenase from circulation. Persistent infection is maintained by replication in newly regenerated permissive macrophages and the escape from all host defenses. The single neutralization epitope located in the middle of GP5 ectodomain (Figure 1) is flanked in the common LDV isolates, represented by LDV-P (10), by

*University of Minnesota, Minneapolis, Minnesota, USA

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                  **      ***** * *** *
LDV    29E NACAAGNSST KNLIYNLTLC ELNVTGFQQ HF....

VR     29L ANASNDSSSH LQLIYNLTLC ELNGTDWLA NK....

LV     30S FADGNGDSST YQYIYNLTIC ELNGTDWLS SH....
```

Figure 1. Amino acid comparison of the GP5 ectodomains of lactate dehydrogenase-elevating virus–P, porcine reproductive and respiratory syndrome virus VR-2332, and porcine respiratory and reproductive syndrome virus Lelystad virus (GenBank accession nos. U15146, U87392, and M96262, respectively). *Indicates amino identity. The neutralization epitope is underlined. N-glycosylation sites are in boldface letters.

two N-glycans that impair the immunogenicity of the epitope and render the viruses completely resistant to in vivo Ab neutralization (18,19). LDV, which is poorly transmitted between mice, is transmitted by biting and perhaps sexually but not via the respiratory route; oral transmission requires high amounts of virus (10,20). All LDVs isolated from tumor-bearing laboratory mice and wild house mice are genetically closely related to LDV-P. Nucleotide differences are largely found in the segments encoding the signal peptides of the glycoproteins or represent mostly translational silent substitutions (21,22), which indicates that LDV-P has attained close to evolutionary stasis (23).

In contrast to LDV, individual field isolates of both the European and North American PRRSVs exhibit great genome variability (e.g., phylogenetic analysis of open reading frame [ORF] 5, Figure 2). However, all PRSSV isolates are closely related to LDV, which is clearly indicated by amino acid comparisons of individual viral proteins. For example, the GP5 ectodomains of LDV, PRRSV VR-2332, and PRRSV Lelystad virus are collinear and contain a segment with approximately 70% amino acid identity (Figure 1). This segment contains the primary neutralization epitopes of LDV and of PRRSV (24,25), two highly conserved N-glycosylation sites and the Cys residue between them that is postulated to disulfide link the GP5 ectodomain to that of the M protein. The branching of the LDV sequence in the phylogenetic tree (Figure 2) in the line connecting the European and North American PRRSVs indicates that LDV is approximately equally related to both. This relationship is also indicated by the finding that some amino acids in all viral proteins are identical for LDV and VR-2332 but not Lelystad virus; vice versa, some amino acids are identical for LDV and Lelystad virus proteins only. For example, in the highly conserved segment of the GP5 ectodomain, two amino acids are identical in LDV and VR-2332 and one is identical in LDV and Lelystad virus (Figure 3; see also the discussion of the N-protein, and ORF1b protein).

The genetic stability of LDV (which contrasts with the high variability of the PRRSV genomes typical for a new virus) and the relationship between the genome of LDV and those of the two PRRSV genotypes (Figure 2) both suggest that PRRSV has been derived from LDV. My hypothesis is that LDV was transmitted from an infected wild house mouse to a Eurasian wild boar (*Sus scrofa*) in central Europe sometime during the 19th century. At that time, wild boars were common throughout Europe, North Africa, and Asia (26). Such transmission would be rare because of species differences and the low transmissibility of LDV. The transmission may have occurred through oral means or wounds and involved a mutant form of LDV able to infect wild boars. The initial replication of this LDV mutant in and transmission between wild boars was likely slow until better host-adapted mutants were selected. The replication of the initially infecting virus might have been limited to tissue macrophages, as in mice. Where the initial virus transmission from wild house mice to wild boars occurred cannot be deduced from genome sequence comparisons. The ORF5 phylogenetic tree (Figure 2) suggests that the transmission could have occurred in Lithuania because the ORF5s of the Lithuanian isolates are more closely related to LDV than those of other European PRRSV isolates, but this relationship is not apparent in a phylogenetic analysis of ORF7 that encodes the N-protein. The initial infection of wild boars likely occurred in the eastern part of Germany (Sachsen-Anhalt), where the first PRRSV-seropositive pigs were discovered in Europe in 1988 and 1989 (9) and infected boars detected in this region in 1991 and 1992. From there the virus may have spread to the United States. Eurasian wild boars were introduced into the United States several times, but the primary introduction that became established occurred in 1912 when 14 wild boars (11 females and 3 boars) were released in a game preserve in the southwestern part of

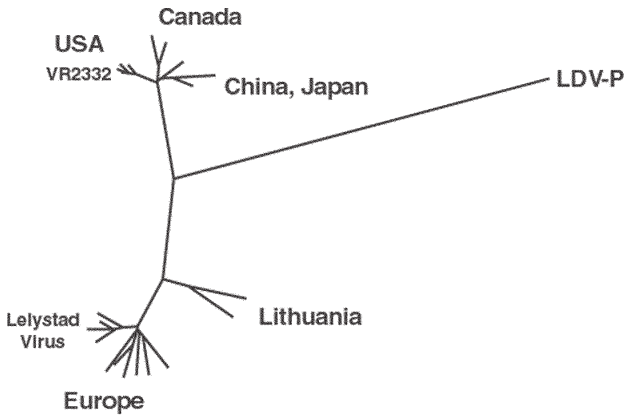

Figure 2. Phylogenetic tree of 432 nucleotide-long open reading frame (ORF) 5 segments of European and North American porcine reproductive and respiratory syndrome viruses and lactate dehydrogenase-elevating virus–P. The sequences correspond to nucleotide 97-526 of ORF5 of the European porcine reproductive and respiratory syndrome virus isolates (provided by T. Stadejek). LDV, lactate dehydrogenase-elevating virus.

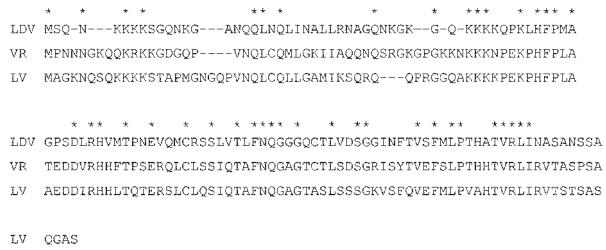

Figure 3. Amino acid alignment of the N-proteins of lactate dehydrogenase-elevating virus–P, porcine reproductive and respiratory syndrome virus VR2332, and porcine respiratory and reproductive syndrome virus Lelystad virus (115, 123, and 128 amino acids long, respectively). *Indicates identical amino acids

North Carolina in Hooper Bald (26). The origin of these wild boars is not entirely certain, but they probably came from the Harz Mountains in Sachsen-Anhalt. I postulate that one or more of these wild boars was infected with a PRRSV precursor virus and thus spread the virus to the United States. In 1912, feral pigs were widely prevalent throughout the United States (26). They were derived from domestic pigs that had escaped from farms, were intentionally released, or were free ranging. Soon after their importation, some wild boars escaped from the enclosed park; hybridization occurred between them and the feral pigs indigenous to the area (26). Most wild hogs now found in many U.S. states appear to be descendants of these hybrids. Wild boars from the North Carolina game farm were introduced into California in 1924, where they became well established. Wild boars were also released in Texas from 1930 to 1933 and became established there; these boars came from the San Antonio Zoo, but their original source is unknown (26).

After the introduction of PRRSV-infected wild boars in the United States in 1912, the virus would be expected to have evolved independently for 70 years in Europe and North America with the selection of mutants with better growth potential in the different wild hog populations on the two continents. Such independent evolution would explain the two distinct genotypes which, however, remain equally, but differently, related to LDV (Figure 2). Such selection of host environment–adapted mutants in this group of viruses is indicated by the rapid development of geographic clades of both the European and North American genotypes of PRRSV (27,28) (Figure 2). Such evolution must also have eventually involved the selection of mutants that efficiently replicate in alveolar macrophages, which is a property of PRRSV. This selection not only increased virulence of the viruses but also improved their replication potential in their hosts and transmission between the latter via the respiratory route.

Eventually, PRRSV variants infected pigs being raised domestically. Direct contact between wild hogs and domestic pigs in outdoor farms is not uncommon, and wild hogs have been intentionally introduced into existing swine herds. However, transmission could also have been mediated by products from hunted or slaughtered infected wild hogs. When and where this transmission might have occurred are unclear. Retrospective analyses found that approximately 1,400 serum samples collected in Iowa in 1980 lacked anti-PRRSV Abs. The first seropositive samples were detected in Iowa in 1985, and the earliest clinical symptoms were observed in herds in North Carolina, Iowa, and Minnesota (29). Thus, the first transmission from wild hogs to domestic pigs in the United States likely occurred in North Carolina, where wild hogs are numerous; the virus further evolved in the domestic pig population there and then spread to various midwestern states. By 1990, PRRSV had spread to 19 western, midwestern, and eastern states (29). In Europe, the first pigs with anti-PRRSV Abs were detected in East German herds in 1988 and 1989 (9) shortly before clinical symptoms of the disease were reported in herds in the central part of western Germany (30). A molecular clock of ORF3 suggests that the common ancestor of the European type of PRRSV infected domestic pigs around 1979 (31). This conclusion is not inconsistent with the relationship between the PRRSV strains in Europe and North America (Figure 2). The infection of the domestic pig populations on both continents may have occurred when the density of the wild hog and domestic swine populations increased considerably, which may have facilitated contact between them. Once virulent variants developed in the domestic pig populations, the spread of PRRSV has been extremely rapid on both continents as well as to other continents. Thus, pinpointing the location of the initial infection of domestic pigs is difficult.

Wild hogs with anti-PRRSV Abs have now been detected in both Europe and the United States. Two serum samples from 482 wild boars shot in 1991 and 1992 in Sachsen-Anhalt were found to have anti-PRRSV Abs by using the indirect immunoperoxidase monolayer assay. The PRRSV-positive boars were shot close to the former border to West Germany (32). Similarly, in northern France, 25 of 303 farmed wild boars tested in 1993 and 1994 and 8 of 603 shot in the same period were found to be seropositive by using the same serologic test or an indirect enzyme-linked immunosorbent assay (ELISA) (33), and 2 of 117 feral hogs tested in Oklahoma were seropositive in a commercial ELISA and a fluorescent Ab-staining test (34). However, no Abs were detected in 24 feral hogs killed in 1993–1994 in the Fort Riley Army Base in Kansas (35), in 44 wild boars shot in 1999 in Croatia (36), in 78 wild boars shot in 1999–2000 in Spain (37), or in >1,000 serum samples collected from wild boars in Eastern Europe (Stadejek, pers. comm.). The assumption is that

seropositive wild hogs became infected by contact with infected domestic pigs (33), but no evidence exists to rule out that this infection may be an endogenous infection of wild boars that served as recent reservoir for the infection of domestic pigs. Regardless, the finding of PRRSV-seropositive wild hogs in both Europe and the United States indicates that wild hogs are susceptible to PRRSV infection. Wild hogs can also carry other viruses, such as those causing classical swine fever, Aujeszky's disease, and pseudorabies; these viruses are considered an important potential source of infection of domestic pigs, especially in the case of classical swine fever.

In any case, the serologic tests available for the assay of anti-PRRSV Abs are likely inefficient or unsuitable for the detection of Abs to LDV-PRRSV intermediates that may be prevalent in wild hog populations. The tests are designed to detect anti-N-protein Abs, which are the primary Abs generated in PRRSV infected domestic pigs (11). Although the N-protein is a relatively conserved arterivirus protein, considerable amino acid differences exist between European and North American PRRSVs. For example, the N-proteins of Lelystad virus and VR-2332 exhibit only 60% amino acid identity (Figure 3). Several linear epitopes have been identified in the N-proteins of Lelystad virus and North American PRRSV, but they differ for the two PRRSVs and little serologic cross reaction occurs between them (11). Furthermore, these epitopes were identified by reaction with N-protein specific mouse monoclonal Abs (mAbs); only limited information is available on the immunogenicity of these epitopes in pigs. In addition, a common conformational epitope has been identified in the center of the N-protein (AA51-69), which exhibits 84% amino acid identity between Lelystad virus and VR-2332 (Figure 3). The corresponding segment of the LDV N-protein, however, exhibits much lower amino acid identity (42%) (Figure 3). Overall amino acid identity of the N-proteins of LDV, Lelystad virus, and VR-2332 is 36% (Figure 3); 15 additional amino acids are identical in LDV and VR-2332 and 10 amino acids in LDV and Lelystad virus, again indicating the close, but distinct, relationship of LDV with both PRRSV genotypes. The LDV/VR-2332 and LDV/Lelystad virus identical amino acids are primarily located in the C-terminal and N-terminal halves of the N-proteins, respectively. These amino acid differences between the LDV and PRRSV proteins may explain a lack of serologic cross-reaction between them; the same may be true for LDV-PRRSV intermediates. In addition, mice infected with LDV or immunized with inactivated virions do not generate Abs to the N-protein (38,39), apparently because the viral envelope or its proteins interfere with the immunogenicity of the N-protein since a mAb to the N-protein was generated by immunizing mice with isolated nucleocapsids (40). The primary Ab response of mice to LDV is to nonneutralization epitopes of GP5, which contrasts with the primary anti-N–protein Ab response of pigs or mice to PRRSV. These differences in Ab responses of pigs and mice may also apply to LDV-PRRSV intermediates and make it unlikely that their infection can be recognized by the available serologic tests for either LDV or PRRSV Abs. Furthermore, nothing is known about the prevalence of potential LDV-PRRSV intermediates in the wild hog population, the pathogenesis and course of infection of such viruses (or of PRRSV) in wild hogs, or the antiviral immune response of wild hogs.

An approach that is more suitable to detect LDV-PRRSV intermediates is reverse transcription–polymerase chain reaction (RT-PCR) with primers to highly conserved genomic sequences, for example, in a segment of the RNA polymerase domain upstream of the nidovirus characteristic SDD protein sequence (Figure 4). This nucleotide segment exhibits 53% identity between LDV, Lelystad virus, and VR-2332 (118/224 nucleotides; amino acid identity of the encoded protein segment is even higher, 75%). This segment contains short segments with complete nucleotide identity (Figure 4, stars); in addition, 25, 30, and 31 nucleotides are identical for LDV and VR-2332, LDV and Lelystad virus, and Lelystad virus and VR-2332, respectively, indicating again the close, but distinct, relationship between LDV and both Lelystad virus and VR-2332. My laboratory has previously used degenerate primer sets of this region designed on the bases of LDV, equine arteritis virus, and Lelystad virus sequence information (Figure 4) that detected not only the genomes of these three viruses but also those of VR-2332 and simian hemorrhagic fever virus, for which no sequence information was available at the time (41). The additional advantages of the RT-PCR approach are that this method can be readily applied to

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          ** ** ** **  * ** ** ** ** **     ** ** ** *** *  * ******
LDV   CTGGAGGCTGACCTAGCCTCATGTGACCGGTCAACCCCGGCGATTATCAGGTGGTTTACA
VR    CTTGAAGCTGATCTCGCATCCTGCGATCGATCCACGCCTGCAATTGTCCGCTGGTTTGCC
LV    CTTGAGGCGACTTGGCCTCCTGTGACCGCAGCACCCCCGCCATTGTAAGATGGTTTGTT

           * ** ** ** * *** * **  *   ******     * **    ** *   * **
LDV   ACAAATCTCCTATTTGAATTGGCTGGTGCTGAAGAATGGATTCCTTCCTACGTTTTGAAT
VR    GCCAACCTTCTTTATGAACTTGCCTGTGCTGAAGAGCATCTACCGTCGTACGTGCTGAAC
LV    GCCAACCTCCTGTATGAACTTGCAGGATGTGAAGAGTACTTGCCTAGCTATGCTCTTAAT

          ** ***** *** *  *    **   *   **      *    **  * ** **  **** *
LDV   TGTTGCCATGACGTGGTCAGCACCATGAGTGGCTGCTTTGATAAGCGGGGAGGTTTGTCAT
VR    TGCTGCCACGACTTACTGGTCACGCAGTCCGGCGCAGTGACTAAGAGAGGTGGCCTGTCGT
LV    TGCTGCCATGACCTCGTGGCAACACAGGATGGTGCCTTCACAAAACGCGGTGGCCTGTCGT

          * ** ** **  * ***    *    *****  * ***    *
LDV   CAGGAGATCCTGTTACCAGCATCAGCAACACAGTTTATAGTTTA
VR    CTGGCGACCCCATCACCTCTGTGTCTAACACCATTTATACTTTG
LV    CCGGGGACCCCGTCACCATGGTGTCCAACACCGTATATTCACTG
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Figure 4. Nucleotide alignment of a segment of open reading frame (ORF) 1b of lactate dehydrogenase-elevating virus–P, porcine reproductive and respiratory syndrome virus VR-2332, and porcine reproductive and respiratory syndrome virus–Lelystad virus beginning at nucleotides 1169, 1165, and 1165, respectively. *Indicates identical nucleotides. Degenerate primer sets for polymerase chain reaction were previously made to the underlined segments (41).

both serum and tissue samples and that the sequence of the PCR-amplified segment allows conclusions about the relatedness of the detected virus to existing viruses and the synthesis of specific gene probes for this virus. Also, degenerate primer sets can be designed to detect specific LDV-PRRSV intermediates. The same approach can be used to examine other species for the presence of arteriviruses.

My hypothesis on the origin of PRRSV encompasses all known facts but will be difficult, if not impossible, to prove, largely because of a lack of suitable materials for experimental investigation. My explanation of the theory serves to elicit interest in this subject and to encourage collaboration between investigators, especially in the search for stored or new materials that can be used to test the hypothesis.

Acknowledgments

I thank T. Stadejek and V. Kaden for personal communications and T. Stadejek for providing the phylogenetic tree of lactate dehydrogenase-elevating virus and porcine reproductive and respiratory syndrome virus isolates.

Dr. Plagemann is professor of microbiology at the University of Minnesota. He has studied arteriviruses for over 40 years, especially the molecular biology, pathogenesis, and immunology of lactate dehydrogenase-elevating virus. He has written several reviews on the subject. Now partially retired, he is continuing research on the immune response of pigs to porcine reproductive and respiratory syndrome virus.

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Address for correspondence: Peter G.W. Plagemann, Department of Microbiology, Medical School, University of Minnesota, 420 Delaware St. S.E., MMC 196, Minneapolis, MN, 55455, USA; fax: 612-626-0623; email: plage001@umn.edu

Detecting Bioterror Attacks by Screening Blood Donors: A Best-Case Analysis

Edward H. Kaplan,*Christopher A. Patton,† William P. FitzGerald,† and Lawrence M. Wein‡

To assess whether screening blood donors could provide early warning of a bioterror attack, we combined stochastic models of blood donation and the workings of blood tests with an epidemic model to derive the probability distribution of the time to detect an attack under assumptions favorable to blood donor screening. Comparing the attack detection delay to the incubation times of the most feared bioterror agents shows that even under such optimistic conditions, victims of a bioterror attack would likely exhibit symptoms before the attack was detected through blood donor screening. For example, an attack infecting 100 persons with a noncontagious agent such as *Bacillus anthracis* would only have a 26% chance of being detected within 25 days; yet, at an assumed additional charge of $10 per test, donor screening would cost $139 million per year. Furthermore, even if screening tests were 99.99% specific, 1,390 false-positive results would occur each year. Therefore, screening blood donors for bioterror agents should not be used to detect a bioterror attack.

The health and economic consequences of an extensive bioterror attack could be severe (1–5); thus, early detection of an otherwise silent bioterror attack is of obvious importance (6). Ongoing developments in rapid testing for potential bioterror agents (7–10) led us to consider whether screening blood donors to detect a bioterror attack with the most feared bioterror agents (11) could prove useful. The rationale for screening blood donors is twofold. First, blood donors are numerous, and donations are uniformly spread over time and throughout the population. In the United States, approximately 13.9 million blood donations are made each year (12); thus, the annual number of donations roughly equals 5% of the 286 million population. Second, in the absence of specific information regarding how such an attack might target the population, we can assume that blood donors are as likely to be infected in a bioterror attack as nondonors. In a sizeable attack, infected donors might donate blood before their infections have been detected medically. Screening donated blood for bioterror agents could therefore serve to detect an attack sooner than would otherwise be possible.

However, the cost of screening donations is proportional to the number of donations tested, in addition to the resources expended investigating false alarms. To investigate these issues, we developed a model for bioterror attack detection under assumptions favorable to donor screening, for if such best-case assumptions fail to justify screening donors, more realistic assumptions will also. In particular, we initially assume that the screening test used is perfectly specific, which removes the possibility of false alarms, and compare the time required to detect an attack through donor screening to the incubation periods for various bioterror agents to see whether donor screening leads to more rapid detection than simply observing symptomatic cases. We then consider tests with imperfect specificity, examine the false-alarm rate that would result from donor screening, and compare this rate to the true-positive rate for blood donations.

Methods

Though blood tests with the ability to detect agents such as smallpox virus or *Francisella tularensis* within days after infection do not exist at present, research to develop such sensitive tests is under way (7–10). To analyze whether screening donors might meaningfully shorten the time required to detect an attack were such tests available, we developed a probabilistic model that joins the workings of a screening test, blood donation, and epidemic spread under assumptions that deliberately favor attack detection through donor screening (see Appendix). In the model, the sensitivity of a screening test is determined by a (random) window period W with mean ω days that must transpire before a person infected at time 0 can be detected as infected. Test sensitivity thus depends on the time from infection until testing. Though the model can accommodate any probability distribution desired, we take W to follow an exponential distribution in our examples, an assumption that favors early detection (since the exponential likelihood is maximized at W=0, that is, no detection delay, and declines as W increases). We assume initially

*Yale School of Management and Yale Medical School, New Haven, Connecticut, USA, †American Red Cross, Arlington, Virginia, USA; and ‡Stanford University, Stanford, California, USA

that the screening test is perfectly specific, though we will relax this assumption later.

A bioterror attack at time 0 infects $I(0)=Np$ persons in a population of size N (where p is the fraction of the population initially infected). We assume that everyone in the population has the same probability p of infection due to the attack, that is, the attack does not target the population in a manner that would make blood donors more or less likely to be infected than nondonors. Given that the total number of blood donations over time results from the independent actions of individual blood donors, the aggregate number of blood donations over time was modeled as a Poisson process (13) with rate $\lambda=kN$, where k is the mean number of blood donations per person per unit of time. If the agent used in the attack is contagious, secondary infections spread according to an epidemic model, governed by a reproductive number R_0 (number of secondary infections per initial index case) and an exponentially distributed duration of infectiousness with mean r^{-1}. To favor donor screening, we deliberately exclude an explicit latent period (during which an infected person is not infectious). These assumptions imply that infections in the population will grow exponentially with rate $(R_0 - 1)r$ postattack (14), an assumption that further favors donor screening as the number of blood donors who are infected (and by ignoring latent periods, infectious) will grow exponentially at the same rate, leading to earlier detection via donor screening than would occur otherwise.

We assume that the attack is detected once a single infected donation tests positive for infection with a bioterror agent, another assumption favorable to donor screening, which enables us to derive the probability distribution of the time required to detect a bioterror attack of a given magnitude. However, to demonstrate the extent to which we have "stacked the deck" in favor of blood donor screening, we relax the assumption of perfect test specificity for noncontagious agents. We assume fixed attack rates and disaster response and recovery periods, which together determine the fraction of time during which infected donations can occur. This assumption allows us to model the rate of false alarms per unit of time and compare this to the rate of true-positive alarms.

Results

For initial attacks ranging from 100 to 1,000 infections, Figure 1 shows the probability distribution of the attack detection delay for a noncontagious agent that would result from using a blood-screening test able to detect infections an average of $\omega = 3$ days after infection (an optimistic assumption, given that such tests do not exist at present), assuming that blood donations arrive at rate $k = 0.05$ per person per year, the average rate for blood donation in the United States (12). The results are not encouraging: for an attack that infects 100 persons, the chance of detecting the attack through blood donor screening within 25 days is 26%; even for a large attack that infects 1,000 persons, the median time to detect the attack is 8 days. Figure 2 (solid curve) shows the mean delay in attack detection as a function of the initial attack size for a noncontagious agent. For an initial attack that affects 1,000 persons, the mean time to detection is 10 days, while for an attack that affects 100 persons, the mean time to detection is 76 days. In most infected persons, symptoms would develop during this period, leading to earlier detection of an attack than blood donor screening would allow, even when potential delay from misdiagnosis or failure to recognize symptoms is accounted for (Table 1; compare to incubation times from infection through symptoms for *Bacillus anthracis* and *Clostridium botulinum*, two noncontagious agents). That we have deliberately made assumptions favorable to blood donor screening strengthens this finding, for the actual time required to detect an attack by means of donor screening would be longer than reported above.

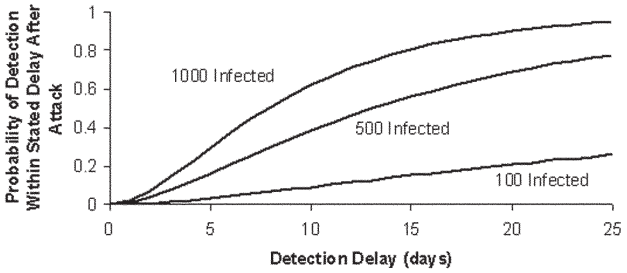

Figure 1. Probability distribution of attack detection delay for a noncontagious agent. Blood donations occur at rate k=0.05 per person per year, the screening test has a mean window period of ω=3 days, and initial attack sizes range from 100 through 1,000 infections.

If we also assume that ω=3 days and k=0.05 per person per year, Figure 3 shows the distribution of delays in attack detection that would result from a contagious agent characterized by R_0=3 and r^{-1}=14 days (parameters suggestive

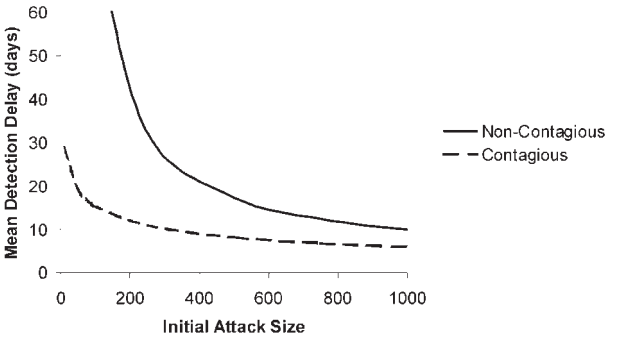

Figure 2. Mean attack detection delays for noncontagious (solid) and contagious (dashed) agents as a function of the initial attack size. Other parameters set as in Figures 1 and 3.

Table 1. Incubation periods from infection through symptoms for Centers for Disease Control category A agents

Agent	Incubation time (days)
Bacillus anthracis	<7
Clostridium botulinum	0.5–1.5
Yersinia pestis	1–6
Smallpox virus	7–17
Francisella tularensis	3–5
Hemorrhagic fever viruses	2–21 (Ebola); 5–10 (Marburg)

Source: (11).

of smallpox [3,11,15] and perhaps Ebola virus [11]). Because additional infections are transmitted to susceptible persons, the probability of detecting an attack within any given period is greater than for a noncontagious agent. Consequently, for a given initial attack size, the attack detection delay distribution is shorter for a contagious agent, as is clear from Figure 3. However, symptoms would develop in many infected persons, and such infections would be recognized before blood donor screening would uncover an attack. Under our best-case assumptions, an attack that initially infects 100 persons would still require 15 days on average before donor screening would detect the attack, while an initial attack infecting 1,000 persons would require 6 days until detection on average (Figure 2).

Treating the range of incubation times from infection through symptoms (Table 1) as 99% probability intervals from agent-specific lognormal distributions, in the case of smallpox one would expect to see five symptomatic cases after 7 days, while more than half of those initially infected with Ebola virus would progress to symptoms within 1 week. The incubation times for plague and tularemia are much shorter (Table 1), but even after increasing r to compensate for this in our model many of those infected would exhibit symptoms before the bioterror event was detected through tests of the blood supply (results not shown). Again, considering that we have made assumptions that favor donor screening—that the test has an exponentially distributed window period that detects infection after 3 days on average, that donor screening detects the attack after the first donor tests positive, that there is no latent period from infection through infectiousness, and that a postattack epidemic grows exponentially—donor screening as a method of attack detection does not seem competitive with simple observation of symptomatic case-patients.

Until now, we have assumed that screening occurs with perfect specificity, which eliminates false-positive results as a consequence. However, if false-positive test results can occur, they will occur frequently. Table 2 reports the false-alarm rates that would occur for tests of different specificities for a noncontagious agent, if one assumes that all 13.9 million annual blood donations are tested, that on average one bioterror attack takes place per year (a rate all would agree is unrealistically high), that on average 1 month is required to respond to and recover from an attack (so infected donations can occur for up to 1 month after an attack), and that each attack infects 1,000 persons. Even with 99.99% specificity, an average of 1,390 false-positive results would occur per year; at 99% specificity, the average would be 139,000 false-positive results per year.

In addition to the resources wasted in investigating so many false alarms, a "crying wolf" mindset could diminish the attention paid to all screening test results, increasing the chance of missing a true-positive test result. That this latter possibility could well occur seems clear because with the attack rate and duration of response and recovery assumed above, one would expect only 3.7 donations with true-positive results each year (again, presuming an exponentially distributed window period with mean ω= 3 days). Also, though lowering the attack rate below one per year to more realistic levels would have no effect on the false-positive rate, the number of donations with true-positive results would fall. Similarly, reducing the duration of the postattack response and recovery during which infected donations can still occur would have essentially no impact on the false-positive rate, while again lowering the number of donations with true-positive results.

Conclusion

We have argued that even under assumptions deliberately favorable to blood donor screening, an attack was unlikely to be detected earlier through donor screening than from observing symptomatic case-patients. We have also shown that imperfect test specificity could overwhelm the blood collection system with false-positive results. In addition, the costs of screening apply to all blood donations tested: even if the cost of screening were as low as an incremental $10 per test, screening all blood donations in the United States to detect a bioterror attack would cost an additional $139 million per year at current donation rates.

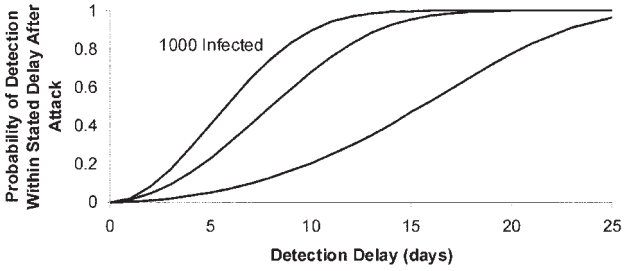

Figure 3. Probability distribution of attack detection delay for a contagious agent. Blood donations occur at rate k=0.05 per person per year, the screening test has a mean window period of ω=3 days, the reproductive number R_0=3, the mean duration of infectiousness r^{-1}=14 days, and initial attack sizes range from 100 through 1,000 infections.

Table 2. False-alarm rates with test specificities as shown

Specificity (*s*)	Annual false-alarm rate (FAR)
0.9	1,390,000
0.99	139,000
0.999	13,900
0.9999	1,390

[a]If one assumes 13.9 million annual blood donations tested, an average of one bioterror attack per year that infects 1,000 persons with a noncontagious agent, and a 1-month response and recovery period during which infected donations continue to arrive.

Total costs would be even higher when the resources that would be expended investigating false-positive results are considered. For all of these reasons, blood donors should not be screened for bioterror agents for the purpose of detecting a bioterror attack.

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Dr. Kaplan is the William N. and Marie A. Beach Professor of Management Sciences at the Yale School of Management and professor of public health at the Yale School of Medicine, where he directs the Methodology and Biostatistics Core of Yale's Center for Interdisciplinary Research on AIDS. His interests include the application of operations research, statistics, and mathematical modeling to public health policy problems such as HIV prevention, and more recently bioterror preparedness and response.

Appendix

We consider a single bioterror attack that infects a proportion p of the population at time 0. To model test sensitivity, we presume that a blood test administered to a person t days after becoming infected will test positive for infection with probability $F_W(t)$, where W refers to the window period of the test. In our examples we assume that W follows the exponential distribution with mean ω days, that is, $F_W(t)= 1 - e^{-t/\omega}$, though the model allows assessment for any window period distribution. We set ω=3 days in our examples.

The probability that a randomly selected member of the population would test positive t days after the attack is then given by

$$\pi(t) = pF_W(t) + \int_0^t \iota(u)F_W(t-u)du \quad (1)$$

where $\iota(u)$, the per-capita rate of infection due to transmission after attack (but before detection) grows exponentially as

$$\iota(u) = pR_0 r \exp((R_0 - 1)ru) \quad (2)$$

as explained following equation 8 below. In equation (2), R_0 is the reproductive number specifying the number of secondary infections transmitted by an initially infected individual early in the outbreak, while r^{-1} is the mean duration of infectiousness (14). We set R_0=3 and r^{-1}=14 days in our contagious examples, parameters suggestive of smallpox (3,15), while results for attacks with noncontagious agents are obtained by setting R_0=0. Note that $\pi(t)$ is proportional to p, the fraction of the population initially infected in the attack.

Due to the superposition of many individually arriving donors (13), we assume that in the aggregate, blood donations occur in accord with a Poisson process with rate λ per unit of time. We set $\lambda = kN$ for some constant k, that is, the blood donation rate is proportional to the size of the population (k=0.05 to represent the average U.S. donation rate [12] in our examples). We further assume that donors are no more or less likely to have been infected than nondonors. The number of blood donations that would test positive within time τ of the attack then follows a Poisson distribution with mean

$$\rho(\tau) = \int_0^\tau \lambda\pi(t)dt. \quad (3)$$

Note that since $\pi(t)$ is proportional to p while λ is proportional to N, $\rho(\tau)$ is proportional to $I(0)=Np$, the initial attack size. Thus the ability to detect a bioterror attack by means of blood donor screening when blood donation occurs at a rate proportional to the population is directly related to the initial number of persons infected in the attack, independently of the size of the population.

The probability that at least one blood donation would test positive and detect the attack within τ days is given by

$$D(\tau) = 1 - \exp(-\rho(\tau)) \quad (4)$$

while the expected time required to detect such an attack equals

$$E[\text{Attack Detection Delay}] = \int_0^\infty \exp(-\rho(\tau))d\tau \quad (5)$$

because the expected value of a nonnegative random variable equals the integral of its survivor function, as is well-known. Since $\rho(\tau)$ is proportional to the initial attack size, the probability of detecting an attack within any fixed time interval increases with the initial attack size, while the expected time required to detect an attack decreases with the size of the attack.

In the event of an attack at time 0 with a contagious agent, we approximate the progress of the resulting epidemic with the standard model

$$\frac{dI(t)}{dt} = \beta I(t)[N - I(t)] - rI(t) \quad (6)$$

where N is the population size, and

$$\beta = \frac{R_0 r}{N} \quad (7)$$

is the disease transmission rate (14). Persons infected in this model immediately become infectious and remain so for r^{-1} time units on average; thus; no latent period occurs during which a person is infected but not infectious. Early in the epidemic we

have $N–I(t)\approx N$, which as usual leads to exponential growth in the number of infections as

$$I(t) = I(0)\exp((\beta N - r)t) = I(0)\exp((R_0 - 1)rt). \quad (8)$$

Note that the per-capita transmission of infection before the detection of the attack in this model is given by

$$\beta NI(t)/N = pR_0 r\exp((R_0 - 1)rt)$$

as in equation 2.

The sensitivity of the attack detection delay to the parameters of this model can be determined directly from the mathematics above. To summarize, the time to detect an attack via blood donor screening will decrease if, *ceteris paribus*, any of the following parameters increase: the initial number of infections, $I(0)$, the per capita blood donation rate (k), the reproductive number (R_0), and the disease progression rate (r). Increasing the mean window period of the screening test (ω) would lengthen the time required to detect an attack.

The screening test employed is perfectly specific in the analysis above, which obviates the problem of false alarms by assumption. We now relax the assumption of perfect specificity and instead assume that an uninfected donation will test negative with probability s, where s is the specificity of the test. With this new assumption, uninfected donations will test positive with probability $1–s$, which leads to false-positive results.

To compare false-positive and true-positive rates for noncontagious agents, we adopt an alternating renewal process model (13) of bioterror attack and recovery (a similar analysis could be conducted for contagious agents, but little insight can be gained from doing so). Under normal circumstances, we assume that attacks occur at a mean rate of α per unit time. Once an attack occurs, we assume that δ time units are required for response and recovery (clearly δ would depend upon the time required to detect an attack, which in turn could be influenced by donor screening, but this effect is minor and not essential for the main results reported). Infected donations can only occur during the response and recovery period, while to simplify the analysis, we presume that no further attacks ensue during the recovery period (indeed, multiple attacks could simply be modeled within this framework as one larger attack). Again for simplicity, we further assume that blood donations occur at the constant rate $\lambda = kN$ over time, and that any attack infects a fraction p of the population.

With these assumptions, it follows immediately that the fraction of time occupied by response and recovery, which coincides with the fraction of time during which infectious donations can occur, is given by

$$f = \alpha\delta/(1+\alpha\delta). \quad (9)$$

It follows that the false-alarm rate, FAR (i.e., the mean number of noninfected donations that falsely test positive), is equal to

$$FAR = kN(1-s)[1-f+f(1-p)] = kN(1-s)(1-fp) \quad (10)$$

for all donations that test positive do so falsely under normal circumstances, while during the response and recovery period, a fraction $(1–p)$ of donations will be noninfected, and of these $(1–s)$ will falsely test positive.

To obtain a simple formula for the true-positive donation rate, note first that the overall attack rate per unit time is given by

$$\alpha' = \alpha(1-f) \quad (11)$$

because, by assumption, attacks do not occur during the response and recovery period. Since $\rho(\delta)$ infected and detected donations will occur on average during the response and recovery period (where $\rho(\delta)$) is given by equation [3]), the overall true-positive donation rate (*TPDR)* is given by

$$TPDR = \alpha'\rho(\delta). \quad (12)$$

In the text, we report results for δ=1 month and α'=1 attack per year, but again the sensitivity of the results to the model parameters is clear from the mathematics: reducing either the attack rate or the duration of response and recovery serves to reduce the true-positive donation rate while marginally increasing the false-positive rate; increasing test specificity obviously reduces the false-alarm rate.

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Address for correspondence: Edward H. Kaplan, Yale School of Management, 135 Prospect Street, New Haven, CT 06511-3729, USA; fax: 203-432-9995; email: edward.kaplan@yale.edu

Legionnaires' Disease Outbreak in Murcia, Spain

Ana García-Fulgueiras,* Carmen Navarro,* Daniel Fenoll,† José García,* Paulino González-Diego,* Teresa Jiménez-Buñuales,* Miguel Rodriguez,* Rosa Lopez,* Francisco Pacheco,* Joaquín Ruiz,‡ Manuel Segovia,§ Beatriz Baladrón,¶ and Carmen Pelaz¶

An explosive outbreak of Legionnaires' disease occurred in Murcia, Spain, in July 2001. More than 800 suspected cases were reported; 449 of these cases were confirmed, which made this the world's largest outbreak of the disease reported to date. Dates of onset for confirmed cases ranged from June 26 to July 19 , with a case-fatality rate of 1%. The epidemic curve and geographic pattern from the 600 completed epidemiologic questionnaires indicated an outdoor point-source exposure in the northern part of the city. A case-control study matching 85 patients living outside the city of Murcia with two controls each was undertaken to identify the outbreak source; the epidemiologic investigation implicated the cooling towers at a city hospital. An environmental isolate from these towers with an identical molecular pattern as the clinical isolates was subsequently identified and supported that epidemiologic conclusion.

Legionnaires' disease (LD) has been an emergent disease since the 1970s. In the last few years, the increased use of a simple test for detecting urinary antigen *Legionella pneumophila* serogroup 1 in patients with pneumonia has facilitated diagnosis (1). Transmission by aerosols has been extensively reported, and evidence of *Legionella* in aerosols derived from cooling towers has been provided (2–5). Although a considerable body of epidemiologic evidence exists for the association of LD outbreaks with aerosols produced by cooling towers, some controversy exists about the role that cooling towers play in LD (6–13).

We describe an explosive outbreak of LD that occurred in July 2001 in Murcia, a municipality with 360,000 inhabitants in southeastern Spain. We also report results of a case-control study performed to identify the source of this outbreak, which turned out to be a cooling tower. The outbreak of pneumonia was first detected on July 7. At the end of the first day of active surveillance, July 8, approximately 100 cumulative suspected cases were reported. More than 800 suspected cases were recorded by July 22, when the last case was treated, 2 weeks after the onset of the investigation. The epidemiologic investigation using a case-control study emphasizes a combination of strategies to measure and analyze an outbreak of LD that occurs in an area with many large potential sources of environmental contamination.

*Regional Health Council of Murcia, Murcia, Spain; †Council of Murcia, Murcia, Spain; ‡Virgen de la Arrixaca Hospital, Murcia, Spain; §Morales Meseguer Hospital, Murcia, Spain; and ¶Health Institute of Carlos III, Madrid, Spain

Methods

Case Detection

An active surveillance system to detect patients with any form of pneumonia was established on July 8 at all hospitals in the region of Murcia. Any reported case of pneumonia was considered a suspected case of LD if this diagnosis could not be ruled out. A confirmed case of LD was defined as a case of pneumonia with laboratory evidence of acute infection with *Legionella* including a) isolation of any species or serogroup of *Legionella* from respiratory secretions, lung tissue, or blood, b) a fourfold or higher rise in antibody titers from 1:128 against *L. pneumophila* SG1 by immunofluorescence or microaglutination in paired acute- and convalescent-phase serum specimens, or c) detection of *L. pneumophila* antigen in urine.

An epidemiologic questionnaire to elicit information on clinical aspects, predisposing factors, risk factors, place of residence, and recent urban mobility within the city of Murcia was administered to 662 persons with suspected cases, most within 24 to 48 hours after the case was reported. A computerized database was set up as well as maps showing geographically referenced cases and a spatial analysis by census division that used a geographic information system (14).

Case-Control Study

Inclusion in the study was restricted to patients who had confirmed LD, were residents outside the city of Murcia, and had been reported July 8–20 as case-patients. Each case-patient was matched to two controls according to place of residence, sex, and age. Controls were randomly

selected from the population of the same area of residence and health district as the matched patient.

A standardized questionnaire to interview patients and controls was designed. It focused on urban mobility and exposure to outside air within the northern part of Murcia 2 weeks before the patient's onset of illness. Patients and controls were interviewed in person at home between July 25 and August 8. Itineraries of all participants, including information about means of transport and frequency of trips, were outlined on a map of Murcia. In addition, any travel into or visit to 30 specific zones of the city in which putative sources of contaminated aerosols were located was recorded. The questionnaire also requested information about place of residence and work, occupation, education level, employment status, smoking habit, alcohol intake, chronic lung disease, diabetes, renal or heart disease, malignancy, immunocompromising disease, organ transplant, therapy with corticosteroids, and other risk factors for LD within 2 weeks before illness.

A multivariate analysis that used conditional logistic regression was conducted to calculate odds ratios (OR) with 95% confidence intervals (CI) as estimates of the relative risk for LD associated with a person's travel through each zone; we controlled for the confounding effects of traveling through other zones. Any zone of exposure that was significant in univariate analysis or showed biologic plausibility as a source was entered into the multiple analysis. The frequency with which participants visited Murcia city was also introduced into the multivariate analysis. Statistical analysis was conducted with STATA software (15).

Strategies of Analysis

Exposure zones were analyzed in two ways after codification of the information obtained from each patient or control as he or she traveled or did not travel through a) the area defined by the block around a building with a cooling tower or the block around an ornamental fountain (in this way, 30 zones of the northern part of the city were coded), and b) the area delineated by a circle of 200 m radius around a cooling tower or a large ornamental fountain. Therefore, eight high-risk zones were studied.

In all cases, for each area of exposure, how the patient or control traveled through the area (i.e., walking [a category that also included bicycling or motorbiking] or driving [a car, bus, or truck]) was specified. This information was analyzed for the following: a) walking versus not passing through a zone or b) walking versus not passing through an area or traveling through it by car. Finally, for all possibilities, data were analyzed in two further ways: a) complete, which took into account all persons in the study, or b) restricted, which took into account only the trios of case-patients and their two paired controls in which all three persons stated that they had visited Murcia in the study period.

Environmental Investigation

Possible sources of aerosols were inspected, and water samples were collected from the water supply network and from 339 installations (e.g., cooling towers, storage tanks, and decorative fountains). Cooling towers were identified by aerial inspection because no census of these installations was available. Attack rates by residence were used to determine in which locations inspections and environmental samplings could be conducted.

Microbiologic Study

Environmental samples were processed according to ISO 11731/1998. Environmental and clinical *L. pneumophila* serogroup 1 isolates were typed by monoclonal antibody (MAb) with International and Dresden MAb panels (16,17) and compared by three molecular methods, amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE)-*Sfi*I, and arbitrarily primed–polymerase chain reaction (AP-PCR) (18–20).

Results

Descriptive Epidemiology

The outbreak of pneumonia was detected on the evening of July 7, when the Regional Department was notified that an increase in pneumonia cases had occurred in three hospitals in Murcia. *Legionella* antigen was detected in some patients' urine. Approximately 800 suspected cases were reported July 8–22; confirmed cases numbered 449. We estimate that 636–696 persons were affected. These estimates took into account the sensitivity of the antigen test in urine of 70% (4) and the background number of pneumonia cases estimated from the median of patients admitted with pneumonia in the region's hospitals during the summers from 1996 through 2000.

Onset of illness of the first confirmed case-patient was June 26. Until July 1, only a small number of cases occurred. After this date, the outbreak became explosive, with most cases occurring in <10 days (83% of confirmed cases had onset from July 2 through July 9). The last case-patient became ill on July 19 (Figure 1). The outbreak was considered compatible with massive exposure to a common source of contamination. When LD's incubation period was taken into account, the maximum emission was estimated to have occurred June 29–July 1 and to have ended completely July 9–17.

Hospital admission was necessary for 64% of all reported case-patients and 74% of confirmed case-patients. Six deaths from LD were confirmed to be directly related to this outbreak, five confirmed cases and one suspected.

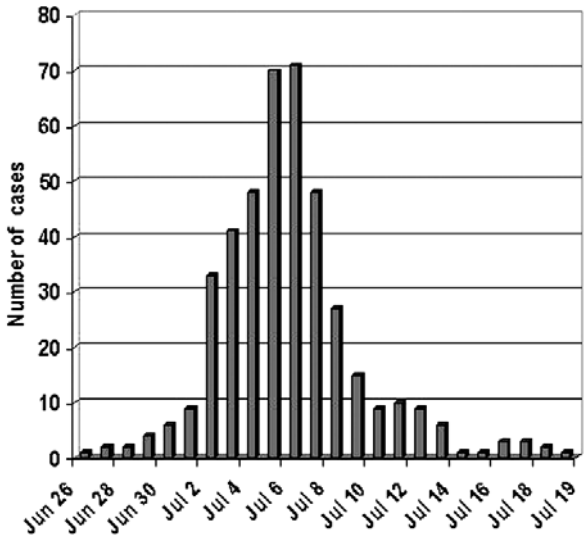

Figure 1. Confirmed cases of Legionnaires' disease by date of onset of illness, Murcia, Spain, June 26–July 19, 2001.

Therefore, the case-fatality rate was 1.1% for confirmed cases only and 0.9% for total estimated cases. For all confirmed case-patients, 74% were men and 26% women. The age range was from 19 to 91 years; 70% were ≥50, and 29% were ≥70. The incidence rate increased with age in both sexes and was higher in men in all age groups (Figure 2).

Of the confirmed case-patients, 68% lived in Murcia city proper, 16% in the satellite districts within the municipality of Murcia, and 16% in other municipalities of the region. To evaluate the risk by quarters within the city of Murcia, the Standardized Incidence Ratio (SIR) was used. Three neighborhoods located in the northern part of the city had the highest incidence rate (4.9–6.7 per 1,000 population), significantly higher than the average for the city of Murcia (Table 1, Figure 3). According to epidemiologic interviews, 95% of the confirmed case-patients lived, worked, or visited in the northern districts in the 10 days before the outbreak began.

L. pneumophila serogroup 1 was recovered from clinical samples of 19 patients; 18 samples were characterized. All were Pontiac (MAb 2+) Philadelphia MAb type and shared an identical molecular pattern by AFLP, PFGE-*Sfi*I, and AP-PCR.

Case-Control Study

The descriptive study showed no common indoor source of exposure and determined that the outbreak was provoked by a common source located in the northern part of the city. The study hypothesis was that the outbreak had its origin in environmental contamination from cooling towers or other installations capable of producing and dispersing large quantities of aerosols potentially contaminated by legionellae.

A total of 85 cases and 170 controls were included in a case-control study. Participation in the case-control study was classified as recently proposed by Olsen et al. (21). The response rate among eligible cases was 89% (85/96) and 96% (85/89) among those eligible who were contacted. The response rate among persons selected as eligible controls was 51% (170/334) and 61% (170/279) among those eligible who were contacted. The distribution of cases and controls according to sex, age, and residence was identical. No significant differences were found between cases and controls in any of the variables considered as a risk for or predisposing factor to the disease, as shown in Table 2. No differences were found with respect to education level or employment situation (Table 3). A strong association between visiting the city of Murcia and being ill with LD was found (OR 14.1, 95% CI 4.2 to 45.9).

The zone of exposure, defined either by the block surrounding hospital H or by a circle of 200 m in radius around hospital H, was significantly associated with illness in all eight models of multivariate analysis (Table 4). This zone of exposure also showed a much higher OR in every model. Thus, LD was 4.8–11.4 times more likely to develop in persons who passed through the zone around hospital H during the risk period than in persons who did not travel through this zone, independent of their having passed through the other zones. These results were also independent of the number of times the patient had visited the city.

An association between the illness and walking through the zone was observed in the multivariate analysis for another three zones of exposure. However, none of these zones appeared in more than two of the eight models, and each had an OR that was lower or much lower than that for the zone around hospital H in the corresponding model.

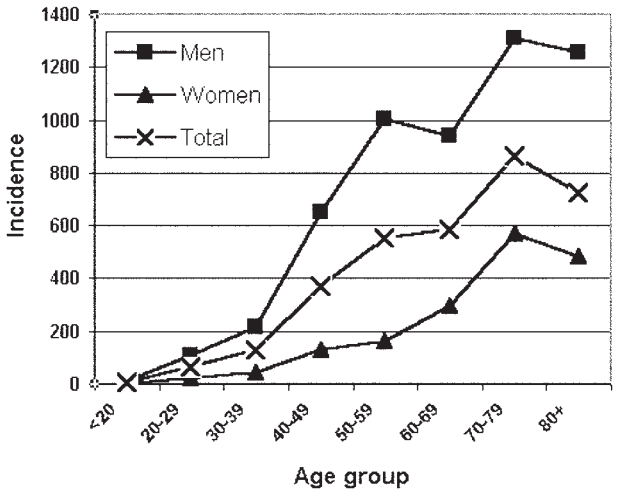

Figure 2. Confirmed cases of Legionnaires' disease within the city of Murcia, Spain. Specific incidence rates by sex and age (per 100,000).

Table 1. Confirmed cases of Legionnaires' disease within Murcia city, Murcia, Spain

Neighborhood	Confirmed cases	Inhabitants	Incidence per 1,000	SIR[a] (95% CI)
Sta. Mª de Gracia	90	13,410	6.7	6.3 (5.1 to 7.8)
Vistalegre	62	12,677	4.9	4.8 (3.7 to 6.1)
San Antón	48	9,373	5.1	5.2 (3.8 to 6.9)
San Miguel	28	9,511	2.9	2.5 (1.5 to 3.3)
San Basilio	17	5,509	3.1	3.0 (1.8 to 4.8)
Santiago Z	8	3,215	2.5	2.5 (1.1 to 4.9)
San Pio X	4	824	4.8	4.2 (1.1 to 10.8)

[a]SIR, standardized incidence ratio by neighborhood within Murcia city. Only results of neighborhoods with SIR >1 are represented; CI, confidence intervals.

Nosocomial Outbreak at Hospital H

During the epidemiologic study of this community outbreak of LD, a nosocomial outbreak of LD at hospital H was discovered. In all 11 definite or probable nosocomial cases, some portion of the previous 10-day period of hospitalization coincided with the period when the cooling towers could have been active.

Environmental Inspection and Microbiologic Study of Environmental Samples

L. pneumophila was not recovered from water samples from the drinking water supply network in the city of Murcia. *L. pneumophila* serogroup 1 Pontiac (MAb 2+) was recovered from 22 installations (cooling towers of 11 buildings in the city and water storage tanks from 3 buildings). Ten of 11 cooling towers contained a Philadelphia MAb-type strain, but only two colonies, recovered in October from a cooling tower of hospital H, were indistinguishable from the patient strains by AFLP (Figure 4). Identical results were obtained when PFGE-*Sfi*I and AP-PCR were applied.

Weather Conditions

Data provided by the Weather Centre of Murcia showed that during the last days of June and early July some atmospheric thermal inversion occurred every day, except one. Winds were predominantly from the northeast quadrant with a very low average speed (9 kph) and very high temperatures (33.5°C–35°C).

Discussion

This LD outbreak is the largest to date in the world, with 449 confirmed cases and an estimated total number of cases of 650. The reported case-fatality rate (1%) is much lower than those observed in other community outbreaks (22,23). This rate can be attributed, at least partially, to the quick detection of the outbreak, early diagnosis of the disease, and appropriate treatment of patients. The explosive quality of the outbreak not only led patients to seek quick assistance at hospital emergency units but also helped clinicians to perform an accurate diagnosis and to immediately initiate adequate treatment, factors reported as linked to low case fatality (24,25). This explosive appearance could also be related to a lower presence of predisposing factors in case-patients in comparison with other community outbreaks (7,9,13), which could also partially explain the low case-fatality rate.

The initial investigation encountered obstacles, such as a large number of potential sources of environmental contamination located in the northern part of the city and the absence of environmental *Legionella* isolates identical to those of patients. The case-control study showed a significant association, with a high consistency between the analyzed models and with a high magnitude of association, between passing through the zone around hospital H and being ill with LD. Results were similar even when the area radius was expanded to 400 m. However, large overlap of areas was observed within this radius, and multicollinearity among zones was a common finding.

The case-control study was designed to select patients residing outside the city of Murcia. We decided on this approach for two reasons. First, the incidence of LD was almost 1% in some neighborhoods, a rate within the 0.1% to 5% attack rate described for this disease (26) Therefore, all the persons living in these quarters could possibly have been exposed to *Legionella*, as has been described in outbreaks of other transmissible diseases (27). If everyone had been exposed, finding incidence differences between persons exposed and those not exposed would have been almost impossible. Second, persons residing outside the

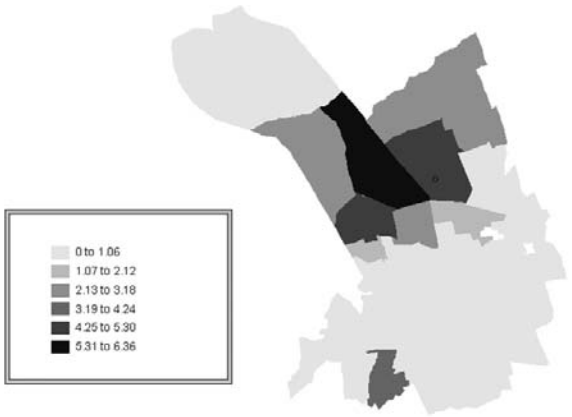

Figure 3. Confirmed cases of Legionnaires' disease within Murcia city, Spain. Standardized Incidence Ratio (SIR) by neighborhood. Circle represents hospital H.

Table 2. Risk factors for Legionnaires' disease patients and controls, Murcia, Spain

Predisposing factors	Cases n=85	Controls n=170
Smoking (%)	43.5	40.6
Alcohol intake (cc/week)	134	106
Chronic illness or immuno-suppressive therapy (%)	16.5	14.7
Previous hospitalization (%)	1.2	1.8
Previous travel (%)	11.8	13.5

city would probably have a more accurate memory of the itineraries they followed in Murcia some weeks previously and would probably have a lesser number of routes than persons living within the city. Conducting 255 personal interviews with questions about itineraries within 2 weeks from the last case and 4 weeks from the outbreak onset may also have been important to our findings.

One concern in case-control studies is that participation rate is not reported consistently (21). Indeed, this information is usually omitted in case-control studies of outbreaks, especially when controls are selected from a population database, as was our situation. A further complication was that the study had to be conducted in July, when many people go on holiday. In spite of achieving the participation of one in two controls whom we initially selected, we evaluated possible selection bias. We determined that it was unlikely to have occurred since neither socioeconomic status nor predisposing risk factors for LD differed significantly among cases and controls. Information bias overestimating this outcome was ruled out since news media did not mention hospital H among the probable sources of the outbreak.

Meteorologic conditions were favorable for the emission of aerosols to be dispersed in a horizontal manner. Low wind speed together with atmospheric thermal inversion between June 29 and July 1 would have facilitated the presence of the aerosols in the environment (9).

The result of the epidemiologic study was subsequently confirmed by the isolation of a strain retrieved on October 30 from a sample from one of the cooling towers of the same hospital; that strain is identical to the strain isolated from the patients. The difficulties found previously in the isolation of this strain were not unexpected. The day after the outbreak was detected, when the first sample was taken, the cooling towers of hospital H were highly chlorinated, which could explain why these first samples gave negative results. Later samples retrieved on four different dates between July 28 and September 13 showed positive results to *L. pneumophila* but were characterized as different strains from those from patients. This strain was only isolated upon the restarting of one tower after it was shut down for more than 1 month, a condition that favors the reappearance of *Legionella* (8,12,13). The fact that the same clone of *Legionella* can be found in an installation for long periods is also documented (28,29). The possible contamination of the tower by new *Legionella* from the water supply was ruled out since the strain linked to the outbreak was not found in samples collected from many other installations during the same period, including July to November.

The coincidence of a nosocomial LD outbreak in hospital H reinforces the previous hypothesis. A nosocomial outbreak of LD as part of a wider community outbreak of the disease has been described (12,30), although in other outbreaks originating in the cooling towers of a hospital no cases of nosocomial LD were identified (7). The use of double HEPA filters on air-intake vents in some hospitals could justify, at least in part, these contradictory observations.

Our research indicates that the cooling towers of a hospital located in the northeastern part of the city of Murcia were the origin of this community outbreak. This study underlines important risk factors that must be taken into account to prevent a new LD outbreak. First, cooling towers had to be identified by aerial and direct inspection in the absence of any census of such installations. Second, the size, location, and state of maintenance of cooling towers are very important. In contrast with epidemics associated with relatively small systems (8), this outbreak was related to a large refrigeration system that seems to have infected patients up to 1.3 km downwind to the west from the cooling tower; this finding suggests that airborne infection with *L. pneumophila* may extend over a large distance from the dissemination source, as has been reported elsewhere (9,10). Although most of the installations in the area showed inadequate maintenance, the cooling towers from hospital H were poorly maintained and had a high-risk size and location. Once the outbreak was identified, urgent measures were undertaken to clean, disinfect, or close possibly contaminated sources. The cooling tower that was the source of the outbreak was subsequently replaced by an air-cooled system.

Before June 2001, no specific national legislation existed in Spain concerning LD, although a recommendation guide and legislation existed in several Spanish

Table 3. Educational level and employment status for Legionnaires' disease cases and controls, Murcia, Spain

Education and employment	Cases (n=85)	Controls (n=170)
Educational level (%)		
Primary	52.9	51.5
Secondary	38.8	37.3
University	8.2	11.2
Employment status (%)		
Employed	65.5	63.5
Unemployed	5.9	3.6
Retired	19.1	21.1
Housewife/husband	8.3	8.4
Student	1.2	3.0

Table 4. Association between Legionnaires' disease and a patient's traveling through specific areas of the northern part of the city of Murcia, Spain[a]

	Block-area study				Circle area study			
	Complete analysis		Restricted analysis		Complete analysis		Restricted analysis	
Area of city	OR (95% CI) Walking vs. not passing through	OR (95% CI) Walking vs. not passing through or passing by driving	OR (95% CI) Walking vs. not passing through	OR (95% CI) Walking vs. not passing through or passing by driving	OR (95% CI) Walking vs. not passing through	OR (95% CI) Walking vs. not passing through or passing by driving	OR (95% CI) Walking vs. not passing through	OR (95% CI) Walking vs. not passing through or passing by driving
Hospital H	10.2 (3.6 to 28.8)	9.7 (3.9 to 23.6)	10.7 (2.5 to 45.5)	6.0 (1.9 to 18.4)	6.9 (1.8 to 26.0)	6.4 (2.5 to 15.7)	11.4 (3.2 to 40.1)	4.8 (1.5 to 5.2)
Garden P	5.2 (1.0 to 25.8)	4.6 (1.2 to 17.0)						
Car-park X					5.1 (1.7 to 14.9)	3.6 (1.4 to 9.2)		
Commercial building						2.9 (1.1 to 7.4)		

[a]Areas associated to the disease in a multivariate analysis of data from eight different models. OR, odds ratio; CI, confidence interval.

autonomous regions that had had community LD outbreaks (31). As an immediate consequence of this outbreak, a national law about prevention and control of LD was enacted in Spain 20 days after the outbreak began (32). The extent of this outbreak is useful to assess the relative role of cooling towers as a source of LD and highlights the importance of prioritizing control measures related to cooling towers among strategies to prevent LD in the community. Compliance with these measures would help to reduce not only community outbreaks but also, perhaps, sporadic cases that could be due to infected cooling towers (33).

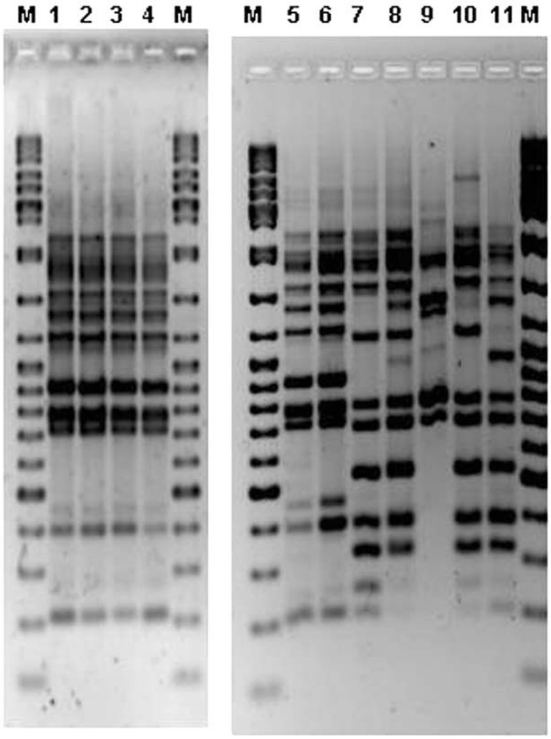

Figure 4. Amplified fragment length polymorphism (AFLP) gel containing outbreak human and environmental *Legionella pneumophila* serogroup 1 isolates. M, molecular weight marker (Ladder Mix, MBI Fermentas, UK). Lanes 1 and 2, two colonies from a cooling tower of the hospital H. Lines 3 and 4, human isolates. Lanes 5 and 6, human isolates. Lanes 7–11, different environmental isolates from several Murcia installations.

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Dr. García-Fulgueiras is an epidemiologist at the Murcia Regional Health Council in southeast Spain. She coordinates the regional epidemiologic surveillance network. Her primary research interests include the epidemiology of infectious diseases and outbreaks.

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Address for correspondence: A. García-Fulgueiras, Servicio de Epidemiología, Consejería de Sanidad, Ronda de Levante 11, 30008 Murcia, Spain; fax: +34 968366656; email: anam.garcia9@carm.es

Survival of *Batrachochytrium dendrobatidis* in Water: Quarantine and Disease Control Implications

Megan L. Johnson* and Richard Speare*

Amphibian chytridiomycosis is an emerging infectious disease of amphibians thought to be moved between countries by trade in infected amphibians. The causative fungus, *Batrachochytrium dendrobatidis*, produces aquatic, motile zoospores; infections have been achieved in experiments by exposing amphibians to water containing zoospores. However, the ability of this fungus to survive in the environment in the absence of an amphibian host is unknown. We show that *B. dendrobatidis* will survive in tap water and in deionized water for 3 and 4 weeks, respectively. In lake water, infectivity was observed for 7 weeks after introduction. The knowledge that water can remain infective for up to 7 weeks is important for the formulation of disease control and quarantine strategies for the management of water that has been in contact with amphibians.

Batrachochytrium dendrobatidis, a member of the fungal order Chytridiales, causes amphibian chytridiomycosis, an emerging infectious disease (1). Amphibian chytridiomycosis has been responsible for massive illness and death in amphibian populations in Australia, New Zealand, Europe, United States, Central America, and South America (2–7). Amphibian chytridiomycosis in several continents has appeared in one region and subsequently spread as an epidemic wave. Data obtained by retrospective examination of museum specimens have identified the date of first occurrence as 1974 in the United States (8), 1978 in Australia (9), 1986 in Eduador (10), and 1999 in New Zealand (7). In 2001, amphibian chytridiomycosis was placed on the Office Internationale des Epizootes Wildlife Diseases List, the first time amphibian diseases had been listed.

B. dendrobatidis is an aquatic organism with two life stages: a sessile, reproductive zoosporangium and a motile, uniflagellated zoospore released from the zoosporangium. Frogs can be experimentally infected by zoospores of *B. dendrobatidis* (11,12) or by contact with skin harvested from infected animals (2). No resting stage has been identified for *B. dendrobatidis* in laboratory culture, and whether one occurs in nature is unknown as yet (13). The organism does not survive desiccation (11), so infected amphibians have been identified as the major means in which *B. dendrobatidis* could be moved within and between countries. Amphibians carrying *B. dendrobatidis* have been detected in the pet trade in the United States (14), Europe (4), and Australia (15); in frogs for scientific purposes, particularly *Xenopus laevis* and *X. tropicalis* (16–18); and in frogs for food, particularly *Rana catesbiana* (19). *B. dendrobatidis* has been hypothesized to have been introduced into new areas by movement of infected amphibians or in contaminated water or soil containing zoospores, but little is known about the epidemiology of amphibian chytridiomycosis (15,20,21).

Recent discussions have highlighted the need for research on the ability of this fungus to survive in the environment in the absence of a suitable host, since the fungus may be capable of a saprophytic life cycle (13). No data exist on the survival of *B. dendrobatidis* in water after an infected frog has been removed. We describe the survival of *B. dendrobatidis* introduced into autoclaved water from different sources. Autoclaved water was used since no currently available technique selects *B. dendrobatidis* from natural water bodies complete with bacteria, fungi, algae, and protozoa. We hypothesized that sterile water would represent the best possible opportunity for survival of *B. dendrobatidis* owing to the absence of competitor microorganisms.

Methods

Water was collected from three sources in Townsville, North Queensland: deionized water, tap water collected from the chlorinated reticulated supply, and lake water from a 1-hectare lake in the suburb of Hyde Park. All water was then autoclaved at 121°C for 15 min. In duplicate 25-cm^2 culture flasks (TPP, CSL Biosciences Ltd., Australia) containing each of the three water types, we added approximately 1 x 10^5 spores/mL of 98-1810/3 of *B. dendrobatidis*, obtained from a wild adult of *Nyctimystes dayi* from Tully, Queensland, in 1998, or *B. dendrobatidis* strain 98-1469/10 isolated from a captive juvenile *Limnodynastes dumerilii* from the Amphibian Research Centre,

*James Cook University, Townsville, Australia

Melbourne, Victoria, in 1998. Strains have been maintained by serial passage in TGhL broth (16 g tryptone, 2 g gelatin hydrolysate, 4 g lactose in 1,000 mL distilled water) approximately every week. Flasks containing water were held at 23°C in an incubator and observed during a 10-week period by using an inverted Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Evidence of growth included attachment of inoculated zoospores, change in size and form of zoosporangia, and release of new zoospores into the water. Signs of viability included movement of free zoospores in the water or movement of zoospores inside the zoosporangia. From weeks 2 to 7, when activity of zoospores was no longer visible and growth appeared to have ceased, 0.5 mL of water from each flask was added into flasks containing TGhL media and incubated at 23°C. These newly inoculated cultures were then observed for growth and activity, with the observations terminating at week 10.

Results

The duration of survival of *B. dendrobatidis* in water varied with the source of the water and the strain of fungus (Table). For all three water samples, zoospores attached to the plastic of the flasks and grew into zoosporangia, but new zoospores appeared to be released only into the lake water. After 1 week in tap and deionized water, no further growth of zoosporangia or activity by zoospores was apparent. However, viability of *B. dendrobatidis* was demonstrated in tap water until week 3 by growth of both strains on addition into culture media. For deionized water, viability of strains differed with growth on inoculation into culture media up to week 3 and week 4 for 98-1810/3 and 98-1469/10, respectively. The lake water contained dead microscopic algae, arthropods, protozoa, and plant debris, and zoosporangia were attached to these organic bodies as well as to the plastic of the flask (Figure). Growth of zoosporangia was apparent at week 1, and motile zoospores of both strains were present in lake water cultures for 1 to 7 weeks. However, during this period, no activity of zoospores was apparent at times. Subcultures from lake water into TGhL broth showed viability to week 6 and week 3 for the strains 98-1810/3 and 98-1469/10, respectively.

Discussion

Zoospores of *B. dendrobatidis* were highly active when placed into all three types of water and progressively attached to the flask to form sessile zoosporangia. With routine culture in nutrient media, zoosporangia release zoospores into the medium as they mature. This cycle continues until the culture becomes overgrown and inhibition of growth occurs, possibly as nutrients become limiting or metabolic waste products accumulate. One cycle of normal growth for both these strains of *B. dendrobatidis* takes up to 5 days at optimum temperatures and nutrient conditions. However, in low nutrient conditions, such as exists in tap, lake, and distilled water, growth is inhibited and the life cycle takes longer to complete. To allow for a slower life cycle, observations were continued for 10 weeks. In our study zoospores developed to zoosporangia in all three water types, but new zoospores were not released in two of the three water types, tap water, and deionized water. Previous research had indicated that *B. dendrobatidis* did not survive well in distilled water (11). However, this study confirmed that the absence of activity by zoospores, or growth of new zoosporangia, is not a reliable indicator of a nonviable or dead culture. Zoosporangia may be able to survive in a state of arrested or nondiscernible development for a long period in environments not conducive to growth, and on inoculation into nutrient broth, the life cycle may then recommence. Results from inoculation of TGhL broth showed that *B. dendrobatidis* in tap water remained viable for 3 weeks. In deionized water strain 98-1810/3 survived for 3 weeks and strain 98-1469/10 survived 4 weeks. *B. dendrobatidis* in lake water was more successful: both strains were able to release new zoospores, or less likely, the original zoospores were capable of surviving for extended periods.

We presume that the longer survival time of *B. dendrobatidis* in lake water could be due to the higher level of

Table. Survival of *Batrachochytrium dendrobatidis* in artificial water environments

	Tap water	Deionized water	Lake water
Duration of growth in water[a]			
Strain 98-1810/3	1 wk	1 wk	1 wk
Strain 98-1469/10	1 wk	1 wk	1 wk
Release of zoospores			
Strain 98-1810/3	No	No	Yes (wk 5–7)
Strain 98-1469/10	No	No	Yes (wk 2,3,7)
Duration of viability[b]			
Strain 98-1810/3	3 wk	3 wk	6 wk
Strain 98-1469/10	3 wk	4 wk	3 wk

[a]Growth, zoospores attach to flask and develop into zoosporangia.

[b]Viability, growth occurs when aliquots from water are injected into TGhL broth.

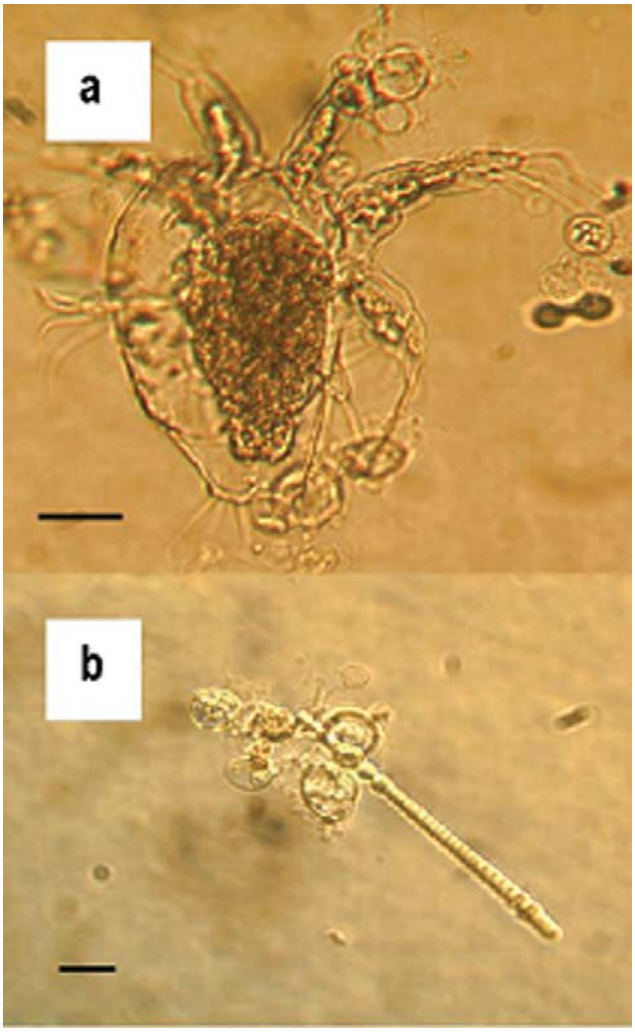

Figure. Zoosporangia of strain 98-1810/3 visible as transparent spherical bodies growing in lake water on (a) freshwater arthropod and (b) algae. Bars = 30 μM.

nutrients and possibly the nonliving organic substrate offered by algae and other microorganisms. Attachment and growth of zoosporangia on skeletons of algae and invertebrates support this theory. However, no evidence of digestion of these bodies by the chytrid was visible. Repeated observations (Figure a) did not show any appearance of degradation. We do not know the effect of these microorganisms, if alive, on the growth and survival of *B. dendrobatidis*. Autoclaved water was used because of the limitations of our current culture system. Although active zoospores of both strains were observed in lake water cultures after 7 weeks, no growth occurred when subcultured into TGhL. Low numbers of zoospores were present. Therefore, when aliquots were removed to inoculate TGhL broth, zoospores may not have been included. Alternatively, zoospores may have been subject to osmotic shock when transferred from water to broth. *B. dendrobatidis* grows best when clusters of zoospores form (13), and too few may have survived inoculation to successfully establish a new culture.

These results have immediate relevance for disease control and quarantine strategies. Water in contact with amphibians should be regarded as contaminated with *B. dendrobatidis* for up to at least 7 weeks after last contact with the amphibian. For quarantine purposes, all water, moist soil, and wet fomites imported into a country with amphibians should be regarded as infectious for *B. dendrobatidis* unless the amphibians are shown to be uninfected. A similar strategy should be adopted when introducing new amphibians into a captive colony or collection. Similarly, water and any items coming into contact with amphibians moved within countries should be regarded as infectious for *B. dendrobatidis*. In practical terms, storage alone for a period of time should not be used as a means of ensuring water that has been in contact with an amphibian is not contagious. All water and wet soil in contact with an amphibian should be disinfected before discharge into the wastewater system or the natural environment. Amphibians should not be placed into enclosures with water used previously by other amphibians without prior disinfection. Any other wet objects that have been in contact with amphibians should either be disposed of or disinfected before repeat use. Various disinfection strategies have been described (M. L. Johnson et al., unpub. data). The most effective strategies for disinfection are heat (>47°C for 30 min), didecyl dimethyl ammonium chloride at >0.0012% final concentration for 2 min, or sodium hypochlorite (>1% for 1 min). To comply with the intentions of Office Internationale des Epizootes listing, amphibians, when moved between countries, should be placed in a different container on arrival; all water, soil, plants, and litter in contact with the amphibian during transport should be adequately disinfected by using techniques capable of killing *B. dendrobatidis*.

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Dr. Johnson is a researcher in the Amphibian Diseases Group at James Cook University, Townsville, Australia. Her research interests include parasitology, particularly of humans and amphibians, and molecular biology.

Dr. Speare is an associate professor and deputy head of the School of Public Health and Tropical Medicine, James Cook University, Townsville, Australia. His research interests include diseases of wildlife, particularly of wild amphibian populations, and control of communicable diseases in humans and animals.

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Address for correspondence: Megan Johnson, Amphibian Diseases Group, School of Public Health and Tropical Medicine and Rainforest CRC, James Cook University, Townsville, Queensland, Australia, 4811; fax: +61 7 4722 5788; email: Megan.Johnson@jcu.edu.au

Enzootic Transmission of Yellow Fever Virus in Peru

Juliet Bryant,* Heiman Wang,* Cesar Cabezas,† Gladys Ramirez,‡ Douglas Watts,* Kevin Russell,§ and Alan Barrett*

The prevailing paradigm of yellow fever virus (YFV) ecology in South America is that of wandering epizootics. The virus is believed to move from place to place in epizootic waves involving monkeys and mosquitoes, rather than persistently circulating within particular locales. After a large outbreak of YFV illness in Peru in 1995, we used phylogenetic analyses of virus isolates to reexamine the hypothesis of virus movement. We sequenced a 670-nucleotide fragment of the prM/E gene region from 25 Peruvian YFV samples collected from 1977 to 1999, and delineated six clades representing the states (Departments) of Puno, Pasco, Junin, Ayacucho, San Martin/Huanuco, and Cusco. The concurrent appearance of at least four variants during the 1995 epidemic and the genetic stability of separate virus lineages over time indicate that Peruvian YFV is locally maintained and circulates continuously in discrete foci of enzootic transmission.

Yellow fever (YF) is an important reemerging arboviral disease and a cause of severe illness and death in South America and Africa. In South America, transmission of yellow fever virus (YFV) is characterized by two types of cycles: an urban pattern of interhuman transmission vectored by *Aedes aegypti* and a sylvatic cycle involving monkeys and forest canopy mosquitoes of *Haemogogus* and *Sabethes* genera. Urban YF has not been reported in South America since 1954 (1). However, the reinfestation of many densely populated coastal cities with *Ae. aegypti* indicates that surveillance and monitoring of endemic or epidemic YF viral activity remain critically important public health objectives. Central questions surrounding the ecology of YF have been the underlying factors that explain the cyclic appearance and disappearance of virus activity from certain locales and the means by which the virus survives between epidemics. Despite extensive efforts to study the vector and host cycles of YFV within South America (2) and reported associations of climatic variables with epizootic activity (3), the parameters that influence the sylvan transmission cycle and the factors that trigger emergence of outbreaks have been poorly understood.

The most widely accepted hypothesis of YFV ecology in South America is that the virus is maintained by "wandering epizootics" of nonhuman primate species that move continuously throughout the Amazon region or along gallery forests of the river courses. Virtually all New World primate species are highly susceptible to YFV infection, and many neotropical species die of the infection. The acute viremic phase in monkeys is followed by solid immunity, and although persistent infection has been documented for some primate species in the laboratory, such infections are probably not accompanied by viremia levels sufficient to infect vectors (4,5). In Panama, Trinidad, and Brazil, finding dead monkeys (particularly *Alouatta* sp.) near forested regions has signaled the onset of epizootics. Many researchers have suggested that epizootics are cyclical events recurring at fairly regular intervals; the length of interepidemic intervals has been interpreted as the time required for reconstitution of susceptible monkey populations (6,7). Efforts to show evidence of alternative vector and host cycles (other than primates) have not been successful (1,2).

Vertical transmission of YFV in the mosquito vector may contribute to virus maintenance in nature. Field studies in eastern Senegal (8) provided indirect evidence for vertical transmission of YFV by *Aedes furcifer-taylori* through isolation of virus from male mosquitoes; similar attempts to demonstrate vertical transmission in field-caught *Haemagogus* mosquitoes from Trinidad were unsuccessful (1). Because experimental studies demonstrating vertical transmission of YFV by *Aedes aegypti* (9,10) and *Haemagogus* (11), have demonstrated very low rates of vertical transmission, researchers have suggested that a primary arthropod-vertebrate cycle is still necessary for virus amplification in nature. Ecologic monitoring for YFV is extremely difficult and expensive and has rarely been implemented for the vast area of the Amazon River Basin. In particular, YFV has never been isolated from any mosquito or wild-caught vertebrates in the Peruvian

*University of Texas Medical Branch, Galveston, Texas, USA; †Instituto Nacional de Salud, Lima, Peru; ‡Ministry of Health, Lima, Peru; and §U.S. Naval Medical Research Center Detachment, Lima, Peru

Amazon; hence, isolates collected from this region consist exclusively of human isolates from sporadic cases of disease.

In the absence of ecologic data regarding YFV infection rates in vector and vertebrate host populations, we have adopted molecular genotyping of the existing collection of human YFV isolates as a method to gain insight into the possible geographic dispersal patterns of YFV variants. We investigated the genetic diversity of YFV isolates obtained from infected humans in Peru over 22 years. We interpret the phylogenetic data in light of the concept of virus traffic in wandering epizootics. Our data suggest that the YFVs circulating in Peru have differentiated into several subpopulations, and rather than circulating as one intermixing (wandering) population, the variants appear to persist within discrete geographic foci of the Andean/Amazonian region.

Materials and Methods

Virus Strains and Origins

Twenty-five virus isolates were obtained from the World Arbovirus Reference Collection, at the University of Texas Medical Branch (UTMB), Galveston, Texas; the U.S. Naval Medical Research Center Detachment (NMRCD), Lima, Peru; and the Centers for Diseases Control and Prevention, Fort Collins, Colorado (Table 1). The viruses were originally isolated within the laboratories of the Instituto Nacional de Salud (INS) and NMRCD in Lima, at intervals from 1977 to 1999; with the exception of isolate #1914 from a sentinel mouse, the source material consisted of serum samples and tissue biopsy specimens from infected humans. To our knowledge, the 25 strains used in our study represent all strains of YFV isolated in Peru to date (12). The isolates represent 7 of the 14 hydro-

Table 1. Peruvian yellow fever isolates used in this study[a]

Strain ID	Date of illness onset	Sequence ID	Department	Community	Elevation[b]	Ecozone	Passage history[c]
1362/77	6/1977	PERU77A	Ayacucho	San Francisco	1,000–2,000	df-S or df-LM	c6/36#2
1368	6/1977	PERU77B	Ayacucho	Tribolina	1,000–2,000	vhf-S	SM1, Vero1, C6/36#2
1371	6/1977	PERU77C	Ayacucho	Chontacocha	0–1,000	hf-S	SM1, Vero1, C6/36#2
287/78	2/22/1978	PERU78	Ayacucho	San Francisco	1,000–2,000	sf-S	SM1, Mosq 2
R 35740	2/1979	PERU79	Ayacucho	Alto Montaro	0–1,000	vhf-S	SM1, Mosq 2
1899/81	6/19/1981	PERU81A	Cusco	Cusco	2,000–3,000	hf-M	SM1
1914[c]	6/12/1981	PERU81B	Cusco	Cusco	2,000–3,000	hf-M	LLCMK2, Vero 1, C6/36#1
ARVO544	1995	PERU95A	San Martin	Tocache Huaquisha	0–1,000	hf-T near vhf-S	SM1, Vero1, C6/36#2
HEB4224	1995	PERU95B	San Martin	Tocache Nuevo Progresso	2,000–3,000	hf-T near vhf-S, vhf-LM	SM1, C6/36#1
HEB4236	3/2/1995	PERU95C	Pasco	Oxapampa Villa Rica	1,000–2,000	hf-M	C6/36#1
149	3/95	PERU95D	Pasco	Oxapampa Villa Rica	1,000–2,000	hf-M	SM1, C6/36#1
Cepa#2	9/95	PERU95E	Puno	No data	2,000–3,000	hf-S	SM1, C6/36#1
Cepa#1	9/95	PERU95F	Puno	No data	2,000–3,000	hf-S	C6/36#2
OBS 2240	2/95	PERU95G	Huanuco	Hermil	1,000–2,000	vhf-LM	C6/36#2
OBS 2250	5/16/1995	PERU95H	Huanuco	Hermil	1,000–2,000	vhf-LM	SM1, C6/36#1
HEB 4240	1/30/1995	PERU95I	Junin	Chachamayo	1,000–2,000	hf-LM	C6/36#1, SM1
HEB 4245	3/6/1995	PERU95J	Junin	Chachamayo	1,000–2,000	hf-LM	SM1, C6/36#1
HEB 4246	3/8/1995	PERU95K	Junin	Chachamayo	1,000–2,000	hf-LM	SM1, C6/36#1
OBS 2243	2/95	PERU95L	Huanuco	No data	1,000–2,000	vhf-LM	SM1, C6/36#1
ARV 0548	3/19/1995	PERU95M	San Martin	Tocache Huaquisha	0–1,000	hf-T near vhf-S	SM1, C6/36#1
OBS 6530	3/26/1998	PERU98A	Cusco	Echarate	1,000–2,000	df-S	SM1, C6/36#1
03-5350-98	3/13/1998	PERU98B	Cusco	Kanaiquinaba	2,000–3,000	sf-S	C6/36#2
OBS 6745	3/29/1998	PERU98C	Cusco	Minsa/C.S. Moronacocha	1,000–2,000	hf-M	C6/36#2
IQT 5591	1/19/1998	PERU98D	Loreto	Belen, Tihuensa	0–1,000	hf-T	C6/36#2
OBS 7904	5/5/1999	PERU99	San Martin	Tarapoto	2,000–3,000	hf-S and vhf-S	Vero1, C6/36#3

[a]SM, suckling mouse; df-S, dry forest-subtropical; df-LM, dry forest-lower montane; vhf-S, very humid forest-subtropical; hf-S, humid forest-subtropical; sf-S, shrub forest-subtropical; hf-M, humid forest-montane; hf-T, humid forest-tropical; vhf-LM, very humid forest-lower montane; hf-LM, humid forest-lower montane.

[b]Elevation, range of meters above sea level for the ecozone immediately surrounding the place of viral origin.

[c]Passage history of seed strain in collection.

[d]Strain 1914 was obtained from a sentinel mouse.

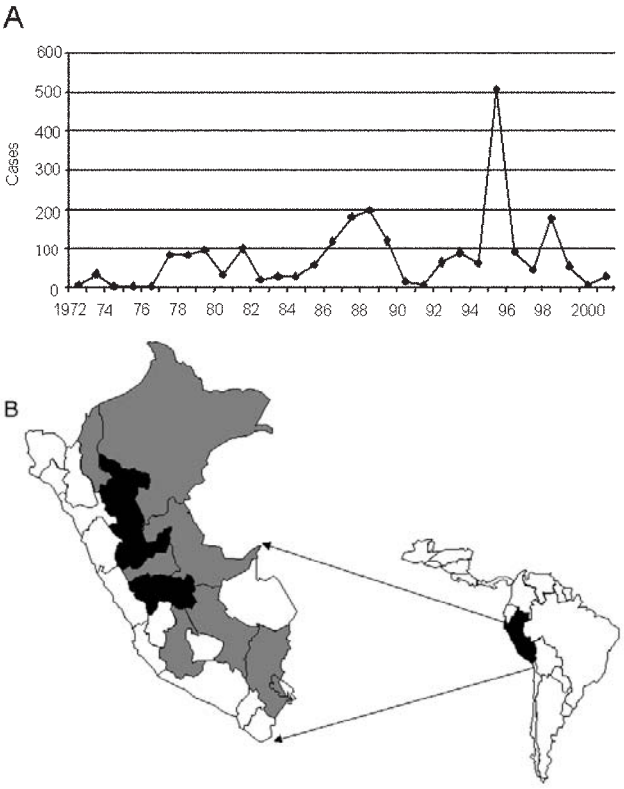

Figure 1. A) Annual incidence of confirmed cases of yellow fever in Peru, 1972-2001. B) Peruvian river basins in which yellow fever virus is endemic.

graphic river basins identified as YF-endemic zones in Peru (Figure 1) (13). Thirteen of the 25 isolates (52%) were collected during the 1995 outbreak in Peru. Geographic coordinates for the YFV isolates were determined from case histories and reflected the communities in which the patients resided at the time of infection or the regional hospital at which they were treated. In cases for which neither the community nor the regional hospital was known, the largest population center of the locale was chosen as a reference. The ecozones and elevations associated with each of the locations of viral origin were obtained by using the original classification system of Holdridge (14) and the Mapa Ecologico de el Perú (15).

Sequence Determination

After transfer of the low passage isolates to the World Arbovirus Reference Center, viruses were grown for a single passage in Vero cells to obtain sufficient quantities for RNA extraction. Methods for viral growth, genomic RNA extraction, and amplification of viral sequences by reverse transcription polymerase chain reaction (RT-PCR) have been previously described (16). The genomic region under analysis comprised 670 nt of the premembrane (prM) and envelope (E) glycoprotein genes, using the genomic-sense primer (5′-CTGTCCCAATCTCAGTCC) and genomic-complementary primer (5′- AATGCTTCCTTTCC-CAAAT). PCR products were screened by electrophoresis, recovered from gels using the QIAGEN gel extraction kit (QIAGEN, Valencia, CA), and sent for sequencing at the UTMB Protein Chemistry core facility. Sequences were obtained from both strands of each RT-PCR product for verification. The prM/E gene sequences obtained from the Peruvian YFV isolates have been deposited in GenBank (accession no. AY161927–AY161951).

Phylogenetic and Statistical Analyses

Sequence editing and alignments were performed with Vector NTI (InforMax, Inc., Frederick, MD), and phylogenetic analysis was conducted by using PAUP* (17) and MRBAYES (18). Support for individual clades was determined by Bayesian inference with monte carlo markov chain simulation (18), as well as by nonparametric bootstrapping (19). To discern whether the pattern of genetic divergence was more closely related to geographic location or time of isolation, we generated matrices of pairwise comparisons of genetic, geographic, and temporal distances, and used Mantel's test to evaluate correlations between the matrices. Geographic distances were calculated by using latitude and longitude coordinates and ArcView mapping software. The pairwise temporal-distance matrix was prepared by counting the months separating each pair of YF cases. Genetic distance matrices were generated by using the Kimura 2-parameter substitution model implemented in PAUP*. Mantel's Z statistic and Pearson's correlation coefficient (r) were calculated with MatMan version 1.0 (Noldus Information Technology, Wageningen, the Netherlands, 1998), and the significance of the Z statistic was computed by permutation analysis (10,000 repetitions).

To determine whether Peruvian YFVs were characterized by a single homogeneous rate of nucleotide substitution (i.e., a molecular clock), we performed a series of likelihood ratio tests using the PAUP* software analysis package. Maximum likelihood trees generated for the full dataset, as well as trees based exclusively on the third codon position, or on the first and second codon positions were also evaluated under the null model (no clock) and alternative models (molecular clock enforced).

Results

Sequence Variation among Peruvian YFVs

Figure 2 shows a maximum likelihood phylogeny based on the nucleotide sequences of the prM/E genes of 25 Peruvian YFV isolates. The Peruvian dataset contained 69 variable nucleotide positions, with a maximum of 7.3% nucleotide variability in all pairwise comparisons (average

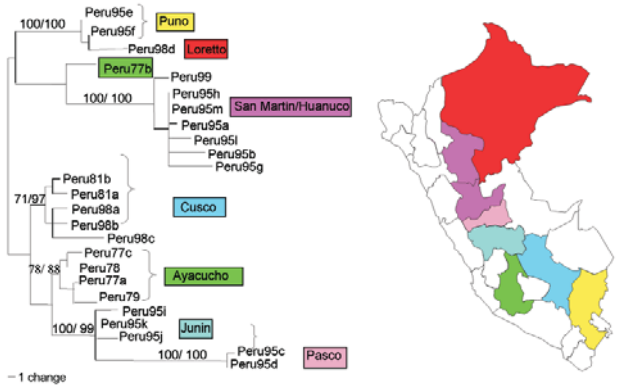

Figure 2. Maximum likelihood phylogeny of prM/E sequences of Peruvian yellow fever isolates constructed using PAUP*, 4.0b4a (17). Horizontal branch lengths represent genetic divergence, and numbers above the branch lengths denote support for individual clades as determined by nonparametric bootstrap analysis with 1,000 replicates (first value) and Bayesian posterior probabilities (second value). Only the values relevant for the interpretation of results are given. The strains used are listed in Table 1.

of 4.03%). Sixty of the nucleotide positions were parsimony informative; 48 informative sites occurred at third codon positions, whereas 12 informative sites occurred at first and second codon positions. Fifteen variable amino acids positions (6.7% of the 223 codons) were scattered throughout the prM, M, and E proteins (Figure 3). Pairwise comparisons showed a mean of 1.87% amino acid variation (range 0% to 3.7%).

The phylogenetic tree of Peruvian YFV prM/E sequences showed six different clades that corresponded very closely with the geographic region of virus isolation and represented the states of Puno, Pasco, Junin, Cusco, Ayacucho, and San Martin/Huanuco (Figure 2). Three of the clades were distinguished by signature amino acid substitutions (i.e., coding changes in nucleotide sequences shared by all members of the group). The Puno strains shared a substitution in the premembrane protein (R→K prM102); the Pasco strains showed a triplet residue substitution motif in the membrane protein (A→T M48, A→V M50, and L→F M52); and the San Martin/Huanuco strains shared two residue substitutions within the envelope protein (I→V E72 and H→N E90) (Table 2). The remaining three clades (Ayacucho, Cusco, and Junin) were distinguished by silent nucleotide substitutions.

Because the sequence from one isolate did not group with those of its geographic neighbors (Ayacucho strain 1368, Peru 77b), the identity of this strain was subjected to additional scrutiny. Resequencing from original stock material, as well as consensus sequencing of populations that had been serially passaged three times in Vero cell culture, confirmed that the passaged strain was 100% identical to the parental population and that the sequence shared one of the signature amino acid substitutions characteristic of the San Martin/Huanuco clade. The single isolate from Loreto also showed an anomalous position on the tree; it appeared most closely related to the two Puno strains, which are paradoxically the most geographically distant. Reasons for the anomalous phylogenetic clustering of these isolates remain unclear.

Correlation of Genetic Distance with Geography and Time

Following the approach taken by Bowen et al. (20), we used Mantel's test to assess the strength of correlation between genetic variability, geographic distribution, and the times of virus isolation. We hypothesized that in a virus population circulating as a wandering epizootic, most of the genetic variation in isolates would be because of differences in the times of virus isolation. This finding is in contrast to the pattern of genetic variation expected from an enzootic transmission cycle; if subpopulations of viruses are isolated in discrete enzootic foci, one would expect genetic variation to correlate more closely to geographic rather than temporal distances. Our analysis of the variability among the 25 Peruvian YFVs showed a significant correlation between genetic and geographic distance ($r=0.56$, $Z=22898.51$, $p<0.0001$), as well as between genetic and temporal distances ($r=0.127$, $Z=147.04$, $p<0.05$). The possibility of an interaction effect between geographic and temporal factors clearly exists, which was tested by performing a modified version of Mantel's test. We calculated the partial rowwise Mantel Zr and partial Pearson's r and found that after controlling for geographic distance, the correlation between the genetic and temporal matrices was no longer significant ($r=0.057$, $Zr=0.073$, $p>0.1$). In contrast, the correlation between genetic and geographic distances remained highly significant after controlling for temporal variation ($r=0.55$, $Zr=0.55$, $p<0.0005$). These results suggest that temporal factors alone do not adequately explain the divergence pattern of the Peruvian YFV phylogenetic tree. In contrast, the strong correlation between genetic and geographic distances indicates a high degree of population substructure and suggests that the clades of Peruvian YFVs may be evolving as separate distinct lineages.

Molecular Evolution of YFV

Tests of "clocklike" behavior or uniformity in rates of evolution were introduced into our study as an additional method of assessing the amount of temporal structure in the sequence dataset. Under the molecular-clock assumption, the maximum likelihood branch lengths from the root of the tree to each branch tip are expected to be equal (i.e., evolutionary changes in any lineage are expected to be proportional to elapsed time since divergence from a com-

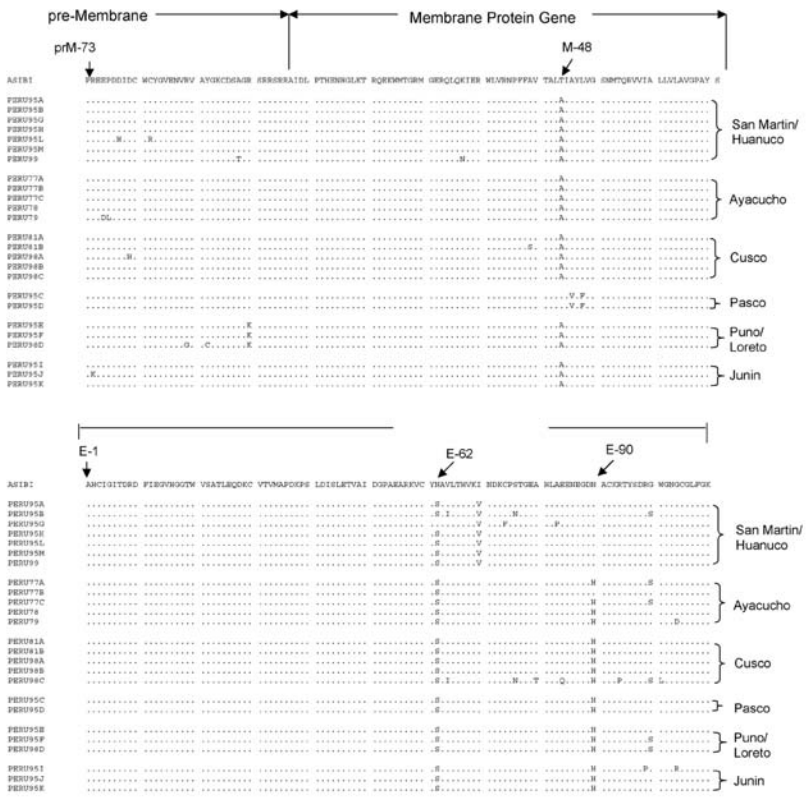

Figure 3. Amino acid alignment for the 25 Peruvian yellow fever virus. Dots indicate identity with prototype Asibi strain (from Ghana, 1927).

mon ancestor). Results of the likelihood ratio tests for a molecular clock indicated that Peruvian YFVs in different subclades are evolving at different rates; trees constructed under the null model, allowing for rate heterogeneity, were assigned a statistically higher likelihood score than trees reconstructed with the molecular clock enforced. The lack of clocklike behavior in the phylogenetic reconstructions is a further indication of the lack of consistent temporal associations among Peruvian YFVs. Small populations are prone to genetic drift and "founders effects," such as transmission bottlenecks; the combined effects of geographic isolation and small population size are the most likely explanations for the different branch lengths for each of the Peruvian clades. Therefore, the observation of different evolutionary rates (i.e., highly variable branch lengths) supports our hypothesis of limited gene flow and intermixing between of the YFV subpopulations in Peru.

Discussion

Previous studies of the molecular epidemiology of YFVs have demonstrated that the virus genome exhibits remarkable stability, with low rates of genetic drift (21–22). The envelope gene region of YFV has been the focus of numerous molecular studies (21–25), and variability within the prM/E gene fragment appears to be representative of the virus genome (24,25). This study supports the observation that the coding sequence of YFV is highly conserved. The prM-E fragments, however, still retained sufficient phylogenetic signal to resolve several distinct geographic clades among the collection of Peruvian isolates. One unanticipated result of our study was the high degree of overall sequence divergence (7.3%) observed among the strains of the 1995 epidemic. The isolates collected during the 1995 epidemic varied at 71 nucleotide positions (10.7%) and represented four geographically distinct clades that could be distinguished by both silent and coding substitutions. This remarkable level of variability among strains from the same epidemic is unprecedented and approaches the threshold level of pairwise divergence (9%) previously used to distinguish the major genotypes of YFV in Africa (25). Molecular epidemiologic studies of related flaviviruses have previously observed very low levels of divergence among isolates from the same epidemic (26–29). Given the estimated substitution rate for YFV of ~5 x 10^{-4}subs/site/y (based on the prM/E sequences of 38 African strains) (25), the numerous substitutions among the 1995 variants probably could not have accumulated during a single transmission season. Thus, our findings suggest that the large-scale epidemic in 1995 most likely resulted from concurrent emergence events in neighboring regions, rather than distribution and spread through epizootic waves of infection.

To our knowledge, our study has analyzed every available YFV isolate from Peru. The first isolates from Peru

Table 2. Signature amino acid and nucleotide substitutions of Peruvian YFV genetic variants, based upon sequence analysis of prM-E gene fragments of 24 isolates

Department	No. of Isolates	Signature substitutions		Coding changes from consensus of Peruvian strains
		nt	Amino acid	
Ayacucho	5	1	0	None
Cusco	5	1	0	None
Junin	3	1	0	None
Puno	2	7	1	R→K prM102
Pasco	2	3	3	A→T M48; A→V M50; L→F M52
San Martin/Huanuco	7	7	2	I→V E70; H→N E90

were obtained in 1977, and epidemic surveillance and outbreak investigations have yielded a total of only 25 isolates since that time. One of the difficulties of conducting molecular epidemiologic studies on the basis of historical samples is the lack of balanced, representative datasets. Given the severe limitations of the number of available YFV isolates from Peru, considerable caution must be exercised when inferring evolutionary implications from the observed genetic variability. Unfortunately, only three of the six Peruvian clades contain representatives from different time points. Strains collected from Ayacucho, San Martin/Huanuco, and Cusco represent intervals of 2, 4, and 17 years, respectively; isolates from these regions have diverged by <0.3% (Figure 2). The stability of these clades over a period of almost 2 decades suggests limited levels of exchange between virus subpopulations of adjacent watersheds. We propose that our ability to discern separate clades is possible because significant gene flow between the subpopulations has not occurred, as one might expect from virus traffic in wandering epizootics.

The original concept of wandering epizootics to explain YFV transmission cycles in South America grew out of observations of the cyclical occurrence and disappearance of outbreaks within particular geographic areas. The concept of continual virus movement has prevailed over the past years in part because of the failure to identify a vertebrate reservoir, and in part because of the dearth of ecologic data to support or refute alternative modes of transmission (29). Epizootics may appear to be wandering if disease incidence peaks in one area, sweeps through a population, then disappears for a period while the focus of transmission moves onward to a distant location. Epidemiologic data on YF incidence in Peru do not support a pattern of virus movement. Figure 1 presents incidence data for YF cases within different departments of Peru and demonstrates that sporadic cases occur on an annual or semiannual basis within tropical and subtropical montane forests (30). These observations indicate that no interval exists that can be construed as an interepidemic period during which the virus is completely absent; hence, transmission most likely occurs continuously within specific locales. While monkey epizootics are frequently reported in association with Brazilian outbreaks of YF (31), no instances of monkey deaths have been reported during Peruvian epidemics. Population subdivision among YFV from different river basins could be explained as an example of vicariant evolution, and as such, would lend further support to the hypothesis that other non-primate vertebrate hosts or vertical transmission by mosquito vectors, plays a role in maintaining an unbroken virus cycle.

Whether the finding of localized enzootic foci is a unique characteristic of YF transmission cycles within the montane regions of the Andes remains unclear. Patterns of focal endemicity of YFV have been suggested for certain areas of Brazil on the basis of repeated annual isolations of YFV from *Haemagogus janthinomys* during the rainy season along secondary roads of the TransAmazon Highway (3). However, the periodic appearance and disappearance of YF outbreaks observed in the eastern regions of Brazil and the genetic variation among Brazilian YFVs indicate that transmission cycles of that region are more broadly distributed than those of Peru (P.F.C. Vasconcelos, pers. comm.). Future molecular typing of YFV isolates from the widespread 2000 epizootic in Brazil will help clarify patterns of virus dissemination.

Climatic changes with increase in rainfall were previously associated with YF epidemic and epizootic episodes (3). Abundant evidence has accumulated regarding the critical role of rainfall and temperature in altering the vectorial capacity of mosquitoes, and such factors may have played a role in the 1995 outbreaks. Interestingly, however, neither excessive rainfall nor increased temperatures associated with El Nino and southern oscillation events were reported during 1995 in Peru (32). One potential factor that may have influenced the 1995 outbreaks was the increase in internal human migration that began in 1994 in association with the opening up of new agricultural and industrial areas in enzootic areas (33). During the early 1990s, the Peruvian government instituted economic reforms that led to rapid privatization of many government-run mining and agricultural operations. Economic dislocations resulted in acceleration of the ongoing movement of highland Indians into the cities of the coast and the settlements of the lowland forests to the east. Mass migrations and increased clearing of land may have changed the exposure status of human populations within endemic

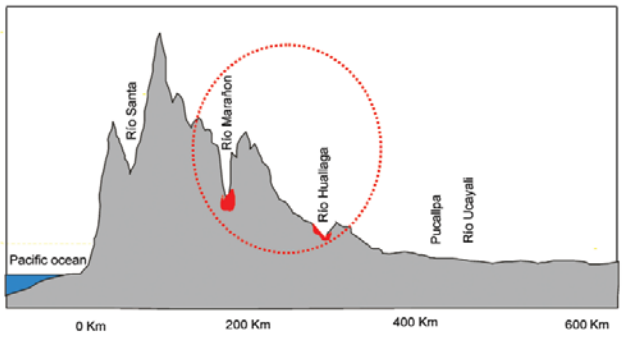

Figure 4. Schematic topographic cross-section through the southern Peruvian Amazon. Areas in red indicate river basins where yellow fever virus is endemic.

regions. Movement of agricultural workers and colonists from areas not endemic for YF into the Amazon Basin is a continuing challenge for public health authorities responsible for immunization programs. The cutting of trees and clearing of forests for agriculture represents a high risk for human infection, since *Haemogogus* species in the rain forest canopy are brought to ground level with subsequent increase in mosquito-human contact. We suggest that the intrusion of humans upon the usually silent sylvatic transmission cycle of the Peruvian Amazon was most likely the underlying reason for multiple concurrent outbreaks that occurred in 1995.

The genetic diversity of Peruvian YFV is best interpreted in light of the remarkable biologic diversity and unique ecologic features of Andean premontane and montane rain forest. Peru is in many respects one of the most diverse countries of the world; the geographic territory of Peru contains 84 of the 103 ecologic life zones proposed by Holdridge for the world (15), and the terrain of the eastern Andean foothills is renowned for its extraordinary topographic complexity and numerous centers where endemic species are found. Estimates published in Instituto Nacional de Salud (30) and Pan American Health Organization (33) documents mention elevational limits of the YF endemic zone from 650 m to 1,000 m or 400 m to 2,000 m, respectively. Our estimates of the geographic origin of YFV isolates in our collection, however, suggest that YF activity may extend to elevations >2,000 m. A considerable portion of the YF endemic zone would therefore be expected to comprise either premontane forest (from 500 m to 1,500 m) or humid montane forest (1,500 m to 3,500 m), as these are the vegetation formations in the states of Amazonas, San Martin, Huanuco, Pasco, Junin, Ayacucho, Cusco, and Puno, and the adjacent portions of Apurimac and Madre de Dio (34). We speculate that the population structure of Peruvian YFV may have resulted from geographic isolation of vertebrate host or mosquito vectors. The complex topography of these regions limits the range and dispersal of many organisms, and may likewise present strong barriers to virus dispersal (Figure 4). As more data become available from biologic surveys of YF-endemic areas, insight into the possible vertebrate reservoirs and arthropod vectors responsible for maintenance of the virus in nature may be gained.

Our study represents an attempt to apply the relatively new techniques of molecular genotyping to address longstanding, intransigent questions regarding YFV transmission cycles in nature. Despite substantial uncertainty regarding the ecologic parameters of the transmission cycle, our results suggest that YFV has a highly restricted pattern of geographic distribution within Peru. The limited number of strains available from Peru indicate that the transmission cycle most likely involves a persistent enzoosis that is either vertically maintained within arthropod populations or that survives as a latent infection within an as yet unidentified vertebrate host. The full extent of the geographic distribution of YFV variants within Peru will become clearer in the future, as additional isolates become available for analysis. The finding that YF transmission remains relatively limited within discrete enzootic foci lends support to the argument for highly focused interventions in the event of future outbreaks.

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Address for correspondence: Alan D.T. Barrett, Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555-0609, USA; fax: (409) 747-2415; email: abarrett@utmb.edu

Molecular Analysis of Echovirus 13 Isolates and Aseptic Meningitis, Spain

Ana Avellón,* Inmaculada Casas,* Gloria Trallero,* Carmen Pérez,† Antonio Tenorio,* and Gustavo Palacios‡

Echovirus 13 (EV13), considered rare, was reported worldwide in 2000, mostly related to aseptic meningitis outbreaks. In Spain, 135 EV13 isolates were identified. The genetic relationships between 64 representative strains from Spain and other reported isolates from the United States, Germany, Italy, Japan, and Sweden were described by analyzing the partial sequence of the major capsid protein (VP1) gene. The strains from Spain were clearly identified as EV13 (79.5% similarity with the EV13 reference strain) and were grouped phylogenetically into two different clusters (by origination on either the Iberian Peninsula or Canary Islands). Isolates from Germany from 2000 clustered with the Canary Islands group. The isolates from other countries obtained before 2000 were genetically distant. Changes in EV13 coding sequence involved several differences in the C-terminal extreme of the VP1 protein. Part of the neutralizing antigenic site III has been described in this genome region in poliovirus and swine vesicular disease virus.

The human enteroviruses (HEVs; *Enterovirus* [ENV] genus, *Picornaviridae* family) are common human pathogens with a worldwide distribution. HEVs are found in temperate climates and especially in the tropics because warm weather favors their spread. Humans are the only known reservoir for HEV, and the main transmission route is fecal-oral. HEV infections may be unapparent or related to different disorders, including encephalitis, pleurodynia, myocarditis, conjunctivitis, or systemic infections in neonates. These viruses are commonly reported in association with aseptic meningitis outbreaks in pediatric patients (1). Although five HEV species exist (2), they are grouped into four clusters (HEV-A, -B, -C, and -D) on the basis of sequence analysis of the major capsid protein (VP1) and the 3′ noncoding region (3). The HEV-B cluster includes coxsackie virus B (CBV), coxsackie virus A9, ENV69, and all echoviruses (EV).

The advent of nucleic acid amplification methods has facilitated the study of the molecular epidemiology of HEV (4–6). Several reverse transcription-polymerase chain reaction (RT-PCR) methods were developed for this purpose, most of which analyze different sequence fragments within the VP1 gene (7–9). Moreover, the amplification and subsequent analysis of the VP1 3′ end partial gene have been successfully used to serotype HEVs (10,11) and to describe the molecular epidemiology of EV30 (12). Analysis of this fragment of the enteroviral genome has permitted the study of important pathogenic structures, such as part of the antigenic sites described for polioviruses (13), CBV, and swine vesicular disease virus (SVDV) (14,15), and part of the canyon structure, related to viral attachment, which has been described in several HEVs and rhinoviruses (16).

The EV strains most frequently isolated in patients with aseptic meningitis are EV30, EV6, EV11, and EV9 (1). Displacement of prevailing lineages on the basis of immune escape (antigenic drift) has been suggested for EV30 as the mechanism to maintain viral circulation levels that increase periodically (12,17,18). Unlike EV30, EV13 has been considered a rare virus (1). Until 1999, isolation data showed very low rates in countries such as the United States (19), Ireland (20), and England and Wales (21). During 2000, EV13 circulation, mostly related to aseptic meningitis, was reported in America (19), Europe (20,22–25), Asia (26), and Australia (27). In Spain, an outbreak of aseptic meningitis attributable to EV13 occurred from February to October 2000 (25), the first identification of this virus in Spain since record keeping began in 1988 (28).

We describe the molecular epidemiology of the EV13 isolates obtained in Spain from the 2000 outbreak by analyzing the VP1 partial sequences of 64 identified viruses. We also compare them with other isolates for which sequences have been deposited in databases. Finally, by analyzing the molecular nature of their changes in nucleotides and amino acids, we attempt to describe the nature of, and reasons for, the actions and epidemiology of EV13.

*Instituto de Salud Carlos III, Madrid, Spain; †Doctor Negrín Hospital, Las Palmas de Gran Canaria, Canary Island; and ‡Columbia University, New York, New York, USA

Materials and Methods

Clinical Specimens

Study specimens consisted of 61 EV13 isolates and three cerebrospinal fluid (CSF) samples obtained as follows. During 2000, the Enterovirus Laboratory (Service of Virology, National Microbiology Centre, Madrid) received 538 isolates for typing from different laboratories within Spain. These viruses were mostly recovered in a human rhabdomyosarcoma cell line with a positive cytopathic effect, which was confirmed by immunostaining with an anti-HEV group-specific antibody (Dako, Glostrup, Denmark). A neutralization test (Lim-Benyesh-Melnick immune serum pools) identified EV13 in 135 (25%) specimens. On the basis of their geographic and temporal distribution (Figure 1), 61 isolates were selected for further study. The selected isolates were obtained from CSF (n=44), stool (n=12), and pharyngeal swab (n=5) samples from patients with aseptic meningitis, except for samples from seven patients who had fever, one who had acute flaccid paralysis, and two healthy persons (contacts of the patients with acute flaccid paralysis). The outbreak was carefully studied in the Canary Islands because of the high rate of infected patients and the context in which the cases occurred, including geographic location, climate, and tourism activity in the area during the year. Three additional CSF samples obtained from patients with aseptic meningitis in March 2000 were supplied by the Microbiology Diagnostic Service (National Microbiology Centre, Madrid, Spain).

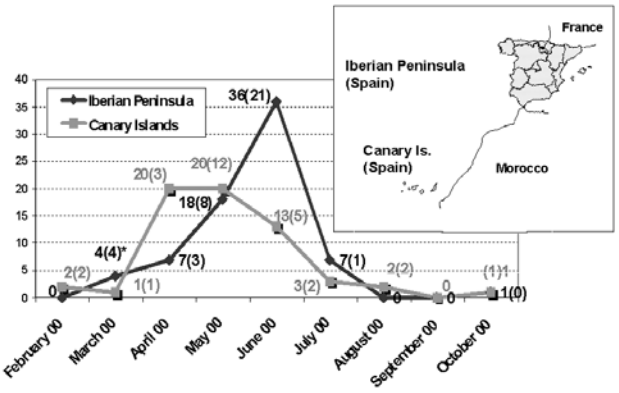

Figure 1. Temporal distribution of the Spanish echovirus 13 isolates during 2000. The isolates were grouped attending to their geographic origin as from the Iberian Peninsula and from the Canary Islands. The number of viruses included in the study (in parentheses) as well as the total isolates per month are shown. *Includes three sequences obtained directly from cerebrospinal fluid.

Amplification

The VP1 3′ end genome region, successfully used to study the molecular epidemiology of other HEVs (7,12), was amplified in all 64 isolates. A 609-nucleotide (nt) fragment was amplified by using the RT-nested PCR method (10) with a total of 5 µL of each 1:10 diluted cell culture isolate. A fragment of the expected length was also obtained in the three additional CSF samples, previously extracted with guanidinium thiocyanate lysis buffer (29).

The VP1 3′ end fragment was useful in differentiating and studying the epidemiology of HEVs. However, since our main goal was to compare the sequences of the Spanish isolates with those of isolates from the rest of the world, we designed a specific PCR to amplify the VP1 5′ end gene, where a collection of isolates from Germany had been sequenced (22). For that purpose, specific primers of EV13 were designed by using the multiple alignments constructed with the available EV13 sequences employing Clustal X software (free software available from: URL: http://www-igbmc.u-strasbg.fr/BioInfo/). The primer sequences were as follows: EV13_VP1sense (5′–3′) TGAGACAGGGCACACATC; EV13_VP1anti (5′–3′) GCTAATGAATGGGATGGACAT. Its relative positions to EV11 polyprotein genome (X80059) were 2560–2577 and 2999–3019, respectively. Single-step RT and amplification was performed by using the Access RT-PCR kit (Promega Corp., Madison, WI). Five microliters of selected 1:10 diluted cell culture isolates was added to the reaction mixture which contained the following: 10 µL of 5X reaction buffer; 2 mM magnesium sulfate; 250 µM each of dATP, dCTP, dGTP, and dTTP; 0.4 µM of each EV13_VP1sense and EV13_VP1 anti-primers; 1 U of avian myeloblastosis virus RT; and 1 U of thermus flavus DNA polymerase and Rnase-free distilled water to a final volume of 50 µL. Amplification was performed in a PTC-200 Peltier thermal cycler (M.J. Research, Inc., Waltham, MA), programmed for a first RT step of 45 min at 48°C, followed by 2 min at 94°C, and for 45 cycles of 30 sec for denaturation at 94°C, 2 min for annealing at 60°C, and 30 sec for elongation at 68°C. Elongation was extended for 5 additional minutes in the last cycle. PCR products were detected by electrophoresis on 2% agarose gels stained with ethidium bromide. A 460-nt fragment was amplified in 14 representative selected Spanish isolates.

Sequencing and Sequence Analysis

Cycle sequencing reactions of products from both RT-PCR assays were performed by using the Big Dye terminator kit (Applied Biosystems, Foster City, CA). Ambiguities were resolved by sequencing both sense and antisense strands.

The 423-nt VP1 3′ end fragment (nt 2912–3334, according to the EV11 polyprotein genome, X80059) was analyzed through the multiple sequence comparison of the 64 viral sequences from Spain, the EV13 and ENV69 pro-

totype strains (Del Carmen and Toluca-1, respectively), and the isolates VA86-6776 (30) and TX95-2089 (7). For the analysis of the 279-nt VP1 5′end fragment (nt 2609–2887, according to the EV11 polyprotein genome, X80059), sequences available from GenBank of the following isolates were included: 12 isolates from Germany, obtained during 1965, 1974, 1976, 1979, 2000, and 2001; one isolate each from Italy (1996) and Sweden (1999), and two from Japan (2001). This second group of sequences was compared with the corresponding VP1 5′ end gene of 14 Spanish representative isolates selected according to their geographic origin (four from the Canary Islands and within the Iberian Peninsula, four from the north, four from the center of the peninsula, and two from the east).

Multiple sequence alignments were built with Clustal X. For the molecular typing, Spanish VP1 3′ end gene sequences were compared pairwise with the HEV prototype strains to obtain the identity score (11). The percentage of similarity of the isolates with respect to the reference strain was calculated through Megalign software (DNASTAR, Madison, WI).

The phylogenetic tree in Figure 2 was reconstructed through the neighbor-joining method (MEGA version 2.1 software package; available from URL: http://www.megasoftware.net) by using Kimura two parameters as substitution model, with statistical significance of phylogenies estimated by bootstrap analysis with 1,000 pseudoreplicate datasets. The transition-transversion ratio employed (TRatio=2) was estimated through the Tree-puzzle version 5.0 software (available from: URL: http://www.tree-puzzle.de)**.** Genetic distances were calculated using the same model of nucleotide substitution. The distance matrix was recorded and the pairwise observed distance values were employed to define different subgroups and to build the histogram in Figure 3. The analysis of variance one-way test, with a statistical significance value of 0.05, was used to compare the means of the pairwise observed distance value matrix. To reconstruct the phylogenetic tree shown in Figure 4, the maximum-likelihood method (DNAML program, PHYLIP software [available from: URL: http://evolution.genetics.washington.edu]), was used, with hidden Markov as the model of nt substitution. A Poisson correction model was used to compare the amino acid sequences.

Three-Dimensional Structure

The VP1 amino acid changes of the Spanish isolates with respect to the EV13 reference strain were projected onto the three-dimensional structure of EV11 (GenBank 1H8TA) (31) by using the program Cn3D (available from: URL: http://www.ncbi.nlm.nih.gov/Structure). Neutralizing antigenic sites previously reported for poliovirus (13), CBV4 (32), and CBV5 and SVDV (14,15) were localized in the structure. Amino acids were numbered according to EV11 sequence.

Nucleotide Sequence Accession Numbers

Fourteen complete gene sequences of Spanish EV13 VP1 were submitted to the GenBank database under accession numbers AY227334–AY227347. Fifty Spanish EV13 VP1 3′ end fragments were also submitted under accession numbers AY227284–AY227333. These EV13 sequences were also included in this study: AF081327 (Del Carmen), AJ309256 (Roma96), AF401360 (Halle74), AF401359 (Postdam76), AF401358

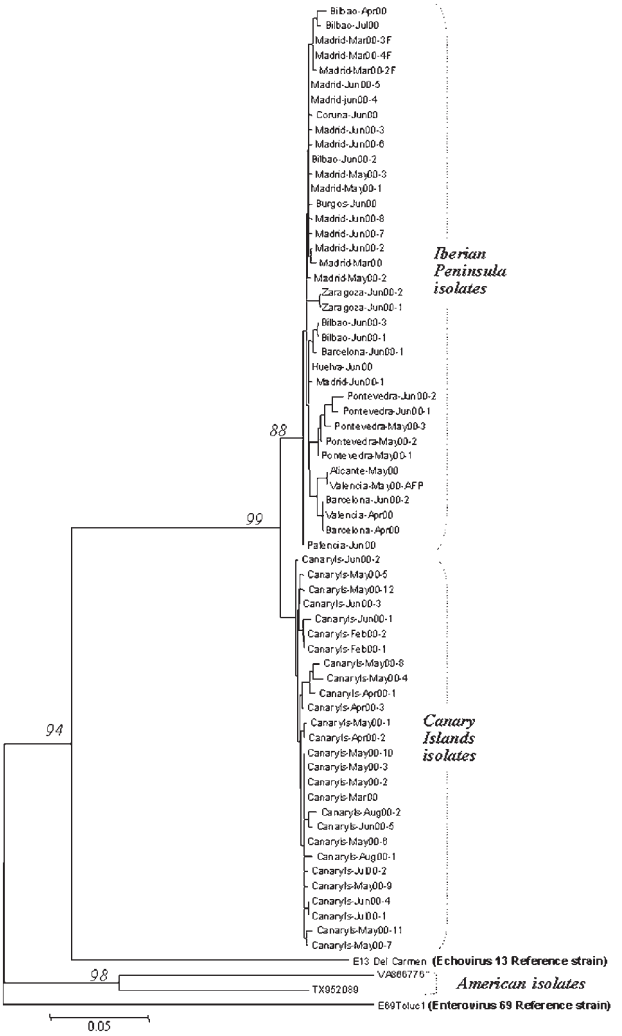

Figure 2. Phylogenetic tree of the VP1 3' terminal region, which identifies the Spanish isolates as echovirus 13 (EV13) and differentiates two clusters (Iberian Peninsula and Canary Islands). The sequences included are, apart from the reference strains, all the Spanish EV13 (61 isolates and 3 sequences obtained directly from cerebrospinal fluid) and two American isolates. Model of nucleotide substitution: Kimura two parameters. Phylogenetic tree reconstructed with the neighbor joining method, and bootstrap analysis with 1,000 pseudoreplicate datasets.

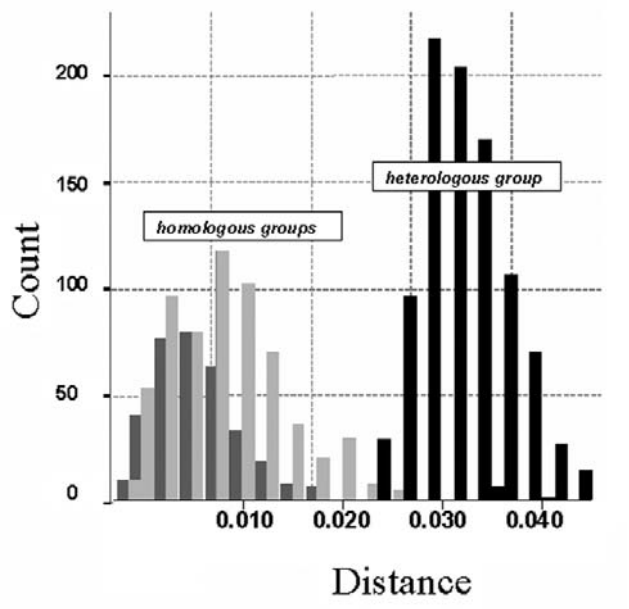

Figure 3. Histogram of the observed distances (Kimura two parameters method) within Canary Islands and Iberian Peninsula groups (dark gray) and between them (light gray). Analysis of variance (ANOVA) (one-way) test results: F=5238 (p=0.000). Distances within homologous groups: Canary Islands: mean 0.008 (standard deviation [SD] 0.004; n=351); Iberian Peninsula: mean 0.010 (SD0.006; n=666). Distances between heterologous groups (black): mean 0.033 (SD 0.005; n=999).

(Postdam79), AF401357 (Berlin65), AF401356 (Chemniz00), AF401355 (Krefeld00), AF401354 (Stuttgart00), AF401353 (Ulm00), AY007223 (Weissenfels00), AF295467 (Sweden99), AF152299 (VA86-6776), AF081635 (TX95-2089), AB092985 (JA1CR0210F), AB092984 (JA1ON022R), AY131288 (KASS31001), AF538285 (DRES41001), and AF538284 (MARD37701). The sequence AF081349, corresponding to the ENV69 strain Toluca-1, was also included as the nearest possible taxon and outgroup. GenBank accession numbers of the rest of the VP1 partial gene of HEVs prototype strains have been detailed previously (7).

Results

Molecular Typing of Isolates

A preliminary multiple sequence alignment, as described by Palacios et al. (11), grouped all the Spanish viruses with the EV13 reference strain with 79.5% identity (SD [standard deviation] 0.37). ENV69 reference strain was the nearest taxon with 74.3% (SD 0.33) identity; the rest of the HEVs shared <71%. In addition, pairwise sequence distance comparison showed that all the isolates from Spain differed from the EV13 and ENV69 reference strains with observed nucleotide distances of 0.260 (standard error [SE] 0.028) and 0.344 (SE 0.032), respectively. Consequently, all the Spanish isolates were classified as EV13, in agreement with the neutralization test results.

Comparison of the Nucleic Acid of the Spanish Sequences

Spanish isolates grouped into two major clusters corresponding to their respective geographic origin (Iberian Peninsula and Canary Islands) with statistical significance (Figures 2 and 3). The 10-nt changes that determined the groups were all in third codon position. The nucleotide distances observed within the Canary Islands sequence group ranged from 0 to 0.022, whereas within the Iberian Peninsula group, the range was from 0 to 0.029. The distance range obtained after comparison of sequences belonging to these different groups was 0.024–0.052. The study of the temporal pattern of the Spanish outbreak suggests that, although all the Spanish isolates have a common source, the group from the Canary Islands might be closer to the ancestor.

Comparison of the Spanish Isolates with Other Available EV13 Sequences

EV13 viruses isolated in 2000 and 2001 from Germany, Japan, and Spain were phylogenetically close. Within all of the studied sequences, the more similar ones, with a nucleotide distance of 0.011, corresponded to the strains isolated in Germany and the Canary Islands in 2000. The

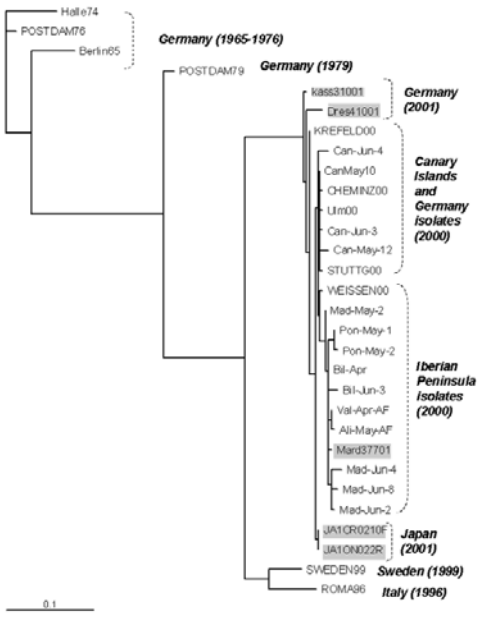

Figure 4. Phylogenetic tree of the 5' VP1 extreme showing the relationship between the Spanish isolates and others. The sequences included are the 14 Spanish echovirus 13 selected isolates as well as the German (n=12), Italian (n=1), Swedish (n=1), and Japanese (n=2) isolates. Phylogenetic tree reconstructed with the maximum-likelihood method, 50 Jumble. Gray sequences correspond to the 2001 isolates.

study of the temporal pattern by the maximum-likelihood approach also grouped the German 2000 isolates with the Spanish ones (Figure 4).

For the European strains, obtained before 2000, the isolates were genetically more distant from the Spanish isolates; the 1999 strain from Sweden was the second most similar to the Spanish isolates, followed by the isolate from Italy (1996) and those from Germany (1979, 1976, and 1965). The two strains from the United States (VA86-6776 and TX95-2089) were the most distant from the Spanish isolates, with a genetic distance of 0.345 and 0.311, respectively (Figure 2). These two viruses seemed to be as similar to ENV69 as to EV13. Further sequence analysis of other strains of American EV13 and additional ENV69 isolates could clarify this point. However, although no other ENV69 sequences were available for comparison, we could confirm that these sequences did not cluster into the same evolutionary lineage of all other published EV13.

Comparison of Amino Acid Sequences

Several amino acid differences could be observed between the Spanish isolates and the EV13 reference strain. The EV13 neutralization sites have not been determined. However, they have been reported for other ENVs and some of them (sites I and III) are in the same position and have the same amino acids involved. For this reason, the amino acid changes noticed in this study and neutralization sites previously reported for other ENVs were projected on the three-dimensional EV11 structure (Figure 5). The possible relationship between them was detailed; however, we can only speculate about whether EV13 neutralization sites were affected or not. Two changes, A100V and F238I, were structurally close to known neutralization site I described for poliovirus (13), CBV4 (32), and CBV5 and SVDV (14). However, in most cases the amino acid differences were located in the VP1-2A junction (S274A, T276P, N284S, P285T, A286G, S287G, K288R, M290V, N291T, and H292N), affecting a region that was considered part of the neutralizing antigenic site III for SVDV (15). The amino acid changes (S274A and T276P) implied a change in the properties from hydrophilic to hydrophobic residues. These two positions were also different from the reference strain in the two U.S. isolates; however, they did not result in amino acids with hydrophobic characteristics (T276D/E).

Apart from the American isolates, the comparison of the amino acid sequence for EV13 isolates with respect to the reference strain was only possible in the amino terminal part of the VP1 protein. In this region, changes were identified in the strains that dated from 1965 to 1979, mostly affecting amino acids that have been considered as part of the neutralizing antigenic site I in poliovirus (D84S and A85D/E) (13). Only one amino acid (M216I) differed between the two Spanish clusters. This position was not related to any known antigenic site.

Discussion

After a 12-years period in which EV13 circulation in Spain was not detected, a virus identified through neutralization test as EV13 began to be isolated in CSF of patients with aseptic meningitis. These isolates were also clearly identified as EV13 serotypes through molecular typing by their substantial homology with the EV13 Del Carmen

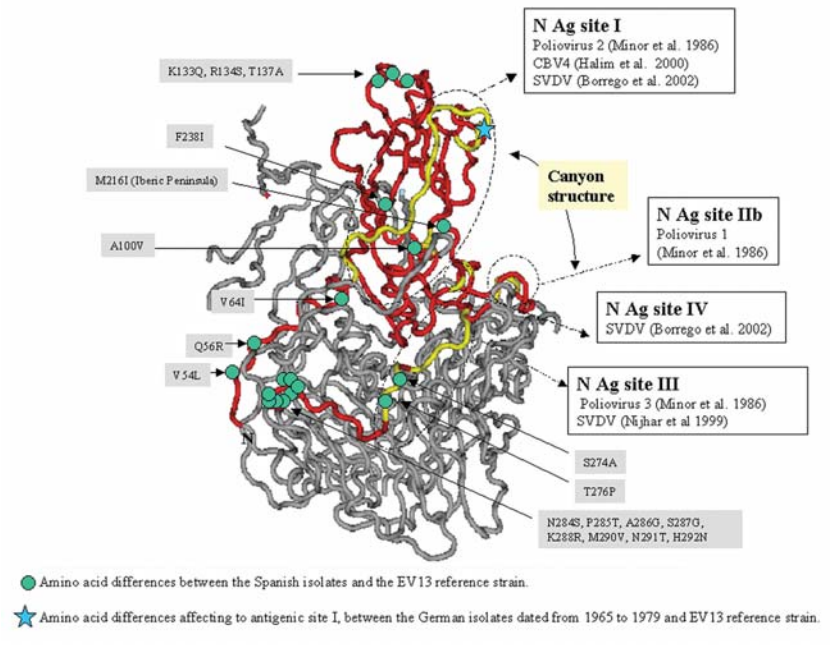

Figure 5. Mapping of the echovirus (EV) 13 VP1 amino acids. The three-dimensional structure is projected onto EV11 structure (GenBank accession no. 1H8TA) with VP1 amino acid numbering according to EV11. The sequenced fragment is shown in red. Previously reported neutralizing antigenic sites for poliovirus, CBV4, and SVDV are shown in yellow. The enterovirus canyon structure is depicted in a similar location to the canyon structure of poliovirus as reported by (40).

prototype strain. The viruses could also be identified directly from most of the CSF samples assayed (three of them included in this study; other data not shown) without the need of isolation, demonstrating the utility and reliability of the ENV molecular typing according to the VP1 homology with respect to the reference strains (11). Indeed, the molecular study was successfully completed, despite the high degree of genetic differences between the EV13 recent isolates and the prototype strains of EV13 and ENV69, which were isolated in the Philippine Islands in 1953 and in Mexico before 1973, respectively.

Phylogenetic analysis of the nucleotide sequences showed that the Spanish strains were clustered into two distinct groups, those from the Canary Islands and those from the Iberian Peninsula, coinciding with their geographic origin. The first aseptic meningitis case caused by EV13 was described in the Canary Islands in February 2000. The distinctness of the groups was indicated by several silent nucleotide changes and one amino acid change, which apparently developed in a short period (the initial isolate of both groups was obtained about 1 month apart); for this reason, we cannot rule out the possibility that both groups could have had a common form but different ancestor. The special climate of the Canary Islands could explain the winter appearance of the cases (first case February 14).

When both Spanish clusters were genetically compared with isolates from other geographic origins, the EV13 strains from the Canary Islands appeared more closely related to the German isolates of 2000. This may reflect the introduction of EV13 from the Canary Islands into northern Europe (or vice versa) through tourist travel. The European isolates (Swedish, Italian, and German) obtained before 2000 presented nucleotide distances in respect to the Spanish isolates that increased over time. On the other hand, the two American strains, obtained in 1986 and 1995, showed the most distance between isolates. Indeed, the Spanish isolates showed larger differences with respect to the American isolates than those identified with the rest of the available EV13 sequences. In addition, the American strains of EV13 seem to be unrelated to the EV13 strains reported in the rest of the world; these American strains may represent another evolutionary lineage of this serotype. As a result, European and American EV13 strains could be assigned to different subgroups, in contrast to EV30 strains, which do not seem to be restricted geographically (12). However, more U.S. isolates from the same period would be necessary to define a consistent subgroup. Yet, preliminary data show that the reported U.S. isolates from 2001 may be genetically close to the European isolates (33).

The correlation between the genetic distance and the time parallels the behavior of such other epidemic viruses as EV30 (12), EV7 (34), EV25 (5), and ENV71 (35). However, these viruses usually maintain their isolation rates and increase their circulation periodically (28). In contrast, EV13 was isolated infrequently throughout the world until 2000, when isolation rates increased dramatically in several countries in association with aseptic meningitis outbreaks. This type of presentation was previously described in other HEVs such as EV4, which caused a large aseptic meningitis outbreak in 1997 in Israel (36). This behavior may be due to a genetic and antigenic drift that changes the virologic properties of the isolates. However, EV13 may simply be rare, and artifacts of surveillance or identification may have led to its seemingly sudden appearance. In consequence, viruses could have increased in virulence or improved their pathogenic mechanisms by antigenic change, despite the fact that they were able to be neutralized in vitro with horse-specific serum. However, further study is needed to investigate their antigenic characteristics, the human immune response to EV13, and whether a change in virulence occurs. The study of the VP1 genome region permitted us to observe the characteristics of the major capsid protein, which may be involved in this type of process, together with the rest of structural proteins. However, other genome changes, including those affecting replication (37,38), may also be implicated; thus, to understand the molecular epidemiology of HEV outbreaks, molecular studies of other regions of the genome are indicated.

Changes in EV13 coding sequence involved several differences in the C-terminal extreme of the VP1 protein, known to be highly variable and containing neutralizing antigenic site III in SVDV (15). Similar alterations have been related to positive selection affecting other ENV as EV30 (12), determining its evolution over the time. These amino acid changes in EV13 VP1 protein could be involved in the notable increase in the number of isolations of these viruses, since they were able to cause aseptic meningitis outbreaks worldwide (19–23,25,27,39). Moreover, although the isolates were successfully neutralized in vitro with the Lim-Benyesh-Melnick immune serum pools, the amino acid changes affecting VP1 should be taken into account during the evaluation of the neutralization test results. The only amino acid change between the two mentioned Spanish groups (M216I), which entails a change of a hydrophilic amino acid into a hydrophobic one, might confer some biologic advantage. It is not related to any neutralizing antigenic site previously described for ENVs, but might be related to the canyon structure, since this position is located in the floor of this structure in polioviruses (40). However, further research on the interaction between the cellular receptor and EV13 canyon should address this point. The canyon structure is a capsid surface depression described in several ENVs and rhinoviruses (16) that has been reported to join different cel-

lular receptors (41). Recently, studies of the EV11 receptor (31) indicate that differences of few amino acid residues of the viral surface affecting the canyon structure may change the infectivity of the virus dramatically.

The molecular epidemiology of EV13 presents similarities and differences with other HEVs. The studies to date have demonstrated an epidemic pattern in viruses related with outbreaks such as ENV70 and EV30. This pattern suggests a unique strain of virus causing outbreaks all over the world similar the epidemics attributable to influenza A and B viruses.

However, since the human population is believed to be the only ENV reservoir, this pattern should be completed with the continuous isolation of the agent over nonepidemic years. This type of circulation has been observed in several HEVs, such as EV30 or EV9 but has not been observed in the case of EV13 or other rare HEVs. However, EV13 may have been circulating in the population at undetectable levels or in a nonpathogenic way. Since HEVs are usually typed only when they are related to unexpected outbreaks, a nonpathogenic virus could circulate in the population for a number of years before causing outbreaks.

The circulation of EV13 in Spain seem to have suddenly stopped. Since October 2000, over 700 ENV isolates have been typed in the Spanish Enterovirus Reference Laboratory, and only 1 imported EV13 isolate (data not shown) was detected in 2001 in a tourist from the Czech Republic who was visiting the Canary Islands.

Acknowledgments

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Dr. Avellón, a physician and medical clinical laboratory specialist, is a postdoctoral fellow at the Spanish National Poliovirus Laboratory. Her research interests include the molecular detection and epidemiology of enteroviruses.

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Address for correspondence: Ana Avellón, Virology Service, National Center of Microbiology, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km. 2, Majadahonda 28220, Madrid, Spain; fax: 91 5097966; email: aavellon@isciii.es

Molecular Characterization of a Non–*Babesia divergens* Organism Causing Zoonotic Babesiosis in Europe

Barbara L. Herwaldt,* Simone Cacciò,† Filippo Gherlinzoni,‡ Horst Aspöck,§ Susan B. Slemenda,* PierPaolo Piccaluga,‡ Giovanni Martinelli,‡ Renate Edelhofer,¶ Ursula Hollenstein,# Giovanni Poletti,‡ Silvio Pampiglione,‡ Karin Löschenberger,¶ Sante Tura,‡ and Norman J. Pieniazek*

In Europe, most reported human cases of babesiosis have been attributed, without strong molecular evidence, to infection with the bovine parasite *Babesia divergens*. We investigated the first known human cases of babesiosis in Italy and Austria, which occurred in two asplenic men. The complete 18S ribosomal RNA (18S rRNA) gene was amplified from specimens of their whole blood by polymerase chain reaction (PCR). With phylogenetic analysis, we compared the DNA sequences of the PCR products with those for other *Babesia* spp. The DNA sequences were identical for the organism from the two patients. In phylogenetic analysis, the organism clusters with *B. odocoilei*, a parasite of white-tailed deer; these two organisms form a sister group with *B. divergens*. This evidence indicates the patients were not infected with *B. divergens* but with an organism with previously unreported molecular characteristics for the 18S rRNA gene.

Babesiosis is a tick-borne zoonosis caused by intraerythrocytic protozoa of the genus *Babesia* (1,2). The world's first well-documented human case of babesiosis was a fatal case in 1956 in an asplenic man in the former Yugoslavia (3). Since then, hundreds of human cases of babesiosis have been reported in the United States, approximately 30 cases have been reported in Europe (4–7), and a few cases have been reported elsewhere.

Most of the reported U.S. cases have been caused by infection with *Babesia microti*, a parasite of small mammals transmitted by *Ixodes scapularis* ticks. Most European cases have been attributed to *B. divergens*, a parasite of cattle transmitted by *I. ricinus*. However, from 1991 through 2000, additional zoonotic *Babesia* and *Babesia*-like pathogens have been identified and characterized with molecular techniques. These pathogens include, in the United States, the WA1- (for "Washington 1") and CA1- (for "California 1") type parasites and the MO1 (for "Missouri 1") parasite (8–10) and, in Europe, the organism we describe here.

We report what to our knowledge are the first described human cases of babesiosis in Italy and Austria and provide evidence that the etiologic agent of the two cases, which is related to but clearly not *B. divergens,* has molecular characteristics that have not previously been reported. Following the precedent we previously established for reports of newly characterized organisms in the United States, we refer here to this organism from Europe as EU1 (for "European Union 1").

Methods

Serologic Testing

Serum specimens from the patients were tested at the Centers for Disease Control and Prevention (CDC) in serial fourfold dilutions by indirect fluorescent antibody (IFA) testing for reactivity to *B. microti* (11), WA1 (8), and *B. divergens* antigens. The antigen sources were human isolates of *B. microti* and WA1 and a bovine isolate of *B. divergens* (the Purnell strain from the Republic of Ireland [12]) that had been passaged in gerbils (Mongolian jirds; *Meriones unguiculatus*) and adapted to culture in bovine erythrocytes. The serum specimens were also tested at the Clinical Institute of Hygiene of the University of Vienna by IFA for reactivity to *B. divergens* antigens (from a bovine isolate from Hanover, Germany, that had been passaged in jirds); the dilutions of serum that were tested were 1:16, 1:64, 1:256, 1:1,000, and 1:4,000.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Istituto Superiore di Sanità, Rome, Italy; ‡University of Bologna, Bologna, Italy; §Clinical Institute of Hygiene of the University of Vienna, Vienna, Austria; ¶University of Veterinary Medicine of Vienna, Vienna, Austria; and #University Hospital for Internal Medicine I, Vienna, Austria

Animal Inoculation

Five jirds, which are competent hosts for *B. divergens* (13), were injected intraperitoneally with 0.5 mL of 1-day old, refrigerated, pretreatment blood from the Austrian patient. Animal experimentation guidelines were followed. The jirds were monitored periodically (at least weekly; 26 times in 17.5 weeks) for parasitemia by examination of Diff Quik-stained (DADE AG, Düdingen, Switzerland) smears of blood obtained either by tail snip or, at the end of the monitoring period, by cardiac puncture after anesthesia with ketamine. The blood obtained by cardiac puncture was also examined by polymerase chain reaction (PCR) (see below).

DNA Extraction, Amplification, and Sequencing

DNA was extracted from EDTA-stabilized whole blood from the two patients by using the QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA); the DNA was stored at 4°C. The complete 18S ribosomal RNA (18S rRNA) gene was amplified by PCR, with a pair of generic apicomplexan 18S rRNA-specific primers: CRYPTOF, the forward primer (5′-AACCTGGTTGATCCTGCCAGT-3′), and CRYPTOR, the reverse primer (5′-GCTTGATC-CTTCTGCAGGTTCACCTAC-3′). PCR was conducted with the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). The conditions for PCR included 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1.5 min. Final extension was done at 72°C for 9 min, followed by a hold step at 4°C. Amplification products were purified by using the StrataPrep DNA Purification Kit (Stratagene, La Jolla, CA). In addition, DNA provided us that had been extracted from two isolates of *B. odocoilei* (i.e., the Brushy Creek and Engeling isolates [14]), a parasite of white-tailed deer (*Odocoileus virginianus*) (15,16), and from *B. divergens* (Purnell strain [12]) was analyzed.

Both strands of the PCR products were sequenced by using a set of internal primers. Sequencing reactions were conducted with the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), and reactions were analyzed on the ABI 377 or ABI 3100 automatic DNA sequencer (Applied Biosystems). The resulting sequences were assembled by using the program SeqMan II (DNASTAR, Inc., Madison, WI). The GenBank accession numbers for the complete sequences we generated of the 18S rRNA gene for the various organisms are as follows: *B. odocoilei*, AY046577; *B. divergens*, AY046576; and EU1, AY046575.

Phylogenetic Analysis

The complete sequences of the 18S rRNA genes for *B. bigemina*, *B. bovis*, *B. caballi*, *B. divergens*, *B. gibsoni*, *B. odocoilei*, and *Babesia* sp. (isolated from *Bos taurus*) were retrieved from the GenBank database (see Figure 1 legend for GenBank accession numbers) and aligned with the sequence for EU1 by using the program CLUSTAL W version 1.83 (17). The 18S rRNA sequence for *Theileria annulata* was included as the outgroup for the phylogenetic analysis. This analysis was performed with the following programs: the PHYLIP package, which includes version 3.573c of CONSENSE, DNADIST, DNAML, NEIGHBOR, and SEQBOOT (18); and version 5.1 of TREE-PUZZLE (19). The phylogenetic trees inferred by these programs were drawn by using the program TreeView, version 1.6.6 (20). The trees were statistically evaluated by using bootstrap (18) and quartet puzzling methods (19).

Case Reports

The Italian and Austrian patients were 55- and 56-year-old men, respectively, who had undergone splenectomy in

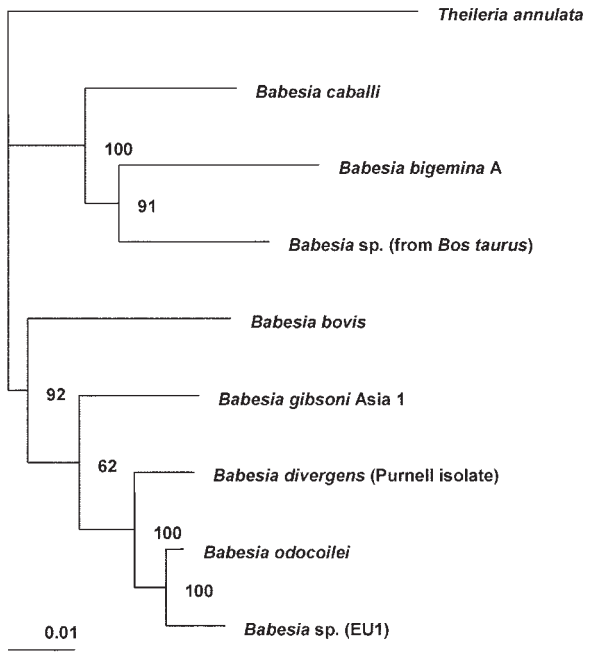

Figure 1. Phylogenetic tree for the complete 18S rRNA gene from selected *Babesia* spp. The tree was computed by using the quartet puzzling maximum likelihood method of the TREE-PUZZLE program and was oriented by using *Theileria annulata* as the outgroup. Numbers at the nodes indicate the quartet puzzling support for each internal branch. Scale bar indicates an evolutionary distance of 0.01 nucleotides per position in the sequence. Vertical distances are for clarity only. The GenBank accession numbers for the sequences used in the analysis are as follows: *Babesia bigemina* A, X59604; *B. bovis*, L19077; *B. caballi*, Z15104; *B. divergens* (Purnell isolate [12]), AY046576; *B. gibsoni* (genotype Asia 1), AF175300; *B. odocoilei* (Brushy Creek and Engeling isolates [14]), AY046577; *Babesia* sp. (isolated from *Bos taurus*), U09834; EU1 (the etiologic agent of infection in the two cases described here), AY046575; and *Theileria annulata*, M64243.

the 1980s because of stage IA Hodgkin's disease. The Italian patient had recently begun chemotherapy (Table footnote) for stage IIIA diffuse large B-cell lymphoma, which had been diagnosed in June 1998. Both men lived in small towns and hunted avocationally (Table); neither had pets. Only the Austrian patient recalled tick exposure—a tick bite while hunting about 2 weeks before he noticed his urine was dark. Neither patient had traveled extensively: the Italian patient had never left Italy, and the Austrian patient had been in Barbados (1998) and Turkey (1999).

The two cases ranged in severity from quite mild (Austrian case) to moderately severe (Italian case). The salient clinical details of their cases and the relevant laboratory values are provided in the Table. Fever occurred only in the Italian patient (maximum of 39°C), which initially was considered a reaction to one of his chemotherapeutic agents (i.e., bleomycin). He also had marked anemia, for which he received blood transfusions (Table). Both patients had thrombocytopenia, elevated serum lactate dehydrogenase and bilirubin values, and dark urine from hemoglobinuria. The Italian patient's creatinine value also was elevated.

In both cases, babesiosis was diagnosed by noting parasitic inclusions in erythrocytes on peripheral blood smears (Table; Figure 2). The intervals between onset of the symptoms that ultimately were attributed to babesiosis and confirmation of the diagnosis ranged from 2 days (Austrian case) to 10 days (Italian case). Subsequent testing of serum specimens from both patients showed IFA reactivity to *B. divergens* but not to *B. microti* antigens; serum from the Italian patient was also tested for reactivity to WA1 antigens and was negative. Attempts to obtain an isolate of the parasite that infected the Austrian patient, by injecting specimens of his blood into jirds, were unsuccessful; the smears of blood from periodic tail snips and PCR analysis of blood obtained by cardiac puncture of the jirds were negative. Both patients responded to antimicrobial therapy for babesiosis: the Austrian patient was treated with clindamycin, and the Italian patient was treated with both clindamycin and quinine (Table).

Molecular Data

Amplification of the complete 18S rRNA gene, by using generic protozoan primers and the *Babesia* DNA extracted from the patients as the templates, yielded a specific product of approximately 1,700 base pairs for each patient. Sequence analysis showed that the 18S rRNA gene was 1,727 bases long and that the PCR products from the two patients had identical sequences. BLAST (available from: URL: http://www.ncbi.nlm.nih.gov/BLAST/) search showed that the sequence, although clearly from a *Babesia* sp., was not identical to any complete 18S rRNA sequences in the GenBank database. In phylogenetic analysis, EU1 clusters together with *B. odocoilei*, and these two organisms form a sister group with *B. divergens* (Figure 1). The clustering of these organisms was identical, regardless of which phylogenetic method was used. The associations were strongly supported statistically. Support for the internal branch leading to the *B. divergens*, *B. odocoilei*, and EU1 group was 100% with both quartet puzzling and bootstrapped distance analysis; for the internal branch separating *B. divergens* from *B. odocoilei* and EU1, the support was 100% for quartet puzzling and 88% for bootstrapped distance analysis. The alignment of the sequences used to construct the phylogenetic tree (Figure 1) is available from the authors upon request.

Because the complete 18S rRNA sequences in GenBank that were previously determined for various bovine isolates of *B. divergens* were not identical, we reanalyzed the complete 18S rRNA gene from isolates (cultures or DNA) from Ireland (Purnell [12]; GenBank accession no. U16370), Germany (U07885 [21]), and Northern Ireland (Z48751) that were provided to us. The sequences of the 18S rRNA gene we obtained for these isolates were identical, which suggests that no variability is present in this gene among geographically distinct bovine isolates of *B. divergens* (Slemenda et al., unpub. data). In contrast, the EU1 and *B. divergens* 18S rRNA sequences differed by 31 bases.

Similarly, our sequences of the 18S rRNA gene for both isolates of *B. odocoilei* (i.e., Brushy Creek and Engeling isolates) were identical to each other (GenBank accession no. AY046577) and to the *B. odocoilei* sequence with the GenBank accession no. U16369 (14). The EU1 and *B. odocoilei* 18S rRNA sequences differed by 29 bases.

Discussion

We investigated the first reported human cases of babesiosis in Italy and Austria and have provided molecular evidence that the etiologic agent was a previously uncharacterized *Babesia* organism, which we refer to here as EU1. The organism was found in countries in Europe not previously known to have zoonotic babesiosis and had novel molecular characteristics for the genetic marker we analyzed, the complete 18S rRNA gene. Sequence analysis of this gene provides an objective and precise means of species identification and phylogenetic classification. The DNA sequences of the 18S rRNA gene were identical for the *Babesia* organisms from the two patients, which indicates that they were infected with the same organism. Each of the organisms was sequenced in a different country, which indicates that the findings were not artifactual.

The phylogenetic analysis (Figure 1) indicates that EU1 is most closely related to but distinct from *B.*

Table. Characteristics of two men who had babesiosis in 1998 and 2000, respectively[a]

Characteristics	Italian patient[b,c]	Austrian patient
Residence and outdoor activities	Lived in northern Italy, in a small town in the district of Romagna, on ~1 hectare of land; often hunted moles in his garden, even after he started chemotherapy	Lived in northeastern Austria, in a small town in the district of Krems Land, in the province of Lower Austria; had an off-site garden; often hunted in the Dunkelsteinerwald forest (usually wild boars, sometimes foxes and badgers)
Clinical illness and general laboratory data		
Initial clinical manifestations	Fever (39°C) and chills developed on October 14, 1998; hospitalized on October 18 because of fever, headache, confusion, jaundice, and dark urine (discharged on November 6)	Marked fatigue developed on July 23, 2000; dark urine, without dysuria, developed on July 24; hospitalized on July 25 (discharged on August 2)
Hematologic parameters[d]		
Hemoglobin (g/dL)	4.8	15[e] (13.2 on July 27, 2000)
Leukocyte count (x10^9/L)	4.4	7.3 (7.8, with 5% atypical lymphocytes, on July 26)
Platelet count (x10^9/L)	71	15 (8 on July 27)
Values of serum chemistries[d]		
Lactate dehydrogenase (U/L)	7,877 (normal range 230–460)	994 (July 26, 2000) (normal range 120–240)
Total bilirubin (mg/dL)	3.2 (normal range 0.2–1.10)	3.27 (July 26) (normal range 0.2–1.0)
Indirect bilirubin (mg/dL)	2.4 (normal range 0.2–0.85)	2.36 (July 26) (normal range 0.0–1.0)
Direct (conjugated) bilirubin (mg/dL)	0.8 (normal range 0.0–0.25)	0.91 (July 26) (normal range 0.0–0.25)
Creatinine (mg/dL)	2.5 (normal range 0.50–1.20)	1.04 (normal range 0.5–1.3)
Diagnosis of *Babesia* infection		
Parasitemia level (% of erythrocytes that were infected) on first blood smear examined	~30% (October 24, 1998)	1.3% (July 25, 2000) (Figure 2)
Antibody titers in IFA testing for reactivity to *B. divergens* antigens[f]	Titers of 1:64 (specimen from October 28, 1998) and 1:256 (February 15, 1999) in testing at both CDC and the Clinical Institute of Hygiene of the University of Vienna	Titers of 1:256 (July 31, 2000) and 1:1,024 (August 8, 2000) in testing at CDC and titers of 1:64 (July 31) and 1:1,000 (August 8) in testing at the Clinical Institute of Hygiene of the University of Vienna
Therapy for babesiosis		
Antimicrobial therapy	Clindamycin (600 mg thrice daily, by intravenous infusion) and quinine sulfate (650 mg thrice daily, by mouth) for 15 days, from October 24, 2000 (i.e., 10 days after onset of fever), through November 7	Clindamycin (600 mg thrice daily), by intravenous infusion, for 8 days, from July 25, 2000 (i.e., 2 days after onset of symptoms), through August 1, and by mouth, for 15 days thereafter (through August 15)
Blood transfusions	11 U packed erythrocytes, from October 19–31, 1998[c,g]	None
Response to therapy	Fever resolved by day 3 of therapy; no parasites found by blood-smear examination after day 6 of therapy; negative PCR analysis of blood from February 15, 1999	Blood from August 8, 2000, negative by blood-smear examination but positive by PCR analysis; negative PCR analysis of blood from November 7, 2000, and February 8, 2001
Long-term follow-up	Non-Hodgkin's lymphoma remitted during hospitalization in 1998, but the lymphoma relapsed in February 2000; no parasites were found on blood smears during subsequent chemotherapy	Remained well

[a]IFA, indirect fluorescent antibody; PCR, polymerase chain reaction; CDC, Centers for Disease Control and Prevention.
[b]Non-Hodgkin's lymphoma developed in the Italian patient (diagnosis: June 1998). Chemotherapy, begun on September 23, 1998, was stopped prematurely on October 14, after he became febrile. His chemotherapeutic regimen included daily prednisone (75 mg) and weekly administration of various drugs in rotation. He received 4 of the intended 12 weeks of therapy, which included doxorubicin and cyclophosphamide during odd-numbered weeks (weeks 1 and 3) and vincristine and either methotrexate (week 2) or bleomycin (week 4) during even-numbered weeks.
[c]Although the possibility that he became infected by blood transfusion could not be excluded because he had been transfused before blood smears were examined, his febrile illness and hemolytic anemia preceded the transfusions.
[d]Laboratory values were from hospital admission (October 18, 1998, for the Italian patient, and July 25, 2000, for the Austrian patient), unless otherwise specified. Values for the Austrian patient are from testing performed at the hospital to which he was transferred after a brief (<24-hour) stay at a local hospital.
[e]Earlier on July 25, at a local hospital, his hemoglobin value was 16.2 g/dL, which had been his approximate baseline value during the previous 10 months.
[f]IFA testing of serum specimens from both patients was negative for antibodies to *B. microti*. A specimen from the Italian patient (February 15, 1999) was negative for antibodies to WA1.
[g]Plasma exchange was performed on October 23, when he mistakenly was thought to have thrombotic thrombocytopenic purpura.

odocoilei, which infects white-tailed deer (15,16) and is not known to infect humans. EU1 and *B. odocoilei* form a sister group to *B. divergens*, a bovine parasite that has been considered the main *Babesia* pathogen of humans in Europe. We have demonstrated that no variability exists in the 18S rRNA sequences among several geographically distinct bovine isolates of *B. divergens* (Slemenda et al., unpub. data), which is the organism to which the name *B. divergens* legitimately applies, and showed that EU1 clearly is not *B. divergens*.

EU1 is also distinct from the MO1 parasite, which caused a fatal human case of babesiosis in Missouri in

Figure 2. Panel of computer-generated electronic images of photomicrographs of *Babesia*-infected erythrocytes on a Giemsa-stained smear of peripheral blood from the patient who became infected in Austria. The electronic images were edited for uniformity of color, without changing the form or size of the organisms. The image on the bottom shows a tetrad (Maltese-cross form). Three glass slides of the actual blood films have been deposited in the Oberösterreichisches Landesmuseum, Biologiezentrum, Linz (i.e., Biology Center of the Upper Austrian Museum, Linz), with the accession number 2002/9. The slides are labeled "*Babesia* sp. (EU1), patient 001, Austria, Krems Land, July 25, 2000."

1992 and was thought then by the investigators to be *B. divergens*–like but distinct from it (10). The sequence provided in the publication about MO1 (10) was for only a 128–base pair fragment; in that region, the EU1 and MO1 sequences differ by four bases, and three positions in the MO1 sequence were unresolved.

The DNA sequences available in GenBank for *B. divergens* in Europe are from cattle not humans. To our knowledge, molecular data have been reported for only one of the purported human cases of *B. divergens* infection in Europe, a case on the Canary Islands (22,23). However, the data were for an incomplete 18S rRNA sequence (GenBank accession no. AF435415), and therefore were not suitable for the phylogenetic analysis we performed of complete 18S rRNA sequences. Nevertheless, the sequence for the case on the Canary Islands differs by 18 bases with the sequence for EU1 and by 1 base with the *B. divergens* sequence from cattle (AY046576) in the 369-base-long region of the gene that could be compared.

In the absence of molecular data, we are not certain which organisms have caused the human cases of babesiosis in Europe that have been attributed to *B. divergens*. The evidence that particular human cases were caused by *B. divergens* has varied in quantity, quality, and type. The evidence typically has included various combinations of morphologic data, from examination of blood smears; serologic data (usually, but not always, from IFA testing); and data concerning whether jirds or cattle injected with the patient's blood become parasitemic. Although these techniques are useful for detecting *Babesia* infection, they do not necessarily provide reliable species identification (e.g., because of serologic cross-reactivity between EU1 and *B. divergens* in IFA testing [Table]). Although some of the human cases attributed to *B. divergens* may truly have been caused by the bovine *B. divergens*, others might have been caused by EU1. The cases of EU1 infection we reported likely would have been attributed to *B. divergens* had only the traditional methods of characterization, without molecular analysis, been used.

Our molecular characterization also showed that EU1 is not closely related to the other *Babesia* (or *Babesia*-like) agents known to have infected humans (most notably, *B. microti* and the WA1- and CA1-type parasites). *B. microti*, together with *B. rodhaini*, *Cytauxzoon felis*, and *B. equi*, is ancestral to the *Theileria* spp. and perhaps also to the *Babesia* sensu stricto group (depending on which tree topology is used) (24). Reclassification of the *B. microti* group to a new family has been proposed (24). The WA1- and CA1-type parasites, which have caused human cases of babesiosis in the western United States (8,9), also form a well-defined group, whose position in the phylogeny of the piroplasms is uncertain (25).

Although EU1 represents a zoonotic pathogen with previously unreported molecular characteristics, whether it represents a new species per se awaits further evidence.[1]

[1]The issue of what constitutes a new or newly described species requires periodic reevaluation as the techniques for characterizing microbes improve. Although the advent of molecular biology/phylogeny has made it possible to characterize organisms more precisely, the issue of how large a genetic difference in a particular gene(s) constitutes a new or different species is controversial. Bacterial taxonomy is a much more active and advanced field (26,27) than that for characterizing protozoa, in part because of the increasingly large numbers of bacteria being proposed as new species and the challenges posed by such possibilities as genetic rearrangements. In the recommendations published in 2002 by an ad hoc committee that reevaluated the species definition for bacteria (26), scientists were encouraged to use the "*Candidatus*" concept (i.e., to propose candidates for newly described bacterial species) for organisms that had been well-characterized, including the sequencing of the small subunit RNA gene, but had not yet been cultured.

As noted above, we have referred to the protozoan we characterized as EU1. We have not claimed it is a newly identified species, despite having complete, identical, and novel 18S rRNA sequences for the organism from two patients, who were separated in time and space, and having done the sequence analysis for the two cases in different countries. However, although the "*Candidatus*" concept per se does not formally exist now for protozoa, on the basis of the precedent from the field of bacterial taxonomy, we propose that EU1 be considered a candidate species. If additional evidence supports the conclusion that the organism indeed constitutes a newly described species of the *Babesia* genus, we would favor the name *Babesia venatorum*, which now does not constitute an official name. We chose this name because the patients whose cases we reported were avocational hunters; "venator" is the Latin word for "hunter" ("venatorum," the plural genitive case, means "of the hunters").

EU1 might constitute a new species in the sense that it was never previously recognized or characterized in any way or one that was characterized but not with molecular data (e.g., was misnamed *B. divergens* or some other *Babesia* sp.). Because DNA sequence data are not available for most of the *Babesia* spp. found over the past century in nonhuman animals and because data about the morphologic features and host specificity of a parasite are inadequate for definitive species identification, we cannot exclude the possibility that EU1 is one of the many previously described *Babesia* spp. of nonhuman animals, some named and some not, that were not known to be zoonotic.

Although the serologic cross-reactivity between EU1 and *B. divergens* could have resulted in diagnostic confusion in the past, cross-reactivity between these two organisms also could be advantageous. The *B. divergens* IFA could be a useful tool for testing serum from persons who might be infected with EU1 or who participate in serosurveys to determine the prevalence and geographic distribution of EU1 infection. Unfortunately, our attempts to obtain an isolate of EU1 by inoculation of jirds were unsuccessful. One consequence is that we did not generate the homologous antigen needed for development of an IFA assay for EU1. Therefore, we could not contrast the degree of reactivity of our patients' serum specimens with antigens from EU1 and *B. divergens*.

The importance of determining whether the etiologic agent of a particular case of babesiosis is EU1 rather than *B. divergens* or some other *Babesia* sp. depends in part on whether the clinical manifestations of infection and the response to antimicrobial therapy differ. We cannot generalize about such issues from two cases of infection with EU1. However, the range in severity of the two cases, from quite mild (Austrian case) to moderately severe (Italian case), is of interest, particularly because the two patients were similar in some respects (i.e., both were asplenic men in their mid-fifties). Factors that likely placed the Italian patient at increased risk for a more severe case included immunosuppressive chemotherapy for lymphoma and the 10-day interval between the onset of fever and the diagnosis of babesiosis (Table).

Largely from data for *B. microti* infection in the United States, combination therapy with either clindamycin and quinine or atovaquone and azithromycin is recommended for treatment of babesiosis (28), with the addition of exchange transfusion in some situations in severely ill patients. The Austrian patient, whose case was mild, was treated with clindamycin only. Some in vitro data and anecdotal clinical data for purported zoonotic cases of *B. divergens* infection suggest that therapy with clindamycin alone, in combination with exchange transfusion, when indicated, might be effective for treating *B. divergens* infection (4,29,30). However, no clinical trials in humans have evaluated the effectiveness of any antimicrobial regimens for treatment of *Babesia* infection not caused by *B. microti*.

The public health importance of infection with EU1, including such factors as its biology, geographic distribution, ecology, prevalence, risk factors for infection and disease, clinical manifestations, tick vector, and animal reservoir host(s), is not yet known and may take years to determine. The Italian patient likely became infected in a garden habitat and the Austrian patient in a garden or forest habitat. Of interest, an incomplete 18S rRNA sequence (GenBank accession no. AF373333) for a *Babesia* sp. found in *I. ricinus* ticks was recently reported by investigators in Slovenia (31), which borders Italy and Austria. The sequence was reported for only 364 bases and corresponds to positions 433–796 of the complete 18S rRNA sequence for EU1. In this part of the gene, the sequences for the organisms from the Slovenian ticks and EU1 are identical. However, the relatedness of the two organisms cannot be determined without the complete 18S rRNA sequence for the organism from the ticks. The occurrence of two identified cases of EU1 infection in humans in different countries (i.e., Italy and Austria) and years (i.e., 1998 and 2000) indicates that EU1 is not restricted to one geographic area or time. Increased vigilance for zoonotic infection with novel vector-borne pathogens is needed.

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Dr. Herwaldt is a medical epidemiologist in the Parasitic Diseases Epidemiology Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention. Her research interests include the epidemiology of various protozoan parasites, such as *Babesia*, *Leishmania*, *Trypanosoma*, and *Cyclospora* spp.

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Address for correspondence: Barbara L. Herwaldt, Centers for Disease Control and Prevention, Division of Parasitic Diseases, 1600 Clifton Road NE, Mailstop F22, Atlanta, GA 30333, USA; fax: 770-488-7761; email: bxh4@cdc.gov

Severe Tungiasis in Underprivileged Communities: Case Series from Brazil

Hermann Feldmeier,* Margit Eisele,* Rômulo César Sabóia-Moura,† and Jörg Heukelbach†

Tungiasis is caused by infestation with the sand flea (*Tunga penetrans*). This ectoparasitosis is endemic in economically depressed communities in South American and African countries. Tungiasis is usually considered an entomologic nuisance and does not receive much attention from healthcare professionals. During a study on tungiasis-related disease in an economically depressed area in Fortaleza, northeast Brazil, we identified 16 persons infested with an extremely high number of parasites. These patients had >50 lesions each and showed signs of intense acute and chronic inflammation. Superinfection of the lesions had led to pustule formation, suppuration, and ulceration. Debilitating sequelae, such as loss of nails and difficulty in walking, were constant. In economically depressed urban neighborhoods characterized by a high transmission potential, poor housing conditions, social neglect, and inadequate healthcare behavior, tungiasis may develop into severe disease.

Tungiasis is a common, but neglected, health problem in economically depressed communities in South American and sub-Saharan African countries (1–5). This ectoparasitosis is caused by the sand flea (*Tunga penetrans,* Siphonaptera: Tungidae, Tunginae), also called the jigger flea. The female jigger flea penetrates into the skin of its host, undergoes a peculiar hypertrophy, expels several hundred eggs for a period of ≤3 weeks, and eventually dies. The shriveled carcass is then sloughed from the epidermis by host repair mechanisms (6,7). Within 10 days, the flea increases its volume by a factor of approximately 2,000, finally reaching the size of a pea. Through its hindquarters, which serve for breathing, defecating, and expulsing eggs, the flea remains in contact with the air, leaving a sore (240–500 μm) in the skin; the sore is an entry point for pathogenic microorganisms (8). The preferred localization for jiggers is the periungual region of the toes, but lesions may occur on any part of the body (9).

Tungiasis, a zoonosis, affects a broad range of domestic and peridomestic animals, such as dogs, cats, pigs, and rats (10). Where humans live in close contact with these animals and where environmental factors and human behavior favor exposure, the risk for infection is high (3,11).

Numerous case reports detail the clinical aspects of tungiasis. However, they almost all exclusively describe travelers who have returned from the tropics with a mild disease (12). Having reviewed 14 cases of tungiasis imported to the United States, Sanushi (13) reported that the patients showed only one or two lesions and, that except for itching and local pain, no clinical pathology was observed. In contrast, older observations show that indigenous populations and recent immigrants, as well as deployed military personnel, frequently suffered from severe disease, characterized by deep ulcerations, tissue necrosis leading to denudation of bones, and auto-amputation of digits, resulting in physical disability, such as being unable to work and walk (14–19). Tungiasis has also been associated with lethal tetanus in nonvaccinated persons (19–22). In a study in São Paulo State, Brazil, tungiasis was identified as the place of entry in 10% of tetanus cases (23).

We present the clinical findings as well as the demographic and environmental characteristics of 16 persons with severe tungiasis who were identified during a prospective study on *Tunga penetrans*–associated disease at a Primary Health Care Center (PHCC) in a economically depressed neighborhood (*favela*) in Fortaleza, northeast Brazil. The results indicate that in resource-poor populations important disease may frequently occur and seems to be related to a combination of intense transmission, economic deprivation, social neglect, and inadequate healthcare behavior.

Materials and Methods

Study Area

The *favela* Morro de Sandras is on the outskirts of Fortaleza, the capital of Ceará State, northeast Brazil, and is similar to other economically depressed areas there. During the high transmission season (July–December), approximately one third of the population is affected by

*Free University of Berlin, Berlin, Germany; and †Mandacaru Foundation, Fortaleza, Brazil

tungiasis (24). Other ectoparasitic diseases such as head lice, scabies, and cutaneous larva migrans are also very common in the study area (25). This area is built on a dune close to the beach and has a total population of 1,500 persons. Sixty percent of the population has a monthly family income of less than two minimum wages (1 minimum wage = US$80.00). Adult illiteracy is 30%, unemployment rates are high, and crime is common. Ninety-seven percent of the households have electricity, and about 60% have access to running water (26). Many houses are made with improvised construction material and do not have concrete floors. Waste and sewage disposal are insufficient, and hygienic conditions are precarious. Most streets are not paved. Innumerable stray dogs and cats roam the area, in addition to dogs and cats kept as pets. Rodents are numerous; *Rattus rattus* can be seen during the day feeding on organic waste disposed of in backyards or outside family compounds. The prevalence of tungiasis ranges from 5% to 35% according to the season (J. Heukelbach, unpub. data).

Study Population

The study was performed at the PHCC that serves the population of the *favela*. During a 6-week period, 86 persons with tungiasis were identified among patients who visited the center for medical reasons unrelated to the ectoparasitosis. Severe tungiasis was arbitrarily defined as the presence of ≥50 lesions. Sixteen of the 86 patients fulfilled this criterion and are described in this case series. They ranged in age from 2 to 50 years of age.

Clinical Examination

As tungiasis may occur at any topographic site (9), the whole body surface of the patient was examined for the presence of vital, egg-producing, involuting, or dead fleas. Lesions were classified according to the Fortaleza Classification, a recently elaborated staging system (7). The following findings were considered diagnostic for tungiasis: flea in statu penetrandi, stage I, a dark and itching spot in the epidermis with a diameter of 1–2 mm with or without local pain; stage II (early lesion), lesions with as a white halo with a diameter of 3–10 mm with a central black dot; stage III (mature flea), a brownish-black circular crust with or without necrosis of the surrounding epidermis; stage IV (dead parasite), circular residue punched out in the keratin layer of the sole of the foot or irregular thickening of the nail rim; and stage V, lesions altered through manipulation by the patient (such as partially or totally removed fleas, which leaves a characteristic crater-like sore in the skin) and suppurative lesions, mainly caused by using nonsterile perforating instruments such as needles and thorns.

During the examination, location and number of lesions were noted, and the following signs and symptoms were observed: erythema, edema, tenderness, itching, pain, shining skin, desquamation, hyperkeratosis, fissures, pustules, suppuration, ulcers, deformation of the toes (defined as deviation of the normal axis of the toe caused by intense swelling), deformation of nails, loss of nails, and difficulty in walking or gripping.

Clinical pathologic findings were classified as follows: acute inflammation or painful lesion surrounded by erythema, edema, and tenderness; chronic inflammation, edema, tenderness, shining skin with or without desquamation, or deformation of digits; superinfection, presence of pustules, suppuration, or ulcers; and physical disability, difficulty in walking, or gripping (if lesions were located on the hands), based on patients' statements that pain restricted their movements. Lesions tended to occur in clusters, which were arbitrarily defined as a group of five or more lesions that occurred in close proximity (e.g., on the periungual region of the toe, the heel, or the fingertip).

Statistical Analysis

Statistical analysis was performed by using the StatView software package version 1.5 (SAS, Cary, NC). The Wilcoxon signed rank test, the Spearman rank correlation coefficient test, and the Fisher exact test were applied when appropriate.

Ethical Considerations

The study was approved by the Ethical Committee of the Federal University of Ceará State, Fortaleza, Brazil. Before the study, meetings with community health workers, community leaders, and staff members of the PHCC were held in which the objectives of the study were explained. Informed written consent was obtained from each patient after the objectives of the study were explained. In the case of a minor, the caregivers were asked for their consent. After the examination, all patients were treated topically with thiabendazole 5% and, in the case of superinfection, with neomycin ointment. All patients received a pair of tennis shoes and were encouraged not to walk barefoot.

Results

The demographic characteristics of the patients in the study and the number of lesions present are shown in Table 1. Patients had at least 52 lesions with a maximum of 145 and a median of 88 lesions. Of the 1,474 lesions, 1,092 (76%) occurred in clusters. A significant correlation existed between the number of lesions per patient and the number of lesions occurring in clusters (rho=0.94; p=0.003; Figure 1). No significant relationship existed between the number of lesions and age (rho=0.44, p>0.05). However, manipulated lesions were more frequent in patients >15 years of age (38% vs. 13%; p<0.05). No difference existed

Table 1. Demographic and parasitologic characteristics and number of lesions in 16 patients with severe tungiasis

Characteristics	No./total
Median age in y (and range)	12 (2–50)
Female/male	7/9
Median no. of lesions/patient (range)	88 (52–145)
Vital lesions (stages I–III)	43 (10–77)
Dead lesions/residuals (stages IV–V)	38 (11–85)
Manipulated lesions	14 (1–46)
Presence of clusters in individual patients	16/16 (100%)
Lesions occurring in clusters	1,092/1,474 (76%)
Median no. of lesions/cluster (range)	12 (6–30)

in the median number of lesions between female and male patients.

The topographic distribution of lesions is summarized in Table 2. All patients had lesions on the toes and soles of the feet. The periungual region of the toe was clearly a predilection site. Other regions of the toes, such as the tip, the plantar, or the dorsal site were also frequently affected. Fifteen patients (88%) had lesions on their heels, and two patients (13%) had lesions on the dorsum pedis. Lesions on the hands were found in six patients (38%); one person had a lesion in the gluteal area, and another patient had a lesion on the chest.

Clinical findings are shown in Table 3. In all patients, signs of acute as well as chronic inflammation were present. Acute and chronic inflammation occurred simultaneously in different topographic sites or in lesions at different stages of development. In three patients, the entire foot and lower leg were inflamed. Deformation or nail loss was common (69%). All patients had difficulty walking, and half of the patients with lesions at the fingers had difficulty gripping. Intense itching, a common symptom, prevented patients from sleeping soundly. The number of lesions was particularly high in patients with signs of generalized inflammation, when fissures were present or when superinfection had occurred (Table 4). We describe four cases that are typical examples of severe tungiasis.

Case 1

A 2-year-old girl had 90 lesions; 49 of these lesions were located on the toes, 33 on the soles of the feet, and 3 on the heels. Five lesions were at ectopic sites (four of them on the fingertips and one in the gluteal region). One sand flea was trying to penetrate the skin of the chest. Many lesions were superinfected as indicated by pustules or suppuration, and the nails of six toes were deformed or had already fallen off. The lesions on the fingertips were particularly painful and caused difficulty gripping. The child also had pediculosis and was underweight. The family (three children and the parents) lived in a small hut with a sandy floor. The mother was 18 years of age; both parents were illiterate.

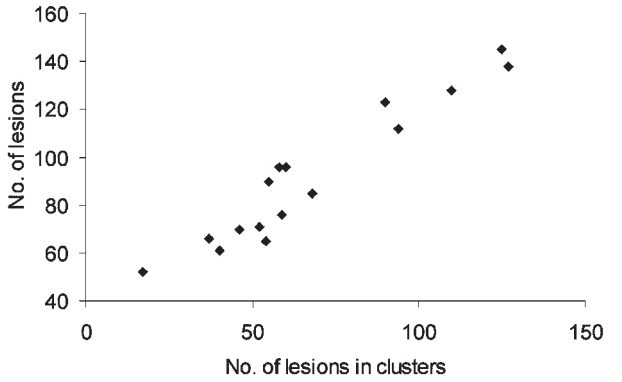

Figure 1. Correlation between total number of lesions and number of lesions occurring in clusters (rho=0.94; p=0.0003).

Case 2

A 6-year-old girl had 96 lesions. Of the 96, a total of 30 were located on the toes; all toes but one were infested with sand fleas; one sand flea was trying to penetrate the skin of on the second toe of the left foot (Figure 2). Forty-eight lesions occurred on the soles of the feet, and eight were located on the heels. Ten lesions were found on the hands and impeded the girl from gripping (Figure 3). Most lesions were surrounded by severe erythema and edema. Pustules and suppurations were frequent. Sleep was reported to be severely disturbed, and the child woke up in the night and cried. The family lived in a hut with sandy soil. The mother devoted most of her attention to two younger siblings. The mother was unmarried and illiterate.

Case 3

A 50-year-old man had 123 lesions. Thirty-six lesions were located on the toes, 33 on the soles, and 54 on the heels. Nine nails were lost (Figure 4). Bacterial superinfection with pustules or suppuration was present on both feet. Nineteen ulcers were also found. Severe desquamation and hyperkeratosis occurred alternately. The patient had persistent pain and could walk only with considerable difficulty. He had manipulated many lesions with a nonsterile needle or a thorn and had also treated his feet with candle wax diluted in used motor oil to get rid of the sand fleas. Presumably, the self-treatment added to the aspect of gen-

Table 2. Distribution of lesions according to topographic site

Topographic site	No. of lesions median (range)	% of all lesions (n=1,474)
Toes	48 (17–101)	56.4
Periungual	29 (9–58)	29.0
Other areas[a]	17 (8–66)	27.4
Sole	20 (3–48)	20.1
Heel	9 (0–62)	20.5
Dorsal area	0 (0–3)	0.3
Hands/gluteal region	0 (0–21)/0 (0–1)	2.7

[a]Such as the tip, the plantar, or the palmar side of the toes.

Table 3. Clinical findings in patients with severe tungiasis (n=16)

Clinical pathology observed	Present in patients (%)
Acute local inflammation	16/16 (100)
Chronic local inflammation[a]	16/16 (100)
Generalized inflammation[b]	3/16 (19)
Superinfection	7/16 (31)
Pustule(s)	3/16 (19)
Suppuration	4/16 (25)
Severe itching	16/16 (100)
Ulcers	8/16 (50)
Fissures	3/16 (19)
Deformation or loss of nails	11/16 (69)
Difficulty in walking	16/16 (100)
Difficulty in gripping[c]	3/6 (50)

[a]Edema, desquamation, shining skin, hyperkeratosis with or without deformation of digits.
[b]Edema and pain of entire foot with or without suppuration.
[c]Six patients had lesions on the hands.

eral inflammation of both feet. The patient was unemployed and lingered around in the *favela* all day. He had no shoes and only wore slippers.

Case 4

A 55-year-old woman had 76 lesions. Forty-eight lesions were located at periungual sites, and 17 in other regions of the toes. Eleven lesions were on the soles of the feet. Both feet were edematous; the edema extended over the entire lower legs. Several toes were deformed and all nails were damaged (Figure 5). Pustules and suppuration occurred in all toes. The patient was unable to walk and had to remain in her hammock. The patient was farsighted and did not have appropriate glasses. She lived alone in a small hut. The sandy compound was littered with waste and organic material.

Table 4. Relationship between number of lesions and selected clinical pathologic findings

Clinical pathology observed	Median no. of lesions
General inflammation	131
Fissures	128
Pustules or suppuration	123
Ulceration	88
Deformation or loss of nails	81

Discussion

With a length of <1 mm, *Tunga penetrans* is the smallest known flea species (27). Once burrowed in the skin of its host, the flea becomes hypertrophic, produces and releases eggs, and eventually dies. Microbiologically, the embedded flea behaves as a foreign body with a continuously enlarging surface (8). The parasite remains buried in the epidermis, except for the posterior parts of the abdomen bearing the anus, the genital opening, and four pairs of large stigmata. The protruding hindquarters create a sore, connecting the surface of the skin to the epidermis and, through

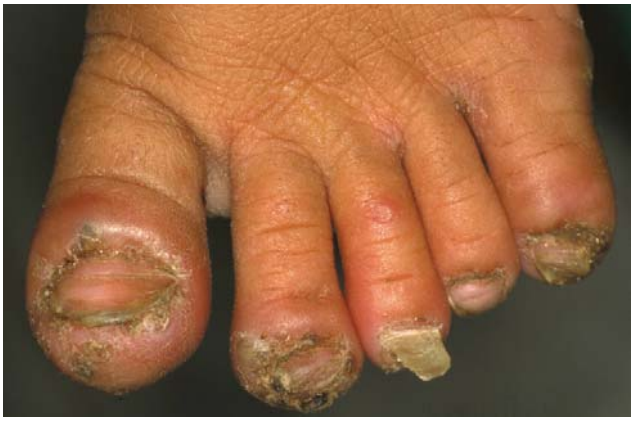

Figure 2. Left foot of a 6-year-old girl. The first, second, third, and fifth toe are infected with *Tunga penetrans*. These toes are inflamed, and the second and the third toe are distorted by severe edema. The first toe shows hyperkeratosis. The nails of the first, second, and fifth toe are deformed, and the nail of the third toe is falling off. A flea is trying to penetrate the skin at the edge of the pustule on the medial side of the second toe (11 clockwise). An ulcer has formed above the proximal phalangeal joint of the third toe.

the proboscis of the flea, to the dermis. When the skin's surface is linked by means of a foreign body to underlying tissue layers, translocation of the normal microflora occurs, leading to a bacterial infection in the epidermis and presumably to the formation of a biofilm on the surface of the foreign body (28). Thus, in the vertebrate host, the infection with *T. penetrans* is a self-limiting process, and the risk for infectious complications is obvious.

In fact, older literature abounds with observations on severe pathologic findings associated with tungiasis and mentions debilitating sequelae such as phagedenic ulcers, gangrene, auto-amputations of digits, and the loss of entire limbs (19,29,30). Death resulting from infection with *Clostridium tetani* is not uncommon in nonvaccinated persons (19–22). Such severe sequelae of tungiasis were not only observed in native South American populations by European travelers (2,15,31) but were also reported by military physicians responsible for local forces in West and East Africa (15,16,29). In 1909, Decle (29) gave a vivid description of the havoc wrought by the ectoparasite on military personnel in Kenya: "At Ford Raymond the garrison consisted of 160 askaris (soldiers) and 70 porters; out of this men 72 soldiers and 30 porters were absolutely unfit for service through ulcers brought on by jiggers, and 30 more were lame." In another garrison, Fort Grant, severe tungiasis caused illness in 50% of the local soldiers (29). During military operations in Cameroon in the first quarter of the 19th century, 5% of all septic admissions were due to complications of the ectoparasitosis (15). These accounts show that severe tungiasis was an important health problem in the underdeveloped rural hinterlands of

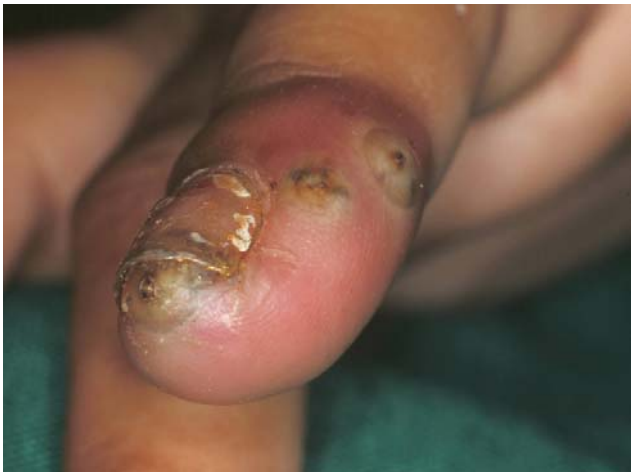

Figure 3. Second finger of the right hand of the same patient. Acute inflammation with intense erythema and a slight edema is shown. Two stage III lesions are located at the lateral side of the finger and another lesion is lifting up the nail.

South America and Africa until the middle of the 20th century.

In the last 50 years, severe tungiasis seems to have disappeared as a disease entity. In current textbooks the ectoparasitosis is mainly mentioned as an exotic nuisance for the casual traveler in South America and sub-Saharan Africa (6). Tourists seldom have more than one lesion and usually seek medical advice within 1 week of flea penetration, resulting in the extraction of the parasite at an early stage (12). However, we observed persons living in a underdeveloped neighborhood in Fortaleza, northeast Brazil, where patients carried up to 145 sand fleas in all stages of development.

As parasites tend to accumulate at certain predilection sites, the pathologic findings should be particularly severe in these sites. In our patients and others in the community, many infected persons have lesions in the periungual region of the toes (9), which explains the high frequency (69%) of nail deformation or nail loss. Furthermore, as the sole of the feet and the heel were other predilection sites, difficulty in walking was also very common.

Various mechanisms exist by which embedded fleas could induce pathologic alterations of the skin in an early stage of development. Acute inflammation with erythema, edema, pain, and itching is conceivably due to tissue damage induced by a metabolically highly active and continuously enlarging parasite. As with other blood-sucking insects, *T. penetrans* releases proteolytic enzymes during penetration and growth, causing an inflammatory response of the skin. In comparison with other ectoparasites that frequently reinfect humans, the immune response of the host might contribute to the intense inflammation observed soon after penetration.

As the lesion develops, bacterial superinfection almost inevitably occurs (8). During penetration, the flea breaks up the stratum corneum, allowing bacterial microcolonies on the skin surface to spread. In addition, pathogenic microorganisms on the outer surface of the flea may be actively carried into the epidermis (8). As the continuously expanding body of the flea (the volume increases by a factor of roughly 2,000) consists of rather smooth intersegmental skin and newly formed chitinous clasps, the embedded flea fulfills the requirement of a structural matrix to which microorganisms could easily adhere (32). In fact, scanning-electron microscopy of extracted fleas showed that pathogens such as streptococci and gram-negative rods formed a biofilm in the tiny grooves of newly built intersegmental skin as well as on the chitinous exoskeleton (Feldmeier and Meckes, unpub. data, 2002).

As the lesion itches immediately after the flea penetrates, patients usually start to scratch, which, in turn, promotes the entry of bacteria through the persistent sore in the epidermis. In fact, we invariably observed microabscesses in histologic sections of lesions only 2 days after penetration (7).

In many of our patients, bacterial superinfection was also the result of an inappropriate manipulation of lesions with nonsterile instruments by the patient or caregiver (Figures 4 and 5). The remarkable desquamation of the skin observed around late-stage lesions (Figure 4) has its histopathologic correlates in hyperkeratosis and parakeratosis of the stratum corneum (7).

Recently, *Wolbachia* species have been identified in the ovaries of *T. penetrans* (33). As antigens of these bacterial endosymbionts have been associated with the pathologic immune response in some filarial diseases (e.g., onchocerciasis), part of the intense immune response in tungiasis might also be evoked by *Wolbachia* antigens being released from decaying fleas (34). At present, a study is

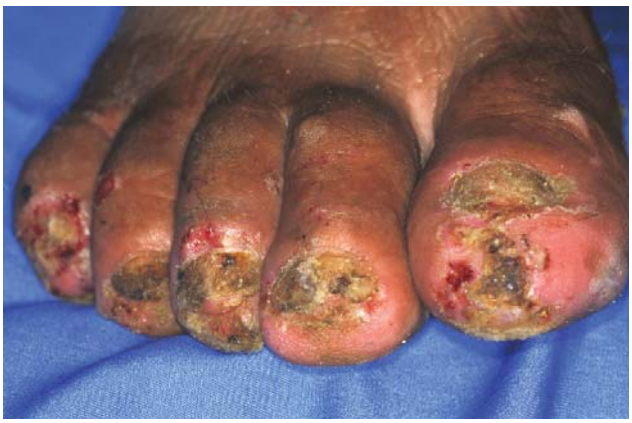

Figure 4. Right foot of a 50-year-old man. All nails have been lost. Embedded fleas have been manipulated by the patient, leaving innumerable sores. Desquamation and ulceration are merged. The skin tends to bleed where the stratum corneum is eroded.

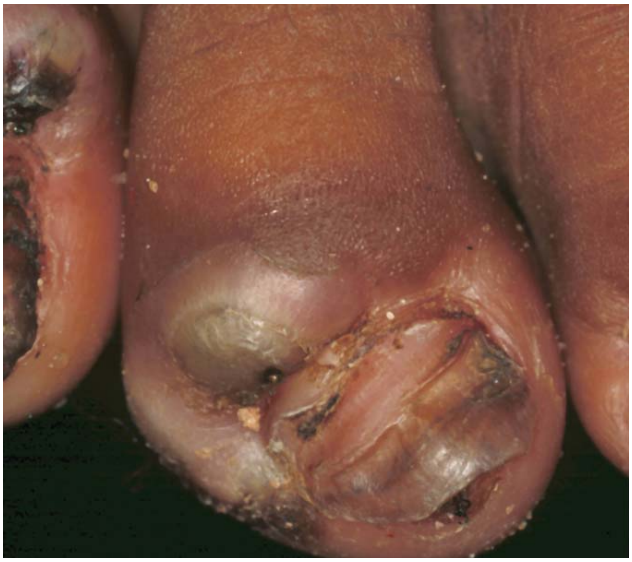

Figure 5. Fourth toe of a 50-year-old women. The nail is lifted up by a lesion. An abcess has formed near the nail wall, and the toe is distorted because of intense edema.

being undertaken to verify this assumption in experimentally infested rats.

Reports on severe tungiasis involve persons with particular risk factors, such as alcoholics or the mentally diseased, who are expected to have prolonged contact with the ground or are unable to care for themselves (35,36). Our data clearly show that severe tungiasis also occurs in persons without such risk factors who live in an impoverished community, when environmental, socioeconomic, and behavioral factors coexist and make frequent reinfection likely or impede the extraction of penetrated fleas in the early stage. Recently, estimates show that in northeast Brazil alone, several million people who live in communities with similar environmental characteristics to those we studied are at risk of tungiasis (2).

Although we cannot give an accurate estimate of severe tungiasis in the general population level, 16 (17%) of 86 patients arrived at the PHCC for reasons unrelated to the ectoparasitoses but showed important sequelae, which indicates that tungiasis is frequent on the community level. This assumption has been corroborated by a study performed in south of Brazil.

Thus, whereas severe tungiasis has disappeared from the underdeveloped rural hinterland where it formerly existed, this disease should be considered as a resurgent health problem of underdeveloped urban areas, where environmental conditions favor a high attack rate and social neglect is intricately linked to poverty and inadequate healthcare behavior. At least in Brazil, the medical profession wholly neglects this ectoparasitosis, and physicians do not diagnose tungiasis during consultation unless the condition is mentioned by the patient (25,37).

Morse (38) has convincingly argued that a reemerging disease is rarely a purely microbiologic event but commonly has causative cofactors such as ecologic changes, changes in human demography, international travel, or breakdown of public health measures. The results of our study suggest that poverty, social neglect, and inappropriate healthcare behavior should be added to this list.

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Dr. Feldmeier is professor of tropical medicine at the Institute of International Health (Free University of Berlin) and a visiting professor at the Federal University of Ceará (Fortaleza). He is a World Health Organization expert for the control of parasitic diseases. His work focuses on gender and social issues in tropical medicine.

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Address for correspondence: Hermann Feldmeier, Institute for International Health, Center for Humanities and Health Sciences, Faculty of Medicine, Free University of Berlin, Fabeckstr. 60-62, Berlin, Germany; fax: ++49-30-84451280; email: feldmeier.fuberlin@t-online.de

Pseudomonas aeruginosa and the Oropharyngeal Ecosystem of Tube-Fed Patients

Arthur Leibovitz,* Michael Dan,* Jonathan Zinger,* Yehuda Carmeli,* Beni Habot,* and Rephael Segal*

We evaluated whether elderly patients fed with nasogastric tubes (NGT) are predisposed to *Pseudomonas aeruginosa* colonization in the oropharynx. Fifty-three patients on NGT feeding and 50 orally fed controls with similar clinical characteristics were studied. The tongue dorsum was swabbed and cultured. *P. aeruginosa* was isolated in 18 (34%) of the NGT-fed group but in no controls ($p<0.001$). Other gram-negative bacteria were cultured from 34 (64%) of NGT-fed patients as compared with 4 (8%) of controls ($p<0.001$). Antibiotic susceptibility of the oropharyngeal *P. aeruginosa* isolates was compared with that of isolates from sputum cultures obtained from our hospital's bacteriologic laboratory. The oropharyngeal isolates showed a higher rate of resistance; differences were significant for amikacin ($p<0.03$). Scanning electron microscope studies showed a biofilm containing *P. aeruginosa* organisms. The pulsed-field gel electrophoresis profile of these organisms was similar to that of *P. aeruginosa* isolates from the oropharynx. NGT-fed patients may serve as vectors of resistant *P. aeruginosa* strains.

The oral cavity has long been considered a potential reservoir for pathogenic microorganisms. It is the only normally accessible site in the body that has hard, nonshedding surfaces for microbial colonization. Those unique tissues allow the accumulation of extracellular products and the formation of biofilms that serve as culture media for bacteria and contribute to the development of antibiotic resistance (1). Within the mouth, distinct habitats provide different ecologic conditions including mucosal surfaces, cheeks, palate, periodontal region, tongue, and abiotic structures (teeth). Ecologic conditions within the mouth may vary and change the ecosystem, facilitating the growth of pathogenic organisms.

Patients on nasogastric tube (NGT) feeding are a growing segment of the frail elderly population. We have recently reported an increased rate of gram-negative bacteria in the nasopharynx of these patients, including a high proportion of *Pseudomonas aeruginosa* (2). *P. aeruginosa* has a predilection for wet sites and respiratory equipment and may create reservoirs that threaten hospitalized patients (3). The oropharynx of NGT-fed elderly patients may provide such an ecosystem and promote the colonization of *P. aeruginosa.* This colonization could be due to several factors such as the papillary structure of the dorsum of the tongue, the lack of mastication and swallowing (eliminating their mechanical cleansing effect), and the tube itself. *P. aeruginosa,* a well-known biofilm-producing microorganism (4,5), may be exploiting the NGT to create a thriving habitat.

The purpose of this study was to reconfirm the high incidence of *P. aeruginosa* isolations from the oropharynx of NGT-fed elderly patients, determine its antibiotic susceptibility, and explore the possibility of biofilm formation on the feeding tube. If these assumptions are true, the oropharynx of NGT-fed patients could constitute a potential reservoir for *P. aeruginosa* in long-term-care facilities.

Methods

This prospective cross-sectional comparative study was conducted in the four skilled nursing wards of a 158-bed geriatric hospital. Skilled nursing wards are licensed for providing care for nursing patients who also have an active disease requiring close medical supervision (e.g., NGT feeding, severe bed sores, advanced cancer, or hemodynamic instability). Eligible for the study were all patients who had been receiving NGT feeding for at least 2 weeks.

The control group comprised matched orally fed patients, with no swallowing disturbances, who resided in the same wards. The orally fed patients received a regular solid hospital diet with occasional supplements. Excluded from both groups were patients who had received any antibiotic treatment up to 2 weeks before the study, patients with advanced cancer, and patients who had received chemotherapy or radiotherapy to the neck. Informed consent was obtained from the patients or their proxies.

Cultures were obtained by applying sterile cotton swabs to the base of the tongue dorsum and rubbing the buccal mucosa. The sample was then placed in transport medium. Samples were taken in the morning, before breakfast and the daily oral cleansing procedure.

*Tel-Aviv University, Israel

Within 1 h of collection, specimens were spread on blood and MacConkey agar plates and were incubated at 35°C for 18 h. Five colonies of each morphotype were selected for identification. Gram-negative bacteria, including *P. aeruginosa,* were identified by using the BBL Crystal ID system (Becton Dickinson, Sparks, MD). Antimicrobial susceptibility testing was performed by the disc-diffusion technique, according to guidelines of the National Committee for Clinical Laboratory Standards (6), with OXOID test discs (OXOID, Basingstoke, Hampshire, England).

Routine oral hygiene for the tuboenteral patients was performed by cleansing the oral cavity before meals three times a day with lemon-glycerine wadding sticks impregnated with a solution of glycerine-citric acid, lemon flavoring, and sodium benzoate 0.1% (7). NGTs in use in our hospital are made from polyvinyl chloride (Duodenal Levin Tube–Maersk Medical, Lynge, Denmark).

For the biofilm study, samples of the oropharyngeal section of the NGT were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, washed with the same buffer, dehydrated in increasing concentrations of ethanol, dried with a critical point drier, and coated with gold (Polaron-sem coating unit E5100, Thermo VG Scientific, Beverly, MA). The outer surface of the samples was examined by a Jeol-840A scanning electron microscope (JEOL USA, Peabody, MA). Biofilm studies were performed on four patients with an oropharyngeal culture that was positive for *P. aeruginosa* 2–4 weeks after the NGT was inserted.

From three NGT-fed patients with isolations of *P. aeruginosa,* samples were taken concomitantly from the oropharynx and the NGT surface for strain typing by pulsed-field gel electrophoresis (PFGE). DNA preparation and cleavage with 20 U of SpeI endonuclease (New England Biolabs, Eldan, Rosh Ha'ain, Israel) were performed, as originally described (8). Electrophoresis was performed in a 1% agarose gel (BMA Products, Hann Woong Yoo, South Korea) prepared and run in 0.5 x Tris-borate-EDTA buffer on a CHEF-DR III apparatus (Bio-Rad Laboratories Ltd. Rishon Le Zion, Israel). The initial switch time was 0.5 s, the final switch time was 35 s, and the run time was 22 h at 6 V/cm with a temperature of 14°C. Gels were stained in ethidium bromide, de-stained in distilled water, and photographed with a Bio-Rad GelDoc 2000 camera. PFGE DNA patterns were compared and interpreted according to the criteria of Tenover et al. (9).

The antibiotic susceptibility of *P. aeruginosa* isolates from the oropharynx was compared to that of *P. aeruginosa* isolates obtained from sputum cultures and recorded in the laboratory logbook in the preceding year. Statistical processing was performed by using SPSS software (SPSS Inc., Chicago, IL). Chi-square test was used for comparative studies; $p<0.05$ was considered significant.

Results

The study group consisted of 53 elderly long-term-care residents who had been receiving NGT feeding for an average of 14 ± 17 months. The control group consisted of 50 counterparts receiving oral feeding. No statistically significant differences in demographic and medical characteristics occurred between the study groups (Table).

Gram-negative bacteria, including *P. aeruginosa,* were cultured from the oropharynges of 34 (64%) of the 53 NGT-fed patients. *P. aeruginosa* was isolated in 18 (34%) patients of this group or in 60% of the patients colonized with gram-negative bacteria. In the control group, gram-negative bacteria were isolated in only 4 (8%) patients ($p<0.001$). *P. aeruginosa* was not isolated in any of the control group samples ($p<0.001$). Two patients from the NGT-fed group had a mixture of other gram-negative bacteria and *P. aeruginosa*. No association was found between predisposing factors such as age, gender, diabetes mellitus, chronic lung disease, presence of residual teeth, and isolation of *P. aeruginosa*.

Antibiotic susceptibility studies of *P. aeruginosa* isolated from the oropharynx showed that the highest susceptibility rates were registered for tazobactam-piperacillin, with 89% of the isolates being susceptible, followed by ceftazidim (79%) and imipenem, (78%). The *P. aeruginosa* isolates from the oropharynx were less sensitive to most antibiotics than those cultured from sputum; for amikacin, this difference was significant ($p<0.03$).

Figure 1 shows the NGT tube embedded in the adjacent anatomical structures. The findings on electron microscopy of an NGT section are shown in Figure 2.

The samples were taken from four patients with cultures positive for *P. aeruginosa,* who had NGTs inserted 2–4 weeks previously. A biofilm with a bacterial organism with a typical form for *P. aeruginosa* was clearly visualized on the outer surface of all four sections. The identity of the bacterium seen in the biofilm was confirmed by culture. Figure 3 shows the results of the PFGE studies on three patients; in each one, the same clone was isolated from both the oral cavity and the NGT.

Table. Demographic data of study groups[a]

Data	NGT-fed patients[a] n=53 (%)	Orally fed patients n=50 (%)
Age	78 ± 9	81 ± 9
Dementia	32 (60)	29 (58)
Stroke	29 (55)	22 (44)
Diabetes mellitus	9 (17)	7 (13)
COPD	8 (15)	5 (9)
Residual teeth	20 (38)	17 (33)
Corticosteroids	6 (11)	4 (8)

[a]NGT, nasogastric tube; COPD, chronic obstructive pulmonary disease.

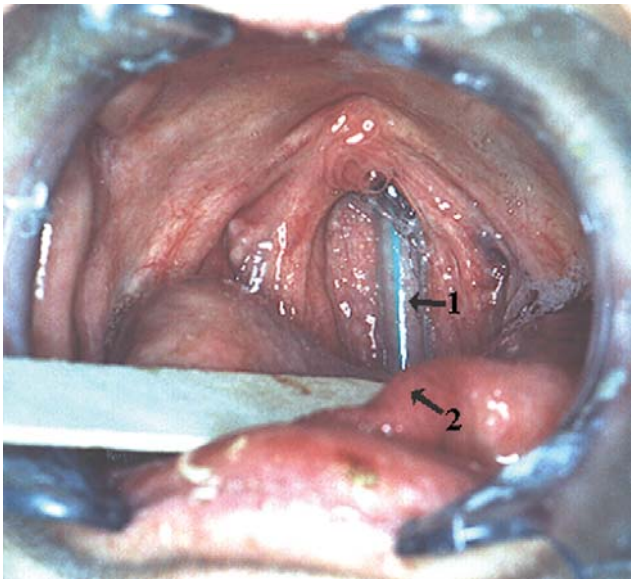

Figure 1. Nasogastric tube embedded in the nasopharynx. 1, nasogastric tube; 2, dorsum of tongue.

Discussion

NGT-fed elderly patients may constitute a human reservoir of *P. aeruginosa.* Determination of the antibiotic susceptibility of these *P. aeruginosa* isolates showed that they had a higher rate of resistance to most antibiotics than to *P. aeruginosa* isolates from sputum obtained from hospitalized patients with bronchopulmonary infections during the same period. This difference reached statistical significance for amikacin. Early studies had reported on the propensity of gram-negative bacteria to colonize elderly patients' oropharynges (10–13). Our recently published study (2) and the present one are the only reports documenting the colonization of the oropharynx in NGT-fed patients by pathogenic florae.

Two factors may explain the high prevalence of *P. aeruginosa* in the NGT-fed patients. One is the lack of mechanical clearance of the mouth provided by chewing and swallowing, an important mechanism in preventing gram-negative bacteria from colonizing the oropharynx of the elderly (14). The other factor is *P. aeruginosa*'s known ability to adhere and form biofilm on plastic tubes, including those made of polyvinyl chloride (15,16). The biofilms formed on NGTs probably play a major role in the persistence of its colonization of the oropharynges of these patients and interfere with its eradication by antibiotics.

The clinical implications of such a reservoir are far reaching. An opportunistic organism such as *P. aeruginosa* in the oropharynx constitutes a threat to NGT-fed patients, who are at risk for aspiration pneumonia and systemic infections (17). Moreover, these elderly NGT-fed patients, most of whom reside in long-term-care facilities, are under "antibiotic pressure" because of frequent clinical infections and may constitute "reservoirs of resistance" (18). The existence of a biofilm in such a reservoir would facilitate antibiotic resistance (19). A similar effect was reported with polyvinyl chloride endotracheal tubes (20). The increased resistance rate of oropharyngeal *P. aeruginosa* isolates to relevant antibiotics in our study as compared with the isolates from sputum is in agreement with this observation.

The epidemiologic importance of a human reservoir of *P. aeruginosa* is not limited to the patient. NGT-fed patients are often transferred to general hospitals and may possibly serve as vectors of resistant organisms to other medical settings (emergency departments, surgical, orthopedic, urologic wards, and intensive care units).

One limitation of our study is that it was performed at a single facility. However, each of the four wards that participated is located in a pavilion separated by 50 m to 100 m from another. Moreover, the factors involved in the oropharyngeal ecosystem colonization by *P. eruginosa* dis-

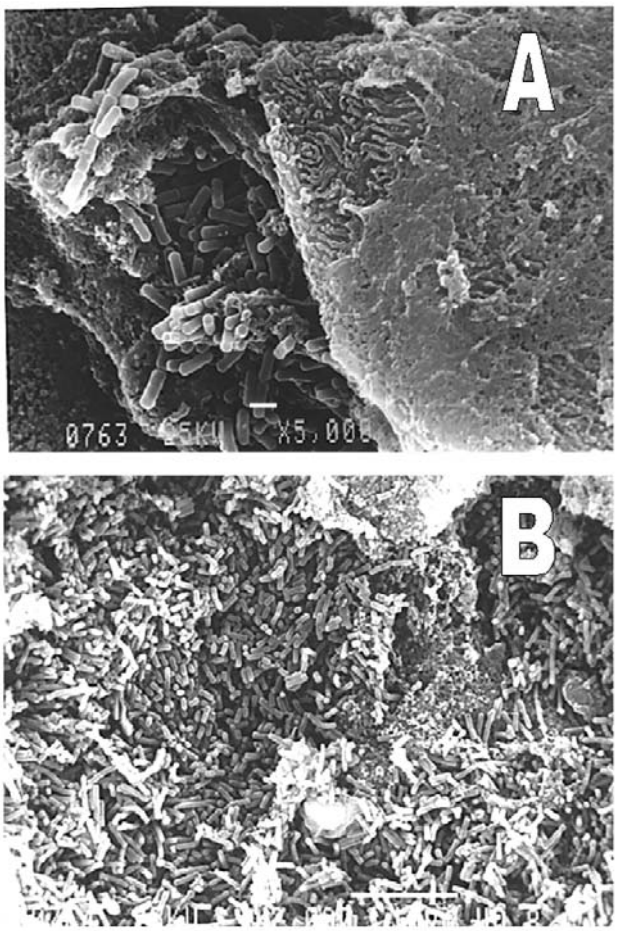

Figure 2. Representative biofilms on nasogastric tubes showing bacterial organisms with typical form of *Pseudomonas aeruginosa*. Scanning electronic microscope. A, scale bar, 1 µm; B, scale bar, 10 µm.

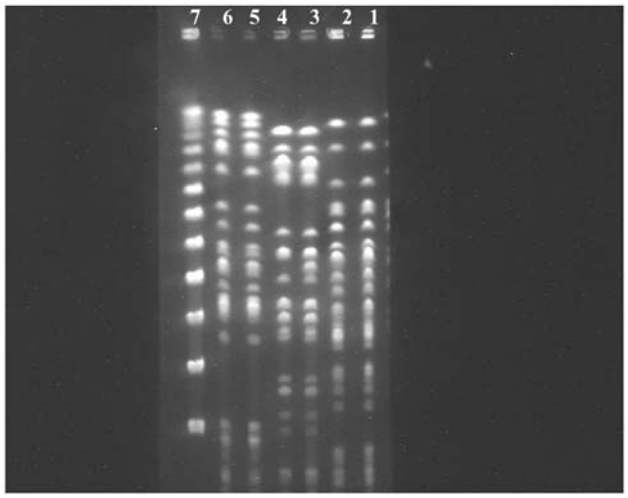

Figure 3. Pulsed-field gel electrophoresis of three pairs of *Pseudomonas aeruginosa* isolates obtained from three patients from nasogastric tubes (lanes 2, 4, 6) and from oropharynx (lanes 3, 5, 7). Lane 1 shows the λ marker size (New England Biolabs, Eldan, Rosh Ha'ain, Israel).

cussed in this study exist everywhere. Similar studies from other long-term-care facilities could provide further evidence.

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Dr. Leibovitz is a specialist in internal medicine and geriatrics and director of a skilled nursing ward at Shmuel Harofe Hospital, Geriatric Medical Center, affiliated with the Sackler School of Medicine, Tel-Aviv University. His present subject of clinical research is the mucosal orifices of frail elderly patients.

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Address for correspondence: Arthur Leibovitz, Shmuel Harofe Hospital, Geriatric Medical Center, POB 2, Beer-Yaakov, Israel; fax: 972-89237156, email: shmuelh@netvision.net.il

Nonmalarial Infant Deaths and DDT Use for Malaria Control

Aimin Chen* and Walter J. Rogan*

Although dichlorodiphenyl trichloroethane (DDT) is being banned worldwide, countries in sub-Saharan Africa have sought exemptions for malaria control. Few studies show illness in children from the use of DDT, and the possibility of risks to them from DDT use has been minimized. However, plausible if inconclusive studies associate DDT with more preterm births and shorter duration of lactation, which raise the possibility that DDT does indeed have such toxicity. Assuming that these associations are causal, we estimated the increase in infant deaths that might result from DDT spraying. The estimated increases are of the same order of magnitude as the decreases from effective malaria control. Unintended consequences of DDT use need to be part of the discussion of modern vector control policy.

After the Stockholm convention in 2001, which called for the gradual ban of the persistent pesticide dichlorodiphenyl trichloroethane (DDT), more than a dozen countries in sub-Saharan Africa requested exemptions for DDT use to control malaria (1). Discussions on the health consequences of DDT use have focused on reducing infant illness and death from vector control. The possibility of observable toxicity has been minimized because only a few studies show that DDT may affect child health and development (2–5). In laboratory experiments, effects of DDT include hepatic and central nervous system toxicity, estrogenic and antiandrogenic effects, and possible carcinogenicity (6,7). Some epidemiologic evidence suggests that DDT exposure increases preterm delivery and small-for-gestational-age births (8) and shortens the duration of lactation (9,10); these conditions could increase the rate of infant deaths (11,12) and thus attenuate any benefits on mortality rates from a reduction in malaria. While the observed associations between DDT and such outcomes might not be causal, the studies are not so flawed that the observations can be dismissed out of hand. We attempted to estimate the consequences for infant deaths if maternal DDT exposure in fact increases preterm births and decreases the duration of lactation with the strength of association seen in North America. If the associations are causal but the estimated effect on death rates is very small compared to the plausible benefits from vector control, then whether the associations are causal does not impact public health decisions. If, on the other hand, the estimated increases in infant death rates are similar to or larger than the expected benefits, whether the association is causal matters a great deal, and further investigation is warranted, especially in areas where DDT is reintroduced.

Although DDT can be found in the lipid of human tissues worldwide, and consequently in the fat of breast milk (13), levels of DDT and its metabolites in breast milk are much higher in areas where this insecticide has been applied for malaria control (14). Here, we use published data on the relationship between DDT spraying and levels in maternal serum and breast milk in Africa to estimate the increased exposure from spraying. We assume that, to obtain the benefit of reduced risk for malaria in the infant, the mother's home must be treated and she must be exposed. We then estimate the effect of that exposure on the frequency of preterm births and on duration of lactation. We assume these relationships to be causal. Whether they are causal, and, if they are, whether the strengths of association seen in North America would occur in Africa is not known. Using infant-mortality rates specific to preterm births, or odds ratios for infant deaths by month-specific breast-feeding status, we estimated deaths attributable to the changed preterm birth rate and to the shortened duration of lactation that we assume would be caused by spraying DDT.

Materials and Methods

The Medline database was searched for literature on DDT home spraying for malaria control and its effect on DDT concentration in serum samples or breast milk; DDT levels in blood or milk and pregnancy outcome or lactation duration; and pregnancy outcome or duration of lactation and infant deaths. Published data were reviewed and reanalyzed, if necessary, to estimate our hypothesized increase of infant deaths from DDT home spraying, consequent to small, early births and shorter lactation.

*National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Results

Effect of Spraying on DDT in Serum and Breast Milk

Three studies on DDT levels in serum or breast milk from Kwa-zulu after DDT application for malaria control showed much higher DDT, DDE (dichlorodiphenyl dichloroethene, the most stable and persistent form of DDT), and DDD (dichlorodiphenyl dichloroethane) levels in the DDT-exposed group (14–16). Dwellings in the exposed area were treated with DDT for vector control (14). In the DDT-treated area, DDE concentration in serum samples was 103±85 μg/L; in the control area, the concentration was 6±7 μg/L (16). The median DDE levels in breast milk fat in the treated area (5.2–7.7 mg/kg) were all much higher than those in the control area (0.38–0.59 mg/kg) (14).

DDE and Preterm Birth

A study based on the U.S. Collaborative Perinatal Project, which included 361 preterm births (<37 completed weeks' gestation) out of 2,380 births (a rate of 151 per 1,000 births), indicated that the adjusted odds ratios (ORs) of preterm birth increased steadily with increasing concentration of maternal serum DDE (ORs=1, 1.5, 1.6, 2.5, 3.1 for DDE concentrations <15, 15–29, 30–44, 45–59, ≥60 μg/L, respectively) (8). The increase in serum DDE levels from spraying is greater than the range seen in the United States; the adjusted OR for the most highly exposed U.S. group is 3.1, but we used a more modest increase for this analysis (see below).

Preterm Delivery and Infant Death

The preterm delivery rate in sub-Saharan Africa ranged from 5% to 22% (17–20) in studies from the 1990s, when DDT was not used or used only in small amounts. A Malawi study (18) showed that children born preterm had a crude relative risk (RR) of 2 for infant death; preterm birth accounted for 17% of infant deaths. Malaria itself might increase preterm birth, but this factor is counted in the contribution of malaria to infant deaths (see below). The observed RR of 2 in Malawi is lower than that seen in the United States and Canada, where mild (birth at 34–36 gestational weeks) and moderate (birth at 32–33 gestational weeks) preterm births were linked to a ≥2.9 fold increase in infant deaths (12). If we assumed that DDT use increased the overall preterm delivery rate from 15% (the midrange of the African rates) before spraying to 25% after spraying (RR 1.7, well below the 3.1 seen in U.S. data), and the RR of preterm birth for infant death is 2.0, we estimated a 9% $(=((p_2*RR+1-p_2)-(p_1*RR+1-p_1))/(p_1*RR+1-p_1)$, p_1=15%, p_2=25%, RR=2) increase in total infant deaths.

DDE and Duration of Lactation

Two birth cohort studies on DDE level in breast milk and duration of lactation both showed a negative relationship between DDE level and breast-feeding duration, whether in North Carolina (10) or Mexico (9). Figure 1 shows similar trends in the decrease of duration of lactation from both of these geographic sites. With a breast milk p,p'-DDE level of 5.0–7.5 mg/kg (fat basis), the median duration of breast-feeding is expected to be 3–4 months, down 40% to 50%, as compared with 7–8 months if p,p'-DDE level falls into the 0–2.5 mg/kg category.

Pooled analysis of data from 17 African countries from the World Fertility Surveys and Demographic and Health Surveys (DHS) in late 1970s and 1980s, when DDT was not used in most of Africa, showed that the mean duration of breast-feeding was 18.1 months (first quartile 12.0 months, median 18.8 months, and third quartile 23.7 months) (21). Another DHS dataset indicated the median breast-feeding duration in Africa from 1986 to 1990 was 19.3 months (22). Thus, if we assume the proportional decrease in duration of lactation attributable to high DDE concentration in milk fat in Africa is similar to that seen in North America, where we observed 40% shorter duration of lactation in women with approximately 6 mg/kg compared to women with approximately 0 mg/kg, and the result of spraying is to increase median DDE in milk fat from 0.4–0.6 mg/kg to 5–8 mg/kg (see above), the median expected duration of breast-feeding in areas with routine

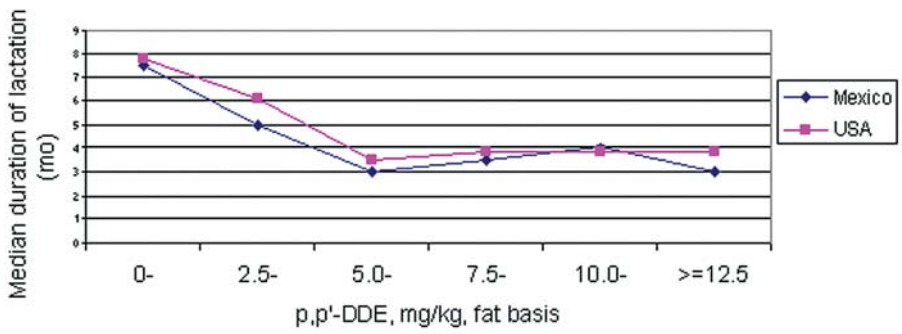

Figure 1. Levels of dichlorodiphenyl dichloroethene (DDE, the most stable and persistent form of DDT), in breast milk and duration of lactation.

DDT application for malaria control should be approximately 11–12 months. The assumption of a similar relative decrease was the simplest. Alternatively, at one extreme, we could assume an absolute decrease of 5 months, from 19 to 13, as we progressed from low to high exposure (Figure 1); at the other extreme, since the median duration of lactation in Mexico was longer than that in North Carolina, but the DDE-level specific durations were the same, we could assume a median duration of 3 to 4 months at the high level of exposure in Africa as well.

Shorter Lactation and Infant Deaths

Shorter duration of lactation increases the risk for infant and childhood deaths in both industrialized and developing countries (23–25). The World Health Organization (WHO) conducted a Mantel-Haenszel pooled analysis to review the effect of breast-feeding on infant and child death rates (11). The analysis identified breast-feeding as a strong protective factor against infant death, especially that caused by infectious illnesses such as diarrhea and acute lower respiratory tract infection. Breast-feeding was most protective in younger infants; nevertheless, it was still protective at 9 to 11 months after birth (Figure 2). If the median duration of lactation were shortened from 19 months to 11–12 months because of high concentrations of DDE, we would expect the proportion of children weaned before 12 months of life to increase from ~25% to 50%. In Africa, where prolonged breast-feeding is the norm, the risk of not being breast-fed continues into the second year of life, with ORs ranging from 8 in Ghana to 2 in Senegal (11). (Death after 1 year of age is no longer considered an infant death, but ORs should not change abruptly between 12 and 19 months of age). In the WHO analyses, the ORs for breast-feeding longer >1 year all were from Africa, and the ORs for breast-feeding <1 year were from Asia or South America. To estimate the effect of decreasing from a median duration of 19 months in Africa to 11 months, we used the most stable African estimate, from Senegal, which is 2.9 at 19 months, and compared it to the pooled estimate from Asia and South America at 11 months, which is 1.4. Thus, if we assume the overall RR of infant death from this degree of DDT-induced shortened lactation to be approximately 2.0, shortened lactation would result in a 20% $(=((p_2*RR+1-p_2)-(p_1*RR+1-p_1))/(p_1*RR+1-p_1)$, $p_1=25\%$, $p_2=50\%$, $RR=2)$ increase in infant mortality caused by infectious diseases.

Increased Rate of Infant Death from Preterm Birth and Shorter Lactation

The reported infant mortality rate (IMR) of sub-Saharan African countries was 108/1,000 in 2000, 110/1,000 in 1995, and 111/1,000 in 1990 (26). If DDT use increases the IMR by 9% because of preterm delivery, IMR will increase by approximately 9.7/1,000 (=108*9%/1,000). Infectious diseases account for more than half of all infant deaths in Africa (27,28); thus, if DDT use increases IMR attributable to infectious disease by 20% (through shorter breast-feeding), another 10.8/1,000 (=(108/2)*20%/1,000) will be added to the IMR in areas with continuous DDT application for malaria control. The results would be a total estimated excess of 20.5/1,000 in IMR. On the other hand, maternal malaria caused 3% to 8% of all infant deaths in areas of Africa with stable malaria transmission (29). Malaria itself caused 20% of deaths in children <5 years of age (175/1,000 [30]) in Africa (31); malaria-specific infant deaths were estimated to be approximately 30% of such deaths (32).

Discussion

When we combine data from North America on preterm delivery or duration of lactation and DDE with African data on DDT spraying and the effect of preterm birth or lactation duration on infant deaths, we estimate an increase in infant deaths that is of the same order of magnitude as that from eliminating infant malaria. Therefore, the side effects of DDT spraying might reduce or abolish its benefit from the control of malaria in infants, even if such spraying prevents all infant deaths from malaria. However, no studies from sub-Saharan Africa show that DDE shortens the duration of breast-feeding there specifically, and studies showing the relationship in the United States and Mexico both come from our group. Neither replications nor replication failures have been reported from other groups with longitudinal data. The relationship has a biologically plausible mechanism, in that both isomers of DDE are weak estrogens (33,34), and estrogen inhibits the stimulatory effect of prolactin on milk synthesis. (A woman's own estrogen production is at its lowest postpubertal level at the beginning of lactation.) Older birth control pills with higher estrogen levels were linked to a decrease in milk volume or shortened duration of lactation (35).

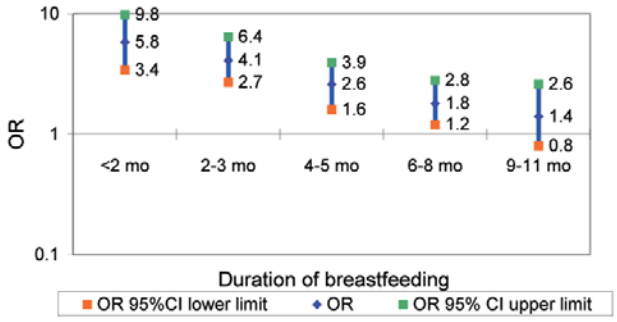

Figure 2. Protection of breast-feeding against infant death caused by infectious disease (not breast-feeding versus breast-feeding). CI, confidence intervals; OR, odds ratio. Source: World Health Organization study team.

In an attempt to replicate the finding on duration of lactation and DDE, Bouwman enrolled lactating women in a cross-sectional study comparing levels of DDE in milk in areas using and not using DDT for malaria control. Even though he did not follow children over time, this researcher reasoned that, if DDE shortened lactation, children of lactating mothers who had higher DDE levels would be, on the average, younger. He found that the DDE levels were much higher in the area where DDT was used than in a control area, but the age of children and the number of women unable to give a milk sample did not differ. This study is informative but cannot be interpreted as a failure to replicate the duration finding, since women were not followed over time, and, obviously, only women still lactating were eligible (15). Roberts argues against the causal nature of the association by observing that, in routinely collected data from Belize, rural women, who might be exposed to DDT, breast-fed longer than urban women, who probably were not exposed (36). However, these women are otherwise probably not similar (e.g., in their occupations or socioeconomic status). Nor is the classification of exposure exact enough to detect even very large effects of DDE. No studies in Africa show a relationship between DDE and preterm birth. The findings from the U.S. Collaborative Perinatal Project, while a confirmation of previous, smaller studies, still need to be replicated in Africa.

Another weakness of this analysis for current day sub-Saharan Africa is that the contrasts in mortality rates between breast-fed and bottle-fed children are derived from older data, when the impact of HIV on infant deaths was lower. In 1990, HIV caused 2% of deaths of children <5 years of age, whereas by 1999 it caused almost 8%. Five countries (Botswana, Namibia, Swaziland, Zambia, and Zimbabwe) had HIV-attributable under-5 death rates of approximately 30 per 1,000 (21% to 42% of total under-5 mortality). An additional 16 countries had HIV-specific under-5 mortality rates between 10 and 25 per 1,000 (8% to 20% of total under-5 death rates). The remaining 18 countries had rates <10 per 1,000 (0.1% to 6.5% of total under-5 deaths) (37). The effect of changing the duration of breast-feeding on HIV mortality is hard to quantify because, while any breast-feeding may increase transmission from infected mothers to infants, prolonged, exclusive breast-feeding may decrease deaths and HIV transmission (38). Such nonlinearities in dose-effect are difficult to reduce to the relatively simple formulae required for the kind of analysis presented here. Our conclusions are robust if shorter duration of lactation does not eliminate transmission of HIV and consequent deaths; even then, HIV mortality rates are likely only high enough in the five most affected countries to cancel the protective effect of breast-feeding entirely.

Our estimation provides a general framework of risk evaluation in sub-Saharan Africa. However, the variation in malaria transmission, illness, death, DDT spraying strategy, incidence of preterm birth, and duration of lactation should be kept in mind before the estimate is applied to a specific country or area. Since we focused on infant deaths, the benefits of DDT to child or adult malaria-specific deaths were not taken into account. Similarly, other potential adverse effects to humans and the environment that might occur from DDT spraying were not considered. However, this analysis counters the assumption that the risk of DDT use is unlikely to outweigh the benefit (39) and requires that such an assumption be tested.

The prohibition of DDT use for malaria control was probably not the sole cause of increasing malaria burden in sub-Saharan Africa (40), and thus DDT will probably not be the sole cure for the malaria epidemic there. Insecticide-treated bed nets, widely used in African households to prevent mosquito bites, are effective (41,42). Synthetic pyrethroid insecticides, cheaper than DDT, are available (43,44). Where DDT is used, all infant deaths, plus birth weights and the duration of lactation, should be counted. Some thought could also be given to a formal trial, since the risk and benefit calculations apply to individual dwellings, and an effective alternative, namely bed nets, is available.

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Dr. Chen is a postdoctoral fellow and Dr. Rogan is a senior investigator in the Epidemiology Branch at the National Institute of Environmental Health Sciences, one of the U.S. National Institutes of Health, in Research Triangle Park, North Carolina. Their research interests include effects of environmental pollutant chemicals on human reproduction and maternal and child health.

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Address for correspondence: Walter J. Rogan, Epidemiology Branch, NIEHS, MD A3-05, P.O. Box 12233, Research Triangle Park, NC 27709, USA; fax: (919) 541-2511; email: rogan@niehs.nih.gov

Multidrug-Resistant Tuberculosis in HIV-Negative Patients, Buenos Aires, Argentina

Domingo Palmero,* Viviana Ritacco,† Martha Ambroggi,‡ Marcela Natiello,‡ Lucía Barrera,† Lilian Capone,‡ Alicia Dambrosi,‡ Martha Di Lonardo,‡ Nélida Isola,‡ Susana Poggi,‡ Marisa Vescovo,‡ and Eduardo Abbate‡

Initial multidrug-resistant (MDR) tuberculosis (TB) in HIV-negative patients treated at a Buenos Aires referral hospital from 1991 to 2000 was examined by using molecular clustering of available isolates. Of 291 HIV-negative MDRTB patients, 79 were initially MDR. We observed an ascending trend of initial MDRTB during this decade (p=0.0033). The M strain, which was responsible for an institutional AIDS-associated outbreak that peaked in 1995 to 1997, caused 24 of the 49 initial MDRTB cases available for restriction fragment length polymorphism. Of those, 21 were diagnosed in 1997 or later. Hospital exposure increased the risk of acquiring M strain–associated MDRTB by approximately two and a half times. The emergence of initial MDRTB among HIV-negative patients after 1997 was apparently a sequel of the AIDS-related outbreak. Because the prevalence of M strain–associated disease in the study population did not level out by the end of the decade, further expansion of this disease is possible.

Multidrug-resistant (MDR) tuberculosis (TB) in patients with a history of previous TB treatment is known as acquired MDRTB and usually reflects shortcomings in current treatment administration such as irregular drug supplies, inadequate treatment regimens, or poor patient compliance (1). On the other hand, MDR among new cases of TB (initial MDRTB) originates from infectious sources that are disseminating MDR bacilli in the community; this resistance is an indicator of treatment deficiencies accumulated over a prolonged period (2).

During the last decade, MDRTB has surged as an important global problem. The AIDS pandemic fostered this emergence by spreading the disease among immunodeficient institutionalized patients (3–5). According to the global antituberculosis drug resistance surveillance program of the World Heath Organization and International Union Against Tuberculosis and Lung Disease, Argentina was identified as an MDRTB "hot spot" in the mid-1990s (6). At that time, initial MDRTB in that country was fueled by contemporary AIDS-related hospital outbreaks, the most important of which originated at Muñiz Hospital (7–12).

Located in Buenos Aires City, Muñiz Hospital is the main Argentinean referral center for infectious diseases and provides for approximately 800 TB patients yearly. An appreciable proportion of the MDRTB cases diagnosed in the country (mainly from the Buenos Aires metropolitan area) are referred to this center for further bacteriologic assessment and clinical management. Soon after the emergence of HIV infection in 1982, TB grew into the most frequent AIDS-associated disease in the country. During the 1990s, the hospital underwent an extensive AIDS-related MDRTB outbreak while continuing to provide care for HIV-negative drug-resistant TB patients.

Throughout the 1990s, a total of 736 AIDS-related MDRTB cases were hospitalized in Muñiz Hospital (1 [1991], 4 [1992], 10 [1993], 58 [1994], 149 [1995], 132 [1996], 159 [1997], 108 [1998], 79 [1999], and 36 [2000]; unpub. data). An IS*6110* restriction fragment length polymorphism (RFLP) study conducted in the first half of the decade of a sample of AIDS-related TB cases resistant to five or more drugs identified the so-called M strain as the main outbreak strain (9). Our RFLP database documents the predominance of the M strain among AIDS-related MDRTB cases investigated in this hospital during the second half of the decade (10). The infection disseminated to other healthcare centers in the metropolitan area, and secondary microepidemics were reported to be associated to the M strain (11,12).

Beginning in 1995, Muñiz Hospital implemented a number of measures to stop the spread of MDRTB. Isolation rooms with portable HEPA filters became available in HIV buildings, and a separate ward was dedicated to HIV-associated MDRTB. Following the recommendations of a visiting team from the Division for TB Elimination at the Centers for Disease Control and

*Hospital F. J. Muñiz, Buenos Aires, Argentina; †National Institute of Infectious Diseases "Carlos G. Malbran," Buenos Aires, Argentina; and ‡University of Buenos Aires, Buenos Aires, Argentina

Prevention, additional measures were gradually implemented beginning in 1996, including smear examination at admission, speeding of bacteriologic diagnosis through radiometric bacteriologic assay, ready availability of second-line drugs, and personal respiratory protection with N-95 masks (13).

Our study was directed at gaining insight into the impact of the MDRTB epidemic that occurred in Buenos Aires in the putatively immunocompetent population. We examined the trend of initial MDRTB in non-HIV patients treated at the Muñiz Hospital during the last decade in the light of molecular clustering of available isolates.

Methods

Population

Medical records of all HIV-negative patients with MDRTB (both inpatients and outpatients) diagnosed at Muñiz Hospital from January 1991 to December 2000 were reviewed. All patients were routinely tested for HIV infection by enzyme-linked immunosorbent assay after giving informed consent. The following patient data were collected: year of MDRTB diagnosis, age, gender, nationality, place of residence, site of TB disease, chest x-ray score of lesion extension (minimal, moderate, advanced), history of previous TB treatment, and underlying illnesses. MDRTB cases were defined as those caused by *Mycobacterium tuberculosis* resistant to at least isoniazid and rifampicin. MDRTB patients with a history of previous TB chemotherapy were defined as acquired MDRTB cases. Initial MDRTB was defined as that diagnosed in a patient without previous TB treatment. Initial MDRTB cases were classified according to possible exposure settings, including hospital (patients with a history of previous hospitalization or hospital attendance and healthcare workers), household, and undisclosed source of infection.

Bacteriologic Methods

Susceptibility to isoniazid, rifampicin, ethambutol, and streptomycin was determined according to World Health Organization standards. Susceptibility to kanamycin, p-aminosalycilic acid, and cycloserine was performed according to the Canetti, Rist, and Grosset method, whereas the pyrazinamidase test was used to infer pyrazinamide susceptibility (14).

DNA Fingerprinting

Available isolates of initial MDRTB cases were typed by IS*6110* DNA fingerprinting by using the standardized protocol for *M. tuberculosis* (15). Briefly, DNA was extracted from bacilli suspensions and digested with the restriction enzyme *Pvu*II. DNA fragments were electrophoresed in 0.8% agarose and vacuum-blotted into a positively charged nylon membrane. The IS*6110* probe was a 245-bp DNA fragment amplified by polymerase chain reaction and labeled by the enhanced chemiluminescence gene detection system (Amersham International plc, Amersham, U.K.). *M. tuberculosis* Mtb 14323 was used as a reference strain. Hybridization patterns were compared visually. Isolates from one patient were considered to be part of a cluster if the IS*6110* fingerprint matched that of a different patient within the study period.

Statistics

We used the Mantel-Haenszel test to compare categoric data from groups of patients. For the comparison of age means, the Student t test was applied. The relative weight of initial MDRTB throughout the study period was analyzed by the Mantel test for linear trend. P values <0.05 were considered significant. The software used was Epi Info version 6.0 (16).

Results

From January 1991 to December 2000, a total of 291 HIV-negative patients treated at Muñiz Hospital were affected by MDRTB. Of these, 212 (72.9%) were acquired MDRTB cases, and 79 (27.1%) were unambiguously identified as initially MDR. The primary site of disease was pulmonary in all patients of both groups with a single exception. An accidental subcutaneous inoculation of a bacteriologist caused the only primary extrapulmonary case, which was obviously classified in the initial MDRTB group.

Demographic, clinical, and bacteriologic characteristics of both groups are compared in Table 1. Compared with acquired-MDRTB patients, patients with initial MDRTB showed significant differences in age, gender, and chest x-ray involvement. The presence of an underlying illness was not identified as a predisposing factor for initial MDRTB. Initial MDRTB patients were significantly younger and their lung lesions less advanced. Female patients predominated in this group. Initial MDRTB increased from 15.0% of total MDR cases in 1991 to 37.8% in 2000 (test for trend, p=0.00033, odds ratio [OR] = 3.45) (Figure 1).

Isolates from 49 of the 79 patients with initial MDRTB were available for DNA typing. Thirty-six (73.0%) of these 49 fit in six molecular clusters with RFLP patterns of six or more bands. The M strain was responsible for the largest cluster involving 24 (49.0%) of the 49 investigated cases. All patients in the M cluster lived and were treated in Buenos Aires City area. A flow tree is shown in Figure 2.

The second largest cluster occurred in four members of a Peruvian family who had immigrated to Argentina in 1999. The remaining four clusters consisted of two cases each. Samples from cases in two of these clusters had

Table 1. Demographic, clinical, and bacteriologic features of acquired versus initial multidrug-resistant tuberculosis (MDRTB) cases among 291 HIV-negative patients, Muñiz Hospital, Argentina, 1991–2000

	Acquired MDRTB (n=212)	Initial MDRTB (n=79)	p value
Age (y ± SD)	39.8±13.2	35.3±15.0	0.013
Gender (male/female)	125/87	32/47	0.005
Origin (Argentinean/foreign-born)[a]	180/32	64/15	0.533
Chest x-ray (minimal/moderate/advanced)	2/52/158	10/38/31	<0.0001
Underlying illness (Y/N)	46/166	15/64	0.731
Diabetes (N)	26	7	–
Alcoholism (N)	9	1	–
Silicosis (N)	2	1	–
Steroid treatment (N)	2	3	–
Substance abuse (N)	1	0	–
Malignancy (N)	1	2	–
Other (N)	5	1	–
Drug resistance, median number of drugs (range)	4 (2–8)	4 (2–6)	–

[a]Neighboring countries (Peru, Bolivia, Paraguay).

matching fingerprints with previously documented outbreak strains; the patients' histories were consistent with such hospital-acquired disease (7). The remaining four case-patients within the last two clusters were household contacts of two clearly identified index MDRTB cases.

Figure 3 shows the biennial number of initial MDRTB cases attributable to the M strain, other strains, and those unavailable for RFLP. Among those available for fingerprinting, the proportion of initial MDRTB associated with the M strain increased significantly in the period 1997–2000 when compared with previous years (21/36 vs. 3/13; p=0.03; OR= 0.21).

The putative exposure setting and RFLP findings of the 79 initial MDRTB cases are described in Table 2. Hospital-related disease attributable to the M strain was detected in 15 cases from seven different health institutions in the metropolitan area. The M strain–associated disease was significantly more frequent among hospital-exposed cases when compared with patients who acquired the disease elsewhere (15/20 vs. 9/29; p=0.002; relative risk [RR] = 2.42; 95% confidence interval [CI] 1.33 to 4.40). The proportion of isolates lost for RFLP typing did not differ significantly between the groups compared above to analyze prevalence of the M strain in different periods (p=0.52) and exposure settings (p=0.11).

Discussion

Initial MDRTB is contracted from a source case, whereas acquired MDRTB is the result of a prolonged history of inadequate treatment. Therefore, patients with initial MDRTB were substantially younger and their lung lesions less advanced. Women predominated in the initial MDRTB group, which might reflect the conventional female role as caregiver in the local population.

The ascending trend of the initial MDRTB in HIV-negative patients may be explained as a consequence of the AIDS-associated outbreak that occurred in the hospital during the 1990s. The M strain was preeminent in this phenomenon. Initial MDRTB cases attributable to this strain appeared at the peak of the AIDS-related epidemic, persisted beyond its decline, and did not reach a neat plateau at the end of the study period. These dynamics reflect the normal latency of infection and hint that a further expansion of the strain is still possible.

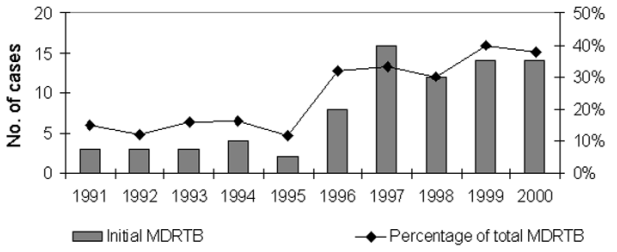

Figure 1. Trend of initial multidrug-resistant tuberculosis among HIV-negative patients at Muñiz Hospital, Buenos Aires, 1991–2000. MDRTB, multidrug-resistant tuberculosis.

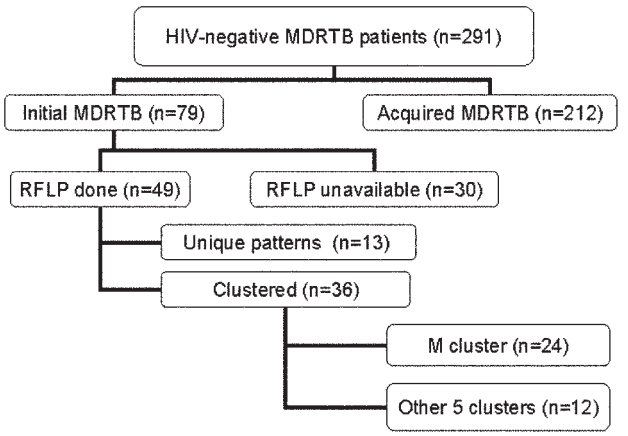

Figure 2. HIV-negative multidrug-resistant tuberculosis groups investigated. MDRTB, multidrug-resistant tuberculosis; RFLP, restriction fragment length polymorphism.

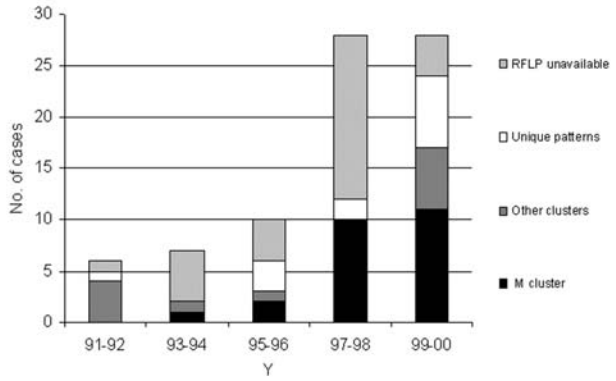

Figure 3. Biennial number of initial multidrug-resistant tuberculosis cases among HIV-negative patients and restriction fragment length polymorphism findings. RFLP, restriction fragment length polymorphism.

Hospital exposure increased the risk of acquiring M strain–associated MDRTB by approximately two and a half times. Thus, the hospital seems still to be the most likely setting for acquiring the M strain of MDRTB. However, 4 of 10 case-patients with an unidentified source of exposure were also affected by this strain. These patients were thoroughly interrogated again, and a previous institutional or household contact was virtually discarded. The failure to trace the index cases for these patients indicates the presence in the community of unidentified infectious cases harboring this highly resistant strain.

However, the marked increase of initial MDRTB cannot be ascribed entirely to the M strain. Other indigenous outbreak strains and strains recently introduced to the country contributed to this emergence, adding complexity to the MDRTB problem in the metropolitan area. Approximately one in every five case-patients with both initial and acquired MDRTB had recently migrated from a neighboring country where TB prevalence is higher than in Argentina, which suggests the influence of regional migration on local MDRTB rates.

The outcome of this investigation cannot be generalized to scenarios different from that of a referral center for infectious diseases. Other possible limitations of our study should be considered. Bias caused by laboratory cross-contamination is improbable because two or more isolates were obtained from every patient and susceptibility patterns were consistent in all cases. Data loss was appreciable: over one third of the isolates were lost for fingerprinting because of inadequate culture maintenance. Therefore, TB transmission might have been underestimated in our population, as shown by computer simulation studies (17,18). Eventually, misclassification of cases might have occurred. Some observations induced the idea of misclassification. A sudden shift from fully susceptible TB (RFLP not available) to M strain–associated MDR disease was documented in six of the cases classified in the acquired MDRTB group. We suggest that these patients were actually reinfected with the M strain while still being treated with standard chemotherapy at the hospital. As demonstrated by van Rie et al., exogenous reinfection is not uncommon among HIV-negative patients in settings with high prevalence of TB and the distinction between initial and acquired MDRTB in such situations becomes ambiguous (19).

The emergence of initial MDRTB among HIV-negative persons assisted in Muñiz Hospital in the second half of the 1990s can be considered a sequel to the MDRTB epidemic that occurred among AIDS patients in Buenos Aires. The M strain has already exceeded nosocomial bounds and might be expanding in the community. The strain appears to be more prosperous than other contemporary MDR strains and is emerging as a persistent strain.

Table 2. Distribution of 79 HIV-negative patients with initial multidrug-resistant tuberculosis (MTDRTB) according to the putative exposure setting and DNA fingerprint findings, Muñiz Hospital, Argentina, 1991–2000

Setting	n (% of total)	RFLP done[a]	M strain
Hospital			
Healthcare workers[b]	20 (25.3)	15	10
Patients	7 (8.9)	5	5
Household	35 (44.3)	19	5
Unknown	17 (21.5)	10	4
Total	79 (100)	49	24

[a]RFLP, restriction fragment length polymorphism.
[b]Physicians, 6; nurses, 5; bacteriologists, 2; and auxiliary staff, 7.

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Dr. Palmero is a pulmonologist at the F. J. Muñiz Hospital, Buenos Aires, and head professor of pulmonology at the Universidad del Salvador. His main research interest is multidrug-resistant tuberculosis.

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Address for correspondence: Domingo J. Palmero, N. Videla 559 (1424) Buenos Aires, Argentina; fax: 54-11-4432-6569; email: djpalmero@intramed.net.ar

Invasive Group A Streptococcal Disease: Risk Factors for Adults

Stephanie H. Factor,*† Orin S. Levine,‡ Benjamin Schwartz,* Lee H. Harrison,§ Monica M. Farley,¶ Allison McGeer,# and Anne Schuchat*

We conducted a case-control study to identify risk factors for invasive group A streptococcal (GAS) infections, which can be fatal. Case-patients were identified when *Streptococcus pyogenes* was isolated from a normally sterile site and control subjects (two or more) were identified and matched to case-patients by using sequential-digit telephone dialing. All participants were noninstitutionalized surveillance area residents, >18 years of age. Conditional logistic regression identified the risk factors for invasive GAS infection: in adults 18 to 44 years of age, exposure to one or more children with sore throats (relative risk [RR]=4.93, p=0.02), HIV infection (RR =15.01, p=0.04), and history of injecting drug use (RR=14.71, p=0.003); in adults >45 years of age, number of persons in the home (RR=2.68, p=0.004), diabetes (RR= 2.27, p=0.03), cardiac disease (RR=3.24, p=0.006), cancer (RR= 3.54, p=0.006), and corticosteroid use (RR=5.18, p=0.03). Thus, host and environmental factors increased the risk for invasive GAS disease.

Invasive GAS infection can lead to dramatic, rapidly-progressive syndromes such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS). An estimated 9,500 cases of invasive GAS disease occurred in the United States in 1999, resulting in approximately 1,100 deaths. The overall case-fatality rate of invasive GAS is estimated to be from 10% to 15%, and the case-fatality rate for STSS can exceed 60% (1). Most of these infections are community-acquired.

Although case series and population-based surveillance have identified several possible host risk factors for the development of invasive GAS disease, including age, Native American ethnicity, HIV infection, diabetes mellitus, cardiovascular disease, alcoholism, and other chronic diseases (1–3), these studies have not been able to assess household risk factors. In addition, surveillance studies identified possible risk factors by comparing prevalence of host factors among GAS patients (by using medical record data) to prevalence of host factors among the general population using population-based estimates (1,2). These comparisons are limited by the completeness and availability of both kinds of data.

Household-based studies conducted in the 1950s demonstrated that school-aged children were most often responsible for introducing a GAS strain into a household and that mothers were more likely to subsequently acquire the bacteria than fathers (4). These studies suggest that exposure to children and duration of exposure to a GAS-infected person influence the transmission of GAS within households. However, community studies have not shown the relative importance of these factors compared to host factors.

We conducted a case-control study to evaluate the importance of previously identified risk factors for invasive GAS infection: contact with other persons in the home, with children, with persons symptomatic with GAS disease in the home, with other persons at work, and with persons symptomatic with GAS disease at work.

Methods

Invasive GAS disease was defined as the isolation of *Streptococcus pyogenes* from a normally sterile site (e.g., blood, cerebrospinal fluid, pleural fluid, peritoneal fluid, pericardial fluid, joint fluid, surgical specimens, bone, and scrotal fluid) in a noninstitutionalized resident, ≥18 years of age, in a surveillance area. Persons who had group A *Streptococcus* isolated from a sterile site more than 48 hours after hospital admission were presumed to have a nosocomial infection (5) and were excluded.

Cases of invasive GAS disease were identified through active, laboratory-based surveillance in three areas: metropolitan Atlanta, Georgia, from July 1, 1997, through June 30, 1999; metropolitan Baltimore, Maryland, from July 1, 1997, through June 30, 1999, and the Toronto-Peel region, Ontario, Canada, from July 1, 1997, through December 31, 1997. The surveillance area population was estimated to include 9 million people (3,627,184 in metropolitan

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †New York Academy of Medicine, New York, New York, USA; ‡National Institute for Allergy and Infectious Diseases, Bethesda, Maryland, USA: §Johns Hopkins University, Baltimore, Maryland, USA; ¶Emory University, Atlanta, Georgia; and #Mount Sinai Hospital, Toronto, Ontario, Canada

Atlanta, 2,436,239 in the Baltimore metropolitan area, and 3,008,570 in the Toronto-Peel region based on 1997 Bureau of Census estimates [6]). All acute-care hospitals and laboratories serving the residents of the surveillance area were contacted biweekly and audited semiannually to identify patients with invasive GAS disease.

A "case algorithm" was used to contact persons infected with invasive GAS infection. For each case-patient identified, up to 15 separate telephone calls were made to contact the patient. To maximize the likelihood of contacting the case-patient, the telephone calls were made on 5 nonconsecutive days, including at least 1 weekend day, during each of three different time periods (8 a.m.–noon; noon–5 p.m.; 5 p.m.–8 p.m.). The party was considered unavailable for that call if the telephone was allowed to ring 10 times or if an answering machine picked up the phone. Persons still hospitalized when the case was identified were interviewed by phone in the hospital when feasible. Case-patients were eligible if their enrollment was complete within 3-months of onset of their GAS disease.

To both maximize enrollment and limit bias, several arrangements were made. Family members of deceased case-patients were interviewed. Non–English-speaking patients were included if the individual surveillance sites had the resources to communicate with the patients in their language.

A population-based sample of matched control subjects was selected through a process of systematic, sequential-digit telephone dialing. Case-patients and control subjects were matched by age group, postal or zip code, and telephone exchange. Age groups were defined as follows: 18–44 years of age, 45–64 years of age, and ≥65 years of age. Matching was done on postal or zip code and telephone exchange to control for socioeconomic status. Because control-subjects were identified by telephone interview, case-patients who did not have a telephone were excluded.

A "control algorithm" was used to identify control subjects. Control identification began after a case-patient had been identified, confirmed as eligible, and enrolled in the study. Phone numbers for control subjects were generated by incrementally adjusting the enrolled case-patient's home phone number. For example, if an enrolled case-patient's telephone number ended in 1234, the first phone number used to identify a control had the same area code and exchange and ended in 1235. The numbers generated for control subjects were called a maximum of four times over several days. Any number of phone numbers were called at one time. Calling continued until 1) a minimum of two control subjects were enrolled per case-patient, and 2) all numbers that had been called at least once completed the control algorithm. As a result, more than two control subjects were enrolled for some case-patients. The purpose of completing the control algorithm was to ensure that the control group was not biased towards households that were easier to contact.

When experienced surveillance personnel reached the case-patient or control subject, they explained the purpose of the study, obtained informed consent, and administered a standardized questionnaire. This study was approved by the Institutional Review Boards at the Centers for Disease Control and Prevention and at each of the sponsoring institutions.

Questionnaire

The questionnaire included demographic information, socioeconomic status, smoking status of the interviewee, smoking status of other persons in the home, medical history, and history of alcohol and injecting drug use. Within the medical history section, we differentiated between "regular" nonsteroidal antiinflammatory drug (NSAID) use and "new" NSAID use. New NSAID use implied that the case-patient had started using NSAIDs in the 2 weeks before their illness or that a control subject had started using NSAIDs in the 2 weeks before their interview. Each case-patient and control subject were allowed to self-define "regular use" of NSAIDS. To screen for alcoholism, we used the CAGE questionnaire in which a subject is asked four questions about his or her relationship to alcohol. The likelihood of alcoholism increases with the number of times the person answers "Yes" (7).

The questionnaire also asked about contact with other persons in the home, contact with children, contact with persons exhibiting symptoms of GAS disease in the home, contact with other persons at work, and contact with persons with symptoms of GAS disease at work. To evaluate contact with other persons in the home, the questionnaire included questions about number of persons in the home, crowding in the home (number of persons per room), type of home, age of all persons living in the home, and types of relationships with all persons living in the home. To evaluate contact with children, the questionnaire asked about the number of children in the home (i.e., persons <18 years of age), amount of time spent with children in the home, frequency of certain types of sharing behavior with children in the home (including eating off the same plate, sharing a beverage, sleeping in the same bed), amount of time spent with children who did not live in the home, and frequency of sharing behavior with children who did not live in the home.

The questionnaire asked whether anyone in the home had been ill in the 2 weeks before the case-patient's illness or the control subject's interview by asking about specific types of illnesses. For example, the questionnaire asked "Has anyone in your household, other than yourself, had a

sore throat?" If the interviewee said "yes," he or she was asked how many children and how many adults had a sore throat during that period. The questions asked whether anyone in the home had a sore throat; fever; new cough; new runny nose; skin infection; a diagnosis of strep throat, an ear infection, or a sinus infection; or died. To measure the severity of the illness, the questionnaire asked whether the ill person(s) had visited a doctor, missed school or work, had taken antibiotics, or had been hospitalized.

To evaluate exposure to both persons at work and symptomatic persons at work, the questionnaire asked which type of environment the case-patient or control subject worked in, how many hours she or he worked, how many persons were in their work environment, and whether anyone at work had been ill in the 2 weeks before the case-patient's illness or control subject interview by asking about specific types of illnesses. The questions were asked in the same manner as those asked of persons in the home.

Statistical Analysis

Odds ratios (ORs) for each potential risk factor were determined by using conditional logistic regression (Proc PHREG, SAS Version 6.12, Cary, NC), controlling for sex and race. These ORs were determined separately for age groups 18–44 years of age and ≥45 years of age to identify age-dependent risk factors. Those variables found to have a p value ≥0.20 in individual analyses were included in multivariable analysis. Computer-assisted and manual forward, backward, and stepwise conditional logistic regression was done to identify risk factors independently associated with invasive GAS disease. ORs with 95% confidence intervals (CIs) that do not include 1.00, and p values <0.05 were considered statistically significant in multivariable analysis.

Results

Surveillance identified 401 episodes of invasive GAS disease among adults ≥18 years of age; 390 persons were traceable and were screened by surveillance personnel for possible participation. Of the 390 persons with invasive GAS disease, 49 were ineligible (36 due to nosocomial infection and 13 due to institutionalization). Of the remaining 341 case-patients, 139 were enrolled, 24 refused to participate, 27 were not reached after exhausting the telephone case algorithm, and the rest were nonparticipants. Reasons for nonparticipation included the following: refusal to allow participation by spouse or surrogate, >3 months had elapsed since the illness, incomplete or incorrect contact information (i.e., wrong phone number, disconnected phone, no phone, homelessness), and difficulty with communication over the phone (i.e., poor communication skills, non–English-speaking patient).

The number of case-patients and control subjects enrolled varied by area: 58 case-patients were from Atlanta, 49 case-patients were from Baltimore, and 32 case-patients were from Toronto. Of the 139 case-patients enrolled, 48 (34%) were 18–44 years of age, 54 (39%) were 45–64 years of age, and 37 (27%) were ≥65 years of age. Of the 139 case-patients, 70 (50%) were men, 75 (54%) were white, and 60 (43%) were African-American (Table 1). Eighteen (13%) of 139 patients died. Primary bacteremia and cellulitis were the two most common diagnoses (Table 2). The organism was most commonly identified from blood.

Several factors were associated with invasive GAS disease among those 18–44 years of age (Table 3). When sex and race were controlled for, HIV seropositivity and history of injecting drug use were significantly associated with invasive GAS disease (p≤0.05) when each variable was analyzed individually. Smoking, presence of children in the home, diabetes, cancer, regular use of NSAIDs, corticosteroid use, and alcohol abuse were not associated with invasive GAS disease. Using multivariable conditional logistic regression and controlling for sex and race, we found that three risk factors were independently associated with invasive GAS disease: having one or more children with a sore throat in the home in the past 2 weeks (RR=4.93, p=0.02), HIV seropositivity (RR=15.01, p=0.04), and history of injecting drug use (RR=14.71,

Table 1. Characteristics of patients with invasive group A streptococcal disease, Atlanta, Baltimore, Toronto, 1997–1999

Characteristic	Patients, N=139 (%)
Geographic area	
Atlanta	58 (42)
Baltimore	49 (35)
Toronto	32 (23)
Age group	
18–44	48 (34)
45–64	54 (39)
65+	37 (27)
Sex	
Male	70 (50)
Female	69 (50)
Race	
White	75 (54)
Black	60 (43)
American Indian or Alaskan Native	1 (1)
Asian or Pacific Islander	2 (1)
Other or not specified	1 (1)
Ethnicity	
Hispanic	2 (1)
Non-Hispanic	137 (99)
Outcome	
Lived	120 (86)
Died	18 (13)
Unknown	1 (1)

Table 2. Clinical syndromes of patients with invasive group A streptococcal disease, Atlanta, Baltimore, Toronto, 1997–1999

Clinical syndromes[a]	Patients, N=139 (%)
Primary bacteremia (without focus)	57 (41)
Cellulitis	36 (26)
Septic arthritis	11 (8)
Necrotizing fasciitis	9 (6)
Pneumonia	8 (6)
Streptococcal toxic shock syndrome	6 (4)
Otitis	4 (3)
Peritonitis	3 (2)
Osteomyelitis	2 (2)
Endometritis	2 (2)
Abscess	2 (2)
Puerperal sepsis	2 (2)
Meningitis	1 (1)
Endocarditis	1 (1)
Urologic syndrome	1 (1)
Other syndrome	18 (13)

[a]Patients may appear in more than one category.

p=0.003). Among those 18–44 years of age, one case-patient had a history of paralysis, and no one had recent varicella infection.

Many factors were associated with invasive GAS disease in adults ≥45 years of age (Table 4). When the results were controlled for sex and race, we found that the following factors were significantly associated with invasive GAS disease (p≤0.05): three or more persons living in the home, any child living in the home, other smokers living in the home, diabetes mellitus, cardiac disease, chronic obstructive pulmonary disease (COPD), cancer, paralysis, regular use of NSAIDs, use of corticosteroids, and history of injecting drug use. Current smoking and alcohol abuse were not associated with invasive GAS disease. When multivariable conditional logistic regression analysis, controlling for sex and race, was used, the following factors were independently associated with GAS disease: three or more persons living in the home (RR=2.68, p=0.004), diabetes mellitus (RR=2.27, p=0.03), cardiac disease (RR=3.24, p=0.006), cancer (RR=3.54, p=0.006), and use of corticosteroids (RR=5.18, p=0.03).

Among adults ≥45 years of age, the only cases of cirrhosis and recent varicella infection occurred in case-patients; five case-patients reported a history of cirrhosis, and one case-patients reported a recent varicella infection. Five (83%) of six persons with paralysis were in the case-patient group. This difference was significant when this variable was evaluated alone but did not reach statistical significance in multivariable analysis. None of the six patients with paralysis in this age group reported the presence of a decubitus ulcer; two of the persons with paralysis indicated that they had open sores in other places.

Of the 47 patients who had a cutaneous focus of invasive GAS infection (36 with cellulitis, 9 with necrotizing fasciitis, and 2 with an abscess), 39 (83%) reported an open sore, bruise, or burn before the onset of invasive GAS symptoms, and 5 (11%) reported having been diagnosed with a skin condition in the past (eczema, psoriasis, or seborrheic dermatitis).

Type of work environment, number of persons at work, number of hours at work, and presence of an ill person at work were not associated with invasive GAS infection in either age group.

Discussion

This study suggests that both host and environmental factors are associated with the risk of community-acquired invasive GAS disease in adults. We found that the host factors of HIV infection, diabetes, malignancy, injecting drug use, and cardiac disease were associated with an increased risk of invasive GAS disease. The environmental factors associated with an increased risk were household size and the presence of a child with a sore throat. These environmental factors highlight the importance of person-to-person transmission of group A streptococcus.

Although previous studies have found host risk factors to be associated with invasive GAS infection, this study found that the importance of host factors varied significantly by patient's age. In adults 18–44 years of age, HIV infection and history of injecting drug use were associated with invasive GAS disease. The association with HIV has been previously identified (1) and suggests that immune suppression increases the risk for invasive GAS disease. The association with injecting drug use has been previously identified also (8) and may be because of the direct injection of group A streptococci from the skin into the blood. The increased risk associated with injecting drug use was independent of that related to HIV infection. Because control subjects identified by random digit dialing may be reluctant to admit certain illicit behaviors and HIV infection, our ascertainment of injecting drug use and HIV infection among controls may be an underestimate; the strong association between these two factors and invasive GAS infection found in our study may be an overestimate of the true value of this association.

In older adults, diabetes mellitus, cardiac disease, cancer, and corticosteroid use are associated with invasive GAS infection. The association with diabetes, cancer, and corticosteroids again suggests that immune dysfunction is important in the development of this disease. An association between cardiac disease and invasive GAS infection was also suggested in a recent 10-year population-based surveillance study done in the San Francisco Bay area (8). The mechanism by which cardiac disease increases the risk of invasive GAS disease is not known but warrants further study.

Our data also suggest that paralysis, cirrhosis, and varicella infections may be risk factors for invasive GAS dis-

Table 3. Individual risk factor and multivariable analysis for risk factors for invasive group A streptococcal disease among case-patients and control subjects matched on age and zip code, 18–44 years of age, Atlanta, Baltimore, Toronto, 1997–1999[a,b,c]

			Individual risk factor analysis		Multivariable analysis	
Variable	Case-patients (n=48) (%)	Control subjects (n=115) (%)	OR (95% CI)	p value	OR (95% CI)	p value
Number of persons living in the home						
3+	38 (79)	80 (70)	1.91 (0.81 to 4.47)	0.14		
1–2	10 (21)	35 (30)				
Smoke exposure						
Current smoker with passive smoke exposure	6 (13)	17 (15)	1.04 (0.32 to 3.37)	0.95		
Current smoker without passive smoke exposure	12 (25)	16 (14)	2.35 (0.85 to 6.48)	0.86		
Passive smoke exposure	6 (13)	12 (10)	1.82 (0.57 to 5.82)	0.31		
No smoke exposure	24 (50)	70 (61)	1.00			
Any child <18 years living in the home						
Yes	30 (63)	68 (59)	1.25 (0.59 to 2.64)	0.57		
No	18 (37)	47 (41)				
≥1 Child with sore throat in the family						
Yes	7 (15)	7 (6)	2.75 (0.87 to 8.70)	0.09	4.93 (1.24 to 19.68)	0.02
No	41 (85)	108 (94)				
Diabetes mellitis						
Yes	6 (13)	5 (4)	2.11 (0.63 to 7.10)	0.23		
No	40 (87)	107 (96)				
Cancer						
Yes	0	3 (3)	0	0.99		
No	47 (100)	108 (97)				
Varicella						
Yes	0	0				
No	48 (100)	115 (100)				
Cirrhosis						
Yes	0	0				
No	48 (100)	115 (100)				
Paralysis						
Yes	1 (2)	0	Undefined			
No	47 (98)	115 (100)				
Regular NSAID use						
Yes	11 (26)	31 (28)	.97 (0.42 to 2.23)	0.93		
No	32 (74)	78 (72)				
New use of NSAIDs						
Yes	9 (19)	10 (9)	2.15 (0.79 to 5.80)	0.13		
No	34 (81)	100 (91)				
Use of corticosteroids						
Yes	0	3 (3)	0	0.99		
No	44 (100)	109 (97)				
HIV+						
Yes	7 (15)	1 (1)	12.66 (1.47 to 108.92)	0.02	15.01 (1.09 to 207.30)	.04
No	39 (85)	110 (99)				
Ever injected drugs						
Yes	10 (22)	3 (3)	11.80 (2.46 to 56.66)	0.002	14.71 (2.52 to 85.70)	.003
No	36 (78)	110 (97)				
Alcohol use (based on CAGE questions)[d]						
CAGE score 0	28 (72)	57 (68)	1.14 (0.69 to 1.89)	0.62		
CAGE score 1	5 (13)	22 (26)				
CAGE score 2	0	3 (4)				
CAGE score 3	5 (13)	2 (2)				
CAGE score 4	1 (3)	0				

[a]OR, odds ratio; CI, confidence interval; NSAID, nonsteroidal antiinflammatory drug.

[b]Due to missing data, for some variables, data for fewer than 48 case-patients and 115 control subjects were available.

[c]Analyses controlled for race and sex.

[d]Source: (7).

Table 4. Individual risk factor and multivariable analysis for risk factors for invasive group A streptococcal disease case-patients and control subjects, matched by age group and zip code, 45+ years old, Atlanta, Baltimore, Toronto, 1997–1999[a,b,c]

			Individual risk factor analysis		Multivariable analysis	
Variable	Case-patients (n=91) (%)	Control subjects (n=196) (%)	OR (95% CI)	p value	OR (95% CI)	p value
Number of persons living in the home						
3+	47 (52)	55 (28)	2.64 (1.46 to 4.75)	0.001	2.68 (1.37 to 5.28)	0.004
1–2	44 (48)	140 (72)				
Any child living in the home						
Yes	31 (34)	33 (17)				
No	60 (66)	163 (83)	2.12 (1.11 to 4.05)	0.02		
Smoke exposure						
Current smoker with passive smoke exposure	15 (16)	16 (8)	2.37 (0.94 to 6.01)	0.07		
Current smoker without passive smoke exposure	12 (13)	32 (16)	1.07 (0.49 to 2.33)	0.86		
Passive smoke exposure	19 (21)	25 (13)	2.34 (1.08, 5.04)	0.03		
No smoke exposure	45 (49)	123 (63)	1.00			
Diabetes mellitus						
Yes	28 (31)	32 (16)	2.33 (1.22 to 4.45)	0.01	2.27 (1.07 to 4.81)	0.03
No	62 (69)	164 (84)				
Hypertension						
Yes	47 (52)	86 (45)	1.61 (0.92 to 2.82)	.10		
No	42 (47)	107 (55)				
Cardiac disease						
Yes	30 (33)	24 (12)	3.09 (1.58, 6.06)	0.001	3.24 (1.40 to 7.51)	0.006
No	60 (67)	172 (88)				
Chronic obstructive pulmonary disease (COPD)						
Yes	8 (9)	2 (1)	7.85 (1.50 to 41.07)	0.01		
No	79 (91)	193 (99)				
Cancer						
Yes	18 (20)	22 (11)	2.58 (1.23 to 5.41)	.01	3.54 (1.44 to 8.70)	0.006
No	71 (80)	173 (89)				
Varicella						
Yes	1 (1)	0	Undefined	.99		
No	85 (99)	191 (100)				
Cirrhosis						
Yes	5 (6)	0	Undefined	0.99		
No	82 (94)	195 (100)				
Paralysis						
Yes	5 (6)	1 (1)	11.61 (1.34 to 100.87)	0.01		
No	84 (94)	195 (99)				
Regular NSAID use						
Yes	43 (48)	64 (34)	1.82 (1.06 to 3.14)	0.03		
No	46 (52)	125 (66)				
New use of NSAIDs						
Yes	14 (16)	14 (7)	2.13 (0.89 to 5.10)	0.09		
No	72 (84)	178 (93)				
Use of corticosteroids						
Yes	9 (10)	3 (2)	4.96 (1.26 to 19.62)	0.02	5.18 (1.14, 23.54)	.03
No	77 (90)	193 (98)				
HIV+						
Yes	1 (1)	2 (1)	.51 (0.04 to 6.14)	0.59		
No	90 (99)	194 (99)				
Ever injected drugs						
Yes	6 (7)	3 (2)	5.48 (1.00 to 30.09)	0.05		
No	78 (93)	190 (98)				
Alcohol use based on CAGE questions						
CAGE score 0	42 (78)	107 (77)	0.95 (0.56 to 1.61)	.86		
CAGE score 1	5 (9)	19 (14)				
CAGE score 2	2 (4)	9 (6)				
CAGE score 3	3 (6)	4 (3)				
CAGE score 4	2 (4)	0				

[a]OR, odds ratio; CI, confidence interval; NSAID, nonsteroidal anti-inflammatory drug;
[b]Due to missing data, for some variables, data for fewer than 91 case-patients and 196 control subjects were available.
[c]Analyses controlled for race and sex.

ease. These conditions were not common among our study participants; thus, the power of this investigation to identify them as significant risk factors was limited. Future research into the association between invasive GAS disease and paralysis should include evaluation of the possible contribution of skin disruption. Varicella infection is a well-documented risk factor in the development of invasive GAS disease among children (1,9–14).

We did not find an independent association between invasive GAS and use of NSAIDs. The hypothesis that NSAID use might increase the risk of necrotizing fasciitis in children with varicella was first suggested by Brogan et al. (11), and later observations by Peterson et al. supported it (12). Several mechanisms by which NSAID use might influence the incidence or severity of GAS infections have been proposed (15). A recent prospective, multicenter case-control study did not find that NSAID use increases the risk of necrotizing GAS infections (16). Instead, their data suggested that children with varicella were apt to take NSAIDS because they were ill from varicella as opposed to NSAIDS being a risk factor for later acquisition of invasive GAS disease.

A large proportion of the patients with invasive GAS disease had a cutaneous form of the disease, and a large portion of those with cutaneous disease reported an open sore, bruise, or burn before the onset of invasive GAS symptoms. This suggests the skin is an important portal of entry for invasive GAS infection.

Other studies have found an association between invasive bacterial diseases and cigarette smoking (17,18). We did not find this association. A true association may have been undetectable because of the difficulty in ascertaining smoking status over the telephone.

Other studies have found an association between invasive GAS disease and alcoholism (1). We did not find this association. The sensitivity of the CAGE questions (7) may be lower in phone interviews than in face-to-face interviews.

Our study found that environmental exposures were important in the development of invasive GAS infection. Specifically, we found that environmental exposures are related to age. In younger adults, exposure to school-aged children with a sore throat increased the risk of invasive GAS disease. Household studies in the 1950s showed that school-aged children are most likely to introduce group A streptococcus into a household and that symptomatic children are more efficient transmitters of infection than are asymptomatic children (4). Surveillance in Toronto demonstrated that the risk of colonization in the household is associated with younger age and 4 or more hours of contact with the infected person per day (1). More recently, the role of transmission among household contacts has been demonstrated in studies of community GAS outbreaks and household contacts of patients with invasive GAS (19,20). These studies suggest that infections among children may represent an important reservoir for infections in adults.

This study has several limitations. The statistical power of this study to detect the association between GAS infection and certain known risk factors, such as concomitant varicella infection, was limited. Because we matched subjects in terms of socioeconomic status, we were unable to examine socioeconomic variables in our analysis. This study included only people who have phones, and risk factors for invasive GAS disease may differ between people who do not have phones and those who do.

The results from this risk factor study can shape current and future strategies to prevent invasive GAS disease. Currently, these findings may help refine recommendations for prophylaxis of close contacts of persons with invasive GAS disease. Vaccines for GAS disease are being developed. Identifying those at greatest risk of invasive GAS disease is important in developing vaccination recommendations. Our data suggest that vaccination should be considered for those with history of injecting drug use, HIV infection, diabetes mellitus, cardiac disease, cancer, and use of corticosteroids. Our data also suggest that vaccination will provide benefits to those who receive the vaccine and to those with whom they live.

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Dr. Factor was studying the epidemiology of blood-borne pathogens at the Center for Urban Epidemiologic Studies at the New York Academy of Medicine, New York City, when she joined the Centers for Disease Control and Prevention's anthrax response effort in the fall of 2001. She is currently a medical epidemiologist, assigned to the New York City Department of Health and Mental Hygiene to develop the smallpox vaccination plan and general bioterrorism response plans for New York City.

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Address for correspondence: Stephanie H. Factor, Department of Disease Intervention, New York City Department of Health and Mental Hygiene, 125 Worth Street, New York, NY 10013, USA; fax: 212-788-4734; email: sfactor@health.nyc.gov

Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Carrying Panton-Valentine Leukocidin Genes: Worldwide Emergence

François Vandenesch,* Timothy Naimi,† Mark C. Enright,‡ Gerard Lina,* Graeme R. Nimmo,§ Helen Heffernan,¶ Nadia Liassine,# Michèle Bes,* Timothy Greenland, Marie-Elisabeth Reverdy,* and Jerome Etienne***

Infections caused by community-acquired (CA)-methicillin-resistant *Staphylococcus aureus* (MRSA) have been reported worldwide. We assessed whether any common genetic markers existed among 117 CA-MRSA isolates from the United States, France, Switzerland, Australia, New Zealand, and Western Samoa by performing polymerase chain reaction for 24 virulence factors and the methicillin-resistance determinant. The genetic background of the strain was analyzed by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). The CA-MRSA strains shared a type IV SCC*mec* cassette and the Panton-Valentine leukocidin locus, whereas the distribution of the other toxin genes was quite specific to the strains from each continent. PFGE and MLST analysis indicated distinct genetic backgrounds associated with each geographic origin, although predominantly restricted to the *agr*3 background. Within each continent, the genetic background of CA-MRSA strains did not correspond to that of the hospital-acquired MRSA.

Methicillin-resistant *Staphylococcus aureus* (MRSA) are identified as nosocomial pathogens throughout the world (1). Established risk factors for MRSA infection include recent hospitalization or surgery, residence in a long-term–care facility, dialysis, and indwelling percutaneous medical devices and catheters. Recently, however, cases of MRSA have been documented in healthy community-dwelling persons without established risk factors for MRSA acquisition. Because they are apparently acquired in the community, these infections are referred to as community-acquired (CA)-MRSA (2). CA-MRSA infections have been reported in North America, Europe, Australia, and New Zealand (3–5). The recent genomic sequence of a CA-MRSA isolate (6) indicated the presence not only of a novel smaller variant of the methicillin-resistance locus (SCC*mec* IVa, according to Baba et al. designation [6]), but also that of the locus for the Panton-Valentine leukocidin (PVL). The PVL locus is carried on a bacteriophage and is present in only a small percentage of *S. aureus* isolates from France, where this locus is associated with skin infections, and occasionally, severe necrotizing pneumonia (7,8).

In a recent study, we found that CA-MRSA infections in France are caused by a single clone producing the PVL (3). Analysis of a set of CA-MRSA strains from the United States and Australia confirmed the presence of SCC*mec* IVa in most of them, and genetic comparison of the CA-MRSA by multi-locus sequence typing (MLST) indicated that they belonged to five clonal complexes, two of which predominated (4). This finding suggested that CA-MRSA have arisen from diverse genetic backgrounds rather than the worldwide spread of a single clone (4).

The aim of this study was to determine whether the PVL gene represents a stable marker of the CA-MRSA strains worldwide and whether any other common genetic traits such as toxin gene and the accessory gene regulator (*agr*) profiles can be identified.

Materials and Methods

Bacterial Strains

A total of 117 different isolates of CA-MRSA were examined. Community acquisition was defined as growth of the isolates within 48 hours after hospital admission in patients who had no risk factors for nosocomial acquisi-

*INSERM E0230, Lyon, France; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ‡University of Bath, Bath, United Kingdom; §Princess Alexandra Hospital, Brisbane, Australia; ¶Antibiotic Reference Laboratory, 12 Wellington, New Zealand; #Laboratoire Bioanalytique-Riotton, Geneva, Switzerland; and **UMR754, Lyon, France

tion, including no hospitalizations or nursing home residence in the year before admission. Thirty-three isolates were from the United States. The U.S. isolates all belonged to the previously identified major CA-MRSA clonal group and included strain MW2 (2). They originated from 12 different facilities in Minnesota; two isolates were from North Dakota. Sixty-seven isolates originated from Europe (61 isolates from France and 6 from Switzerland). The French CA-MRSA isolates originated from 10 different hospitals located throughout France, and the Swiss isolates were from the Geneva area. Seventeen isolates were from Oceania and included two different clones: 1) 13 isolates corresponded to the Western Samoan phage pattern strains, also designated the Southwest Pacific clone (9) and were isolated from Australia (8 isolates), New Zealand (4 isolates), and Western Samoa (1 isolate) (5); 2) 4 other isolates from Australia belonged to a recently described clone designated as the Queensland clone (10). The Southwest Pacific clone has two distinct phage-typing patterns known as Western Samoan Phage Pattern (WSPP) 1 and 2 (5). The Queensland clone, which cannot be phage typed, was first detected in the southeast Queensland city of Ipswich in 2000 (10). Most isolates were from primary skin and soft tissue infections, with some cases of bacteriemia and at least five cases of necrotizing pneumonia (2,3,9,11,12).

A set of representative hospital-acquired (HA)MRSA isolates was included in the study: 24 from France and 33 from the United States. French HA-MRSA isolates corresponded to those of the major clones isolated throughout France as described by Lelievre et al. (13). HA-MRSA isolates were recovered from the same 12 different facilities that CA-MRSA. HA-MRSA from the United States originated from the same geographic area as the CA-MRSA.

Antimicrobial Susceptibility Testing

The MICs of benzyl-penicillin, oxacillin, gentamicin, tobramycin, kanamycin, chloramphenicol, tetracycline, minocycline, erythromycin, lincomycin, pristinamycin, fusidic acid, rifampicin, ofloxacin, co-trimoxazole, linezolid, mupirocin, vancomycin, and teicoplanin were determined for selected isolates (46 European, 22 U.S., and 13 Oceanian isolates) by using the standardized agar dilution technique as recommended by the French Society for Microbiology (14).

Detection of Accessory Genes by PCR

Using polymerase chain reaction (PCR), we determined the presence of accessory gene regulator (*agr)* allele group (1–4), SCC*mec* element (I-IV, according to the designation of Oliveira [15]), and 22 specific staphylococcal virulence genes (including 16 super-antigenic toxins, 3 hemolysins, and 3 leukocidins), as described previously (16).

Amplification of *gyrA* was used as a quality control of each DNA extract and the absence of PCR inhibitors. *S. aureus* strains Fri 913 (*sea*, *see*, *sec*, *tst*, *lukE lukD*, *sek*, *sel*, *sep*, and *hlg*), Fri 1151m (*sed*, *sej*, *lukE lukD*, *hlgv*, and *hlb*), ATCC 14458 (CCM5757) (*seb*, *lukE lukD*, *sek*, and *hlgv*), NCTC 7428 (*sec*, *tst*, *lukM*, *seg*, *sei*, *sem*, *sen*, *seo*, *lukE lukD*, *hlgv*, and *hlb*), A92 0211 (*seg*, *sei*, *sem*, *sen*, *seo*, *eta*, *etb*, *lukE lukD*, and *hlgv*), RN6390 (*lukE lukD*, *hlgv*, *hlb*, and *agr1*), RN6607 (*sed*, *seg*, *sei*, *sem*, *sen*, *seo*, *lukE lukD*, *hlgv*, and *agr2*), RN8465 (*seg*, *sei*, *sem*, *sen*, *seo*, *tst*, *hlg*, and *agr3*), RN4850 (*seg*, *sei*, *sem*, *sen*, *seo*, *eta*, *etb*, *lukE lukD*, *hlgv*, and *agr4*), RN 6911(*lukE lukD*, *hlgv*, *hlb*, *agr* null), E-1 (*seg*, *sei*, *sem*, *sen*, *seo*, *lukE lukD*, *eta*, *hlgv, edinB* and *C*), ATCC 49775 (*seg*, *sei*, *sem*, *sen*, *seo*, *lukS lukF*, and *hlg*), and ATCC 51811 (FRI 569) (*seh*, *lukE lukD*, *hlb*, and *hlgv*) were used as positive controls for PCR (17,18). *S. aureus* COL (SCC*mec* I), PER34 (SCC*mec* IA), BK2464 (SCC*mec* II), ANS46 (SCC*mec* III), HU25 (SCC*mec* IIIA), and HDE288 (SCC*mec* IV) were used as controls for characterization of the *mec* element according to Oliveira and de Lencastre (15).

The overall genetic background of the isolates was evaluated: 1) by digesting whole cell DNA with *Sma*I macrorestriction enzyme and determining the fragment-size patterns obtained on pulsed-field gel electrophoresis (PFGE) using a contour-clamped homogeneous electric field system on a CHEF DR-II apparatus (Bio-Rad Laboratories, Marnes-la-Coquette, France) as previously described (19). Resolved macrorestriction patterns were compared as recommended by Tenover et al. (20). Isolates differing by up to three fragments were considered as subtypes of a given clonal type. MLST was performed as described by Enright et al. (21). Briefly, seven housekeeping genes were used in the scheme; for each isolate, the alleles at each of the seven loci defined the allelic profile, which corresponded to a sequence type (ST). ST designations were those assigned by the MLST database (available from: URL: http://www.mlst.net).

Results

Distribution of Accessory Genes from MRSA in Three Continents

Overall, we detected 12 different virulence genes or gene clusters among the 117 isolates (Table 1). Two gene loci were common to CA-MRSA isolates from all locations. Methicillin resistance was conferred in all 117 CA-MRSA isolates by the truncated SCC*mec* type IV element, and all the isolates contained the PVL locus. In addition, 112 isolates harbored the related *lukE-lukD* genes of another leukocidin frequently recovered from patients with all types of staphylococcal infections (3). Most (113 [97%] of 117) isolates were of *agr* type 3.

Table 1. Distribution of virulence and resistance determinants in 117 CA-MRSA isolates from three continents

Genes[b]	CA-MRSA isolates from France-Switzerland[c] n=67 (%)	USA n=29 (%)	USA n=4 (%)	Oceania[d] Southwest Pacific clone n=13 (%)	Australia Queensland clone n=4 (%)	Total n=117 (%)
Sequence type	80	1	59 or 8	30	93	
PFGE pattern	A1-7	B1-5	D1 & F1	C1-3	E1	
agr type	3	3	1	3	3	
SCC IV	67 (100)	29 (100)	4 (100)	13 (100)	4 (100)	117 (100)
Leukocidins PVL genes	67 (100)	29 (100)	4 (100)	13 (100)	4 (100)	117 (100)
lukE-lukD	67 (100)	29 (100)	3 (75)	13 (100)	0 (0)	116 (99)
Hemolysins[e]						
hlg	0 (0)	0 (0)	0 (0)	13 (100)	0 (0)	13 (11)
hlg-v	67 (100)	29 (100)	4 (100)	0 (0)	0 (0)	100 (85)
hlb	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	1 (1)
Enterotoxins *sea*	0 (0)	23 (79)	0 (0)	0 (0)	0 (0)	23 (20)
seb	0 (0)	8 (28)	1 (25)	0 (0)	0 (0)	9 (8)
sec	0 (0)	20 (69)	0 (0)	0 (0)	0 (0)	20 (17)
sed-sej	0 (0)	0 (0)	3 (75)	0 (0)	0 (0)	3 (3)
seh	0 (0)	29 (100)	0 (0)	0 (0)	0 (0)	29 (25)
sek	0 (0)	24 (83)	0 (0)	0 (0)	0 (0)	24 (21)
egc[f]	0 (0)	0 (0)	0 (0)	13 (100)	0 (0)	13 (11)

[a]PFGE, pulsed-field gel electrophoresis; PVL, Panton-Valentine leukocidin.

[b]Results for toxin genes absent from all community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates (*see, tst, eta, etb, lukM,* and *edinA*) are not presented.

[c]Isolates from France (61) and Switzerland (6).

[d]Isolates from Australia (8), New Zealand (4), and Western Samoa (1).

[e]*hlg*, γ hemolysin gene; *hlg-v*, γ-hemolysin variant gene; *hlb*, β-hemolysin gene.

[f]*egc*: enterotoxin gene cluster, which includes the *seg*, *sei*, *sem*, *sen,* and *seo* gene.

The distribution of other genes varied by the continent of origin. The European and Southwest Pacific isolates had consistent, relatively simple, patterns of virulence-associated genes. In addition to the SCC*mec* type IV element, PVL genes, and *lukE-lukD,* all the European isolates were positive for *hlg-v* (the γ-hemolysin variant gene) and the Southwest Pacific isolates were positive for *hlg* (the γ-hemolysin gene) and *egc* (the enterotoxin gene cluster coding for the enterotoxins *seg*, *sei*, *sem*, *sen*, and *seo*) (Table 1). The Queensland isolates had not been tested positive for the toxin genes, except the PVL locus. In contrast, considerable variability existed among the 33 U.S. isolates. As for the European isolates, the U.S. isolates showed both the presence of *hlg-v* and the absence of *hlg.* However, seven other toxins (the enterotoxins *sea–sed*, *sej*, *she,* and *sek*), which were absent in non–U.S. isolates, were variously found in up to 29 (3% to 87%) of these 33 isolates (Table 1).

Analysis of Genetic Background of CA-MRSA by PFGE

The 117 CA-MRSA isolates clustered into six PFGE clonal types (A to F, Figure). All 67 European isolates grouped into seven related PFGE patterns (subtypes A1-7) distinct from the other isolates. Most of the U.S. isolates belonged to a closely related group of five patterns (subtypes B1-5), although two well-differentiated outliers existed, one (D1) containing three isolates and the other (F1) containing only one isolate, both of which had an *agr* type 1 genotype. The 13 isolates obtained from Australia, New Zealand, and Western Samoa and belonging to the Southwest Pacific clone showed three closely related PFGE patterns (subtypes C1-3) with no geographic association. The four isolates from Australia belonging to the Queensland clone had the same E1 pattern. The phylogenetic tree shown on the left side of the figure confirms the diversity in PFGE patterns between the CA-MRSA from different continents.

CA-MRSA Antibiotic Susceptibility Profiles

MICs were determined on a selected number of isolates (81) that corresponded to the different PFGE patterns. Overall, CA-MRSA isolates from all locations were susceptible to numerous antimicrobial drugs, including tobramycin, gentamicin, lincomycin, pristinamycin, minocycline, chloramphenicol, ofloxacin, vancomycin, teicoplanin, fosfomycin, rifampin, co-trimoxazole, and linezolid (Table 2). For the purposes of assessing differences between locations, isolates from the United States and Oceania were combined since their susceptibility profiles were similar (Table 2). Minor heterogeneity in erythromycin and mupirocin susceptibility was noted among European isolates. In contrast, U.S. and Oceanian isolates were uniformly susceptible to these antibiotics (Table 2).

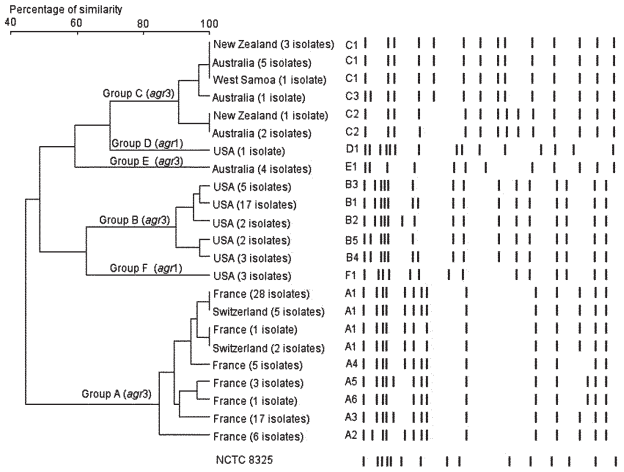

Figure. Pulsed-field gel electrophoresis (PFGE) pattern and phylogenetic tree of 117 community-acquired (CA)-methicillin resistant *Staphylococcus aureus* isolates from three continents. *Sma*I macrorestriction patterns were digitized and analyzed by using Taxotron software (Institut Pasteur, Paris, France) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages (UPGMA) clustering. The scale indicates the level of pattern similarity. Capital letters indicate macrorestriction types based on visual interpretation of PFGE results.

The main difference between the two groups was their susceptibility to kanamycin, tetracycline, and fusidic acid, with the European group of isolates being more resistant than the U.S. and Oceanian group (Table 2).

Comparison of CA-MRSA with HA-MRSA

Using PFGE to compare CA-MRSA isolates with representative HA-MRSA isolates from both France (24 isolates) and the United States (33 isolates), we found that the last isolates grouped into lineages that clearly differed from any of the CA-MRSA isolates of the same continent (not shown). None of the HA-MRSA harbored PVL genes or the SCC*mec* IV element. Moreover, all U.S. HA-MRSA had SCC*mec* type II element; most (31 of 33) of these isolates were *agr* type 2, and the remaining 2 were type 1. Conversely, all French HA-MRSA had an unknown SCC*mec* element according to the method of Oliveira (15). These strains were further designated as SCC*mec* IVc by Hiramatsu (22). Twenty-three of the 24 French HA-MRSA isolates were *agr* type 1, and only 1 was a type 2. None of the HA-MRSA tested was *agr* type 3, the predominant type for CA-MRSA. In addition, unlike the PVL genes, *lukE-lukD* leukocidin genes were found in most HA-MRSA (95%) as well as in CA-MRSA.

Analysis of Genetic Background by MLST

Twenty-one representative isolates of each PFGE pattern of CA-MRSA were further characterized by MLST. Overall, the results perfectly matched those of the PFGE with a unique ST corresponding to each group of related PFGE patterns (Table 1). The STs of CA-MRSA were compared with those in the MLST database (Table 3). Within each continent, the most frequent STs of CA-MRSA (i.e., ST1 for the U.S. clone, ST30 for the Southwest Pacific clone, and ST80 for the European clone)

Table 2. MIC50 and MIC90 of staphylococcal antibiotics against community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) from Europe (46 isolates), United States (22 isolates), and Oceania (13 isolates)

	Isolates from Europe			Isolates from United States and Oceania		
Antibiotics	MIC50 mg/L	MIC90 mg/L	Range mg/L	MIC50 mg/L	MIC90 mg/L	Range mg/L
Benzyl-penicillin	8	8	0.25–8	16	16	4–32
Oxacillin	16	32	4–64	64	64	16–64
Kanamycin	128	128	128	2	2	2
Tobramycin	0.25	0.25	0.25	0.25	0.25	0.25
Gentamicin	1	1	0.5–1	1	1	0.5–2
Erythromycin	0.5	128	0.25–128	0.25	0.5	0.25–128
Lincomycin	0.5	0.5	0.5–32	0.5	0.5	0.25–32
Pristinamycin	0.5	0.5	0.12–1	0.5	0.5	0.12–1
Tetracycline	16	16	0.25–16	0.25	0.25	0.25–32
Minocycline	0.25	0.25	0.25	0.25	0.25	0.25
Chloramphenicol	4	4	4–8	4	8	4–8
Ofloxacin	0.12	0.12	0.12–0.5	0.12	0.25	0.12–1
Fusidic acid	4	4	0.12–64	0.12	0.12	0.12
Vancomycin	0.5	0;5	0.5–1	0.5	0;5	0.5–1
Teicoplanin	0.5	0.5	0.25–0.5	0.25	0.5	0.25–0.5
Fosfomycin	2	2	0.25–2	1	2	0.25–2
Rifampin	0.12	0.12	0.12	0.12	0.12	0.12
Co-trimoxazole	0.5/9.5	0.5/9.5	0.5/9.5	0.5/9.5	0.5/9.5	0.5/9.5
Linezolid	0.5	1	0.25–1	0.5	1	0.25–1
Mupirocin	0.12	0.12	0.12–8	0.12	0.12	0.12

Table 3. Origin and frequency of *Staphylococcus aureus* isolates according to their sequence types

	Present study		Data from the MLST Web site			
Sequence type	Country of origin of CA-MRSA	n	Country of origin of MSSA	n	Country of origin of MRSA	n
1	USA	29	UK, Denmark, the Netherlands, Canada,	20		0
8	USA	3	UK, the Netherlands, Denmark, US, Canada	46	Scotland, Ireland, Australia, U.S., UK, Germany, The Netherlands, France	39
30	Oceania (Southwest Pacific clone)	13	UK, Denmark, Germany	83	UK, Spain, Germany, Sweden	8
59	USA	1	UK	4	U.S.	1
80	France, Switzerland	67		0	Greece	1
93	Australia (Queensland clone)	4		0		0

[a]MLST, multi-locus sequence typing ; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; CA, community acquired.

were different than the STs of HA-MRSA or methicillin-susceptible *S. aureus* (MSSA) strains in the same continent. For instance, ST1 was detected in U.S. CA-MRSA but only in European MSSA. The only correlation within a continent between the ST of MSSA and CA-MRSA was for the two rare a*gr*1 CA-MRSA clones from the United States (STs 8 and 59). Thus, these two infrequent STs of CA-MRSA were also observed in MSSA (ST8) and HA-MRSA (ST8 and 59) in the same country.

Discussion

The characterization of 117 CA-MRSA isolates from three continents indicted four major findings. First, only two genes were unique to CA-MRSA isolates and shared by isolates from all three continents: a type IV SCC*mec* cassette (further designated IVa by Okuma et al. [4]) and the PVL locus. Otherwise, the distribution of the other toxin genes was continent-specific. This finding suggests that PVL and SCC*mec* type IV may confer a selective advantage for community-based MRSA pathogens. Second, CA-MRSA isolates were generally susceptible to most of antibiotics tested apart from β-lactams, although European isolates appeared more resistant (i.e., to kanamycin, tetracycline, and fusidic acid) than U.S. and Oceanian isolates. Third, the genetic background of CA-MRSA organisms was different in each of the three continents, although it was predominantly restricted to the *agr*3 background, which corresponds to one of the three major phylogenetic lineages of pathogenic MSSA previously described (16). This finding demonstrates that dissemination of a single CA-MRSA clone did not occur around the world but rather suggests the possibility of simultaneous co-evolution of CA-MRSA organisms in different locations. Fourth, MLST and PFGE analysis showed that within a continent, the genetic background of CA-MRSA strains did not correspond to that of the HA-MRSA in the same continent, suggesting that CA-MRSA did not emerge from local HA-MRSA.

The STs of CA-MRSA clones were not related to the STs of any described pandemic clones of MRSA, such as the Archaic clone (ST250 with a SCC*mec* I element), the Iberian clone (ST247 with a SCC*mec* IA element), the New York/Japan clone (ST5 with a SCC*mec* II element), the Hungarian clone (ST239 with a SCC*mec* III element), the Brazilian clone (ST239 with a SCC*mec* IIIA element), or the pediatric clone (ST5 with a SCC*mec* IV element) (23). However, analysis of the MLST database indicated that the CA-MRSA of each continent shared a common genetic background with HA-MRSA or MSSA of other continents (Table 3). This suggests that intercontinental exchange of MRSA or MSSA had occurred, possibly followed by the introduction of the SCC*mec* in MSSA and the PVL locus in MSSA or MRSA. However, we cannot rule out the converse hypothesis that, for instance, MRSA of ST8 from France, which do not harbor the PVL locus and are of *agr*1 allele, derive from an ancestor of *agr*1 allele found in the United States, carrying the PVL locus (Table 3 and data not shown). In any case, the association of SCC*mec* IV (SCC*mec*IVa according to Baba et al. denomination [6]) with PVL in the CA-MRSA strains most likely did not result from co-acquisition of the two determinants on a single mobile genetic element because the two loci are widely separated on the *S. aureus* chromosome (6).

The CA-MRSA isolates contained the SCC*mec* type IV element, according to the designation of Oliveira and de Lencastre (15). If MRSA isolates with the SCC*mec* type IV element contained an additional 381–base pair band because of the integration of pUB110, the isolates are said to be SCC*mec* IVA. However, other groups in Japan and the United States have used region-specific primers to define the L-C region of the SCC*mec* element (4). On the basis of L-C polymorphism, researchers have identified three types to date, designated IVa, IVb, and IVc. Thus, the IVa of these latter groups (4) is not the same as the IVA of Oliveira and de Lencastre (15).

The exact nature of the selective advantage conferred by the observed combination of genetic traits remains to be elucidated, but simple antibiotic selection seems unlikely in a community context of widely different populations with various degrees of methicillin exposure. Okuma et al.

(4) suggested that CA-MRSA should display enhanced ecologic fitness, as they had a shorter doubling time than HA-MRSA. The real impact of this in vitro observation needs to be evaluated. Unlike other SCC*mec* elements, SCC*mec* IV and SCC*mec* I do not code for additional resistance determinants; however, SCC*mec* IV does code for *mec*A, a peptidoglycan transpeptidase. This protein is expressed at the external surface of the cytoplasmic membrane, where it could interact with the extracellular protein PVL. We are investigating the possibility of PVL activity or of peptidoglycan formation as a result of such an association.

CA-MRSA infections appear to be an emerging phenomenon worldwide. The PVL locus represents a stable genetic marker of these CA-MRSA strains, which explains the frequency of primary skin infections and occasionally necrotizing pneumonia associated with these strains (2,8,24,25). Although the selective advantage conferred by the combination of genetic traits (i.e., PVL locus and SSC*mec* IV in an *agr*3 background) is not clear, the spread of a limited number of clones in each continent suggests that these CA-MRSA strains are particularly suited to be successful community-based pathogens.

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Dr. Vandenesch is professor of microbiology at the Medical School of Lyon and director of the French National Reference Laboratory for Staphylococci. He is head of an INSERM research unit focused on pathogenesis of staphylococcal infections.

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Address for correspondence: Jerome Etienne, Faculté de Médecine Laennec, National Reference Centre for Staphylococci, IFR62, INSERM E0230, 7 rue Guillaume Paradin, 69372 Lyon cedex 08, France; fax: +33 (0) 478-77-86-57; email: jetienne@univ-lyon1.fr

Candidemia in Finland, 1995–1999

Eira Poikonen,* Outi Lyytikäinen,† Veli-Jukka Anttila,‡ and Petri Ruutu†

We analyzed laboratory-based surveillance candidemia data from the National Infectious Disease Register in Finland and reviewed cases of candidemia from one tertiary-care hospital from 1995 to 1999. A total of 479 candidemia cases were reported to the Register. The annual incidence rose from 1.7 per 100,000 population in 1995 to 2.2 in 1999. Species other than *Candida albicans* accounted for 30% of cases without change in the proportion. A total of 79 cases of candidemia were identified at the hospital; the rate varied from 0.03 to 0.05 per 1,000 patient-days by year. Predisposing factors included indwelling catheters (81%), gastrointestinal surgery (27%), hematologic malignancy (25%), other types of surgery (21%), and solid malignancies (20%). Crude 7-day and 30-day case-fatality ratios were 15% and 35%, respectively. The rate of candidemia increased in Finland but is still substantially lower than in the United States. No shift to non–*C. albicans* species could be detected.

A number of reports indicate a substantial increase in candida infections in the United States during the last 2 decades, including a consequent rise in related deaths and prolonged hospitalization (1–5). *Candida* sp. have been shown to be the fourth most common group of organisms causing nosocomial bloodstream infections (BSI) in the United States (6–9). Reports also suggest an increase in candidemia in Europe and Australia (10–12). Factors contributing to this trend are a growing population of immunocompromised patients and the use of new, aggressive, and invasive therapeutic strategies (6,13). Although most candidemia cases are due to *Candida albicans*, infections caused by non–*C. albicans* species have become more common (8–9,12,14–18).

For the most part, the epidemiology of candidemia has been studied in selected hospitals, which may not be representative of all hospitals serving a population (11–12,19–24). Few population-based studies identifying trends in the incidence of candidemia over time have been published, and the absolute numbers for age- and sex-specific incidence rates have rarely been reported (18,25–28).

*Peijas Hospital, Vantaa, Finland; †National Public Health Institute, Helsinki, Finland; and ‡Helsinki University Central Hospital, Helsinki, Finland

We evaluated trends in the incidence of BSIs caused by C*andida* spp. in Finland from 1995 to 1999, using data on BSIs from laboratory-based surveillance introduced in 1995. We also reviewed the characteristics of candidemia cases that occurred in the largest tertiary-care hospital in Finland during the same period.

Methods

Surveillance and Population

Finland (population 5.2 million) has five tertiary-care hospitals, with well-defined catchment populations of 0.71 to 1.66 million. Since 1995, all clinical microbiology laboratories in Finland have reported all bacterial and fungal isolations from blood, including *Candida* spp., to the National Infectious Diseases Register. Detection and species determination of *Candida* isolates are performed in the notifying laboratories according to standard protocols in use in each laboratory. Data collected with each notification include the date of isolation, date of birth, sex, type of specimen, and place of treatment.

A case of candidemia was defined as a patient with at least one blood culture positive for *Candida* species. Notifications of the same species of *Candida* within 3 months after the first diagnostic sample in the same patient were defined as one case. Isolations of the same species beyond this time period in the same patient were defined as separate cases.

Tertiary-Care Hospital

Helsinki University Central Hospital (HUCH) is a tertiary-care hospital with 1,600 beds that serves a population of 1.66 million living in the Helsinki area in southern Finland. In some specialties, such as bone marrow and solid organ transplantation, HUCH provides national service. All patients with at least one blood culture positive for *Candida* from January 1995 to December 1999 were retrospectively identified from the microbiology laboratory logbooks of HUCH's department of bacteriology. Nosocomial versus community acquisition was defined according to proposed standard criteria (29). The following data were abstracted from patient charts: type of spe-

cialty, underlying conditions, central venous catheters and bladder catheters in place, cultures taken from these catheters, and the outcome of the illness. Immunosuppressive status was defined as cytotoxic therapy or total body irradiation ≤3 months before onset of candidemia or systemic cortisone (≥40 mg per day at onset of cortisone treatment) ≤1 month before onset of candidemia. The annual numbers of patient days and discharges were acquired from the hospital administration. No guidelines for systematic antifungal prophylaxis for any patient groups were in use in HUCH during the study period.

Incidence Rates and Statistical Analysis

Data from the Finland National Population Registry from 1995 to 1999 were used as denominators to calculate age- and sex-specific incidence rates. Average annual incidences during the surveillance period were calculated by using the total number of cases and population during 1995 to 1999. To evaluate secular trends, rates of candidemia in different age and sex groups were calculated for each 6-month period from January 1995 to December 1999. If changes were detected, Poisson regression model was used to assess whether the observed changes in the rates were statistically significant.

Data were analyzed by using Epi Info version 6.04 (available from: URL: http://www.cdc.gov/epiinfo/ei6.htm) and SPSS for Windows version 11 (Chicago, IL). Categorical variables were analyzed with the chi-square test, Yates's correction, or Fisher exact test, as appropriate. Continuous variables were analyzed by Student t test or the Mann-Whitney U test, depending on the sample distribution.

Results

A total of 479 candidemia cases were reported to the National Infectious Diseases Register from 1995 to 1999. The median age of the patients was 59 years of age (range 0–89 years); 266 (60%) were males. The average annual incidence of candidemia was 1.9 per 100,000 population and varied from 1.3 to 2.2 in the five tertiary-care hospital catchment areas. The incidence increased from 1.7 per 100,000 population in 1995 to 2.2 in 1999. The average annual incidence of candidemia was highest in infants <1 year of age and lowest in patients 1–15 years of age (Table 1); infants <1 year of age accounted for only 6% of all candidemia cases. In all age groups, the incidence was higher in males than in females. In males 16–65 years of age, the incidence rose significantly, from 1.0 per 100,000 population in 1995 to 2.4 per 100,000 population in 1999 ($p<0.05$ by Poisson regression); by 1999, the incidence rate for males was three times the rate in females. No trends were identified in other age and sex groups. The highest annual incidence (24.4/100,000 population) occurred in 1999 in infants <1 year of age, which was primarily caused by *C. albicans* (11 cases, 5 of which occurred in one tertiary-care hospital).

The most frequent *Candida* sp. encountered was *C. albicans*, which caused 335 (70%) cases (Table 2). The most common non–*C. albicans* species found was *C. glabrata*, followed by *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. The other species reported were *C. pelliculosa* (five cases) and *C. rugosa* (one case). In 14 (3%) cases, the species were not specified. The proportion of non–*C. albicans* species did not increase during the study period.

Table 1. Annual incidence of candidemia by sex and age group, Finland, 1995–1999

	Rate[a]					
Characteristics	1995	1996	1997	1998	1999	1995–1999
Males (y)						
<1	12.5	10.0	3.3	6.9	27.3	11.9 (18)
1–15	1.2	0.4	0.6	0	0.2	0.5 (12)
16–65	1.0	1.9	1.9	2.1	2.4	1.8 (159)
>65	7.7	6.3	6.1	9.4	7.7	7.4 (97)
All	1.9 (46)	2.1 (53)	2.1 (53)	2.5 (63)	2.8 (71)	2.3 (286)
Females (y)						
<1	6.5	3.4	0	3.6	21.3	6.9 (10)
1–15	0.6	0.4	0.6	0.2	0.6	0.5 (12)
16–65	1.0	0.8	1.1	1.2	0.8	1.0 (84)
>65	4.2	4.3	3.4	3.6	4.2	3.9 (87)
All	1.5 (39)	1.4 (36)	1.4 (37)	1.5 (39)	1.6 (42)	1.5 (193)
All (y)						
<1	9.6	6.6	1.7	5.3	24.4	9.4 (28)
1–15	1.0	0.5	0.6	0.1	0.4	0.5 (24)
16–65	1.0	1.4	1.5	1.7	1.6	1.4 (243)
>65	5.4	5.0	4.4	5.7	5.6	5.2 (184)
All	1.7 (85)	1.7 (89)	1.8 (90)	2.0 (102)	2.2 (113)	1.9 (479)

[a]Cases per 100,000 population (no. of cases).

Table 2. Distribution of *Candida* spp. causing bloodstream infections, Finland, 1995–1999

Candida species	1995[a]		1996[a]		1997[a]		1998[a]		1999[a]		1995–1999[a]	
C. albicans	67	(57)	75	(67)	73	(66)	61	(62)	73	(83)	70	(335)
Non-*C. albicans*	33	(28)	25	(22)	27	(24)	39	(40)	27	(30)	30	(144)
C. glabrata	14	(12)	3	(3)	8	(7)	8	(8)	10	(11)	9	(41)
C. krusei	5	(4)	12	(10)	4	(4)	15	(16)	5	(6)	8	(40)
C. parapsilosis	11	(9)	1	(1)	6	(5)	6	(6)	5	(6)	5	(27)
C. tropicalis	1	(1)	3	(3)	1	(1)	5	(5)	3	(3)	3	(13)
Other	2	(2)	6	(5)	8	(7)	5	(5)	4	(4)	5	(23)

[a]Percent (no.).

In the study hospital, we identified a total of 86 candidemia cases. All but one case were determined to be nosocomial. Four of the 85 nosocomial candidemia case-patients were associated with predisposing treatment in other hospitals, and 2 additional cases-patients, for whom the clinical information was not available, were excluded, leaving 79 patients with cases of nosocomial candidemia for detailed analysis. The median duration of hospital stay before onset of candidemia was 19 days (range 0–177 days). The median age of the patients was 56 years (range 0–≤89 years); 45 (57%) of the patients were male.

The average annual incidence of candidemia at HUCH was 0.17 per 1,000 discharges (range by year 0.12–0.21) and 0.04 per 1,000 patient days (range by year 0.03–0.05). Male patients accounted for 70% of cases <1 year of age and 66% of those 16–65 years of age, whereas 64% of the cases >65 years of age were women. We found no increase in the annual number of cases in men 16–65 years of age, in contrast to the increasing rate identified in the national population-based analysis.

At the onset of candidemia, all cases in the study hospital had at least one predisposing factor. Of 79 cases, 19% were leukopenic (leukocytes <1 x 10E9/L), and 14% were neutropenic (neutrophils <0.5 x 10E9/L); 44% were immunocompromised. Gastrointestinal surgery was the most common underlying condition, followed by hematologic malignancy, other surgery, and solid malignancies (Table 3). Solid organ transplantation preceded onset of candidemia in three cases and bone marrow transplantation in five. None of the case-patients had HIV infection. At the onset of candidemia, 18 cases (23%) were treated in intensive-care units (ICU). Nine (50%) of 18 ICU cases were treated in neonatal ICUs. These 9 neonatal ICU case-patients were preterm and constituted 90% of infants <1 year of age; mean gestational age was 28 weeks (range 23–39), and mean birth weight 1,129 g (range 450–3,340 g). Before onset of candidemia, 64 (81%) of case-patients had a central venous catheter and 33 (42%) had a bladder catheter in place. Central venous catheter tip culture was positive for *Candida* in 28 (44%) of the 64 cases, and urine culture was positive for *Candida* in 7 (21%) of the 33 patients with a bladder catheter. Biopsy-proven deep *Candida* infection was detected in 9 cases (11%); all had central venous catheters, and 8 (89%) were operated before onset of candidemia.

The most common *Candida* species at HUCH was *C. albicans* (55 cases, 70%), consistent with the overall national figure (Table 2). For non–*C. albicans* species, the proportion varied by year from 17% in 1997 to 37% in 1999. In contrast to the national data, *C. parapsilosis* was as common as *C. glabrata* at the study hospital (six cases each).

Among the 79 patients with candidemia at HUCH, 12 (15%) died within 1 week after onset and 28 (35%) within 1 month. Of those who died, one patient had had preceding treatment in ICU. The patients who died were significantly older (median age 51 vs. 60 years of age, $p<0.05$) and were more likely to have hematologic malignancies (60% vs. 27%, $p<0.05$).

Discussion

Our nationwide population-based study shows that the incidence of candidemia in Finland is relatively low. However, we found a consistent year-to-year increase, mainly attributable to an increase in the incidence among men 16–65 years of age. No shift towards non–*C. albicans* species was observed.

We analyzed laboratory-based surveillance data on BSIs caused by *Candida* spp. from nationwide surveillance; therefore, our estimates are representative of the whole population. The rate we found is one third to one fourth of the rates reported from the United States

Table 3. Predisposing factors among 79 patients with nosocomial candidemia, Helsinki University Central Hospital, 1995–1999[a]

Predisposing factor	No. (%)	
Central venous catheter	64	(81)
Urinary catheter	33	(42)
Gastrointestinal surgery[b]	22	(27)
Hematologic malignancy	20	(25)
Other surgery[b]	17	(21)
Solid malignancy	16	(20)
Diabetes	14	(18)
Newborn status	9	(11)
Organ transplantation	8	(10)
Severe trauma	2	(3)

[a]One patient may have several predisposing factors.

[b]Surgery during the same hospital period as candidemia, or within 1 month before the first blood culture.

(6.0–8.0/100,000 population) (18,25–26). Two of the U.S. reports included selected urban areas, and the third one was based on sentinel surveillance implemented in selected laboratories in Iowa (which may not be representative of the general U.S. population). A nationwide study from Iceland also documented an increase in the incidence of candidemia from 1.4 per 100,000 population in 1980 to 1984 to 4.9 in 1995 to 1999 (27). The 1995–1999 rate in Iceland was more than twice as high as the rate we observed in Finland during the same period. Another nationwide study from Norway reporting the annual numbers of fungemia cases did not identify any change in the period 1991–1996 (28). This study also included non-*Candida* yeasts. We did not identify any shift towards non–*C. albicans* species, in contrast to several reports from the United States, Australia, and Europe (12,14,16,18) but in accordance with the nationwide studies from Iceland and Norway (27,28).

We observed the highest age-specific incidence rate in infants <1 year of age. This rate is, however, substantially lower than the rates reported from the Atlanta and San Francisco Bay areas in 1992 and 1993 in the same age group (9.4 vs. 75/100,000 population) (18). We found a substantial increase in candidemia cases in men 16–65 years of age. The reason for this increase remains unknown; the detailed analysis in the largest tertiary-care hospital in Finland during the same period showed no increase or major change of characteristics in this demographic subgroup. The Icelandic study showed that the incidence was highest in the elderly and that the increase occurred most in the youngest age group (27). The male dominance we observed is similar to that found in previous reports (18,25–26,28).

At the largest tertiary-care hospital in Finland, the average annual incidence of candidemia per 1,000 patient days was considerably less than that in the United States (0.04 vs. 2.15), as was the rate per 1,000 discharges (0.17 vs. 0.6) (2,30). Incidences similar to the current study have been reported from European (11,22–24,28) and Australian tertiary-care centers (12). We observed that non-nosocomial candidemia was very rare in this tertiary-care hospital (1/84 cases), which is in strong contrast to reports from the United States, where one fifth of candidemias developed in patients before or on admission to hospital (18).

Differences in candidemia rates between countries may also be attributable to differences in the representativeness of the study population, the prevalence of HIV infection in the study population, and variations in patterns of healthcare delivery and clinical practices, including the frequency of using blood cultures in diagnostics. The differences may also be explained by differences in antibiotic use patterns and resistance situation (28). A previous study from Finland on nosocomial BSIs showed that *Candida* spp. represented only 4% of all findings and the prevalence of antibiotic resistance among bacterial findings was lower than in the United States (31).

The role of fluconazole prophylaxis is well established in neutropenic patients; however, among patients without neutropenia, such as surgical ICU patients, this role is less definitive (32–34). Clinical practices in prophylaxis policies may vary a great deal between institutions and countries. While the prophylaxis effectively reduces the incidence of infections caused by fluconazole-sensitive species, the drug has an impact on the distribution of causative *Candida* species (11,15,32–35). Although national data on fluconazole usage are available from Iceland and Norway, they are not comparable (27,28).

Our study confirms the importance of surgery, cancer, and hematologic malignancies as factors contributing to nosocomial candidemia. Only 23% of our candidemia patients were being treated in ICUs. Approximately twice the proportion of patients with candidemia who had had preceding treatment in ICU was reported from Italy (21,24), but a similar proportion to ours was reported from France (23). Our patient population included no HIV patients, which reflects the low prevalence of HIV infection in Finland (10–16/100,000 population in 1995–1999) (36). This low prevalence may substantially contribute to both the lower overall incidence of candidemia and the low proportion of non-nosocomial *Candida* infection in Finland, since in the United States the proportion of candidemia cases with HIV infection to all candidemia cases varied from 10% to 15% (18,25). The contribution of HIV infection as a predisposing risk factor for candidemia is further emphasized by a report from Italy, where *Candida* spp. was the third most common cause of nosocomial BSI in HIV patients (37). In France, among cases of nosocomial candidemia, 13% of patients were reported to have HIV infection during 1990–1995 in one institution (38).

The high case-fatality ratio we observed in the older age groups and in patients with a hematologic malignancy reflects the combination of serious underlying diseases and the intensity of treatments modifying host defense that leads to candidemia. In our study, the overall case-fatality ratio of 15% during 1 week and 35% within 1 month after the onset of candidemia are similar to ratios reported from Europe (21,24) and the United States (39), with case-fatality ratios ranging from 35% to 39%, respectively, within 1 month after onset of candidemia.

The results of this study demonstrate a low but consistently increasing incidence of candidemia in Finland. The high case-fatality ratio emphasizes the need for continuous surveillance to identify changes in predisposing factors for optimizing prevention policies, including the use of antifungal prophylaxis.

Dr. Poikonen is a clinical hematologist at Peijas Hospital, Vantaa, Finland. Her research interests include invasive candida infections and candidemias and the risk factors and outcome of these infections, specifically the epidemiology of invasive candida infections.

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Address for correspondence: Eira Poikonen, Peijas Hospital, Department of Medicine, Sairaalakatu 1, 01400 Vantaa, Finland; fax: +358-9-471 67 579; email: eira.poikonen@hus.fi

Severe Acute Respiratory Syndrome: Temporal Stability and Geographic Variation in Case-Fatality Rates and Doubling Times

Alison P. Galvani,* Xiudong Lei,* and Nicholas P. Jewell*

We analyzed temporal stability and geographic trends in cumulative case-fatality rates and average doubling times of severe acute respiratory syndrome (SARS). In part, we account for correlations between case-fatality rates and doubling times through differences in control measures. Factors that may alter future estimates of case-fatality rates, reasons for heterogeneity in doubling times among countries, and implications for the control of SARS are discussed.

Concern over the emergence of severe acute respiratory syndrome (SARS) has persisted as epidemic continues in the first months of 2003, despite control efforts. As of May 12, 2003, the World Health Organization (WHO) had reported 7,447 cases with 552 deaths in >30 countries. The most affected locations are China, Hong Kong, Singapore, Viet Nam, Taiwan, and Canada. (Our study focuses on Canada.) Doubling times (i.e., the period required for the number of cases in the epidemic to double) and case-fatality rates (CFRs) are fundamental to the epidemiology and potential public health impact of SARS. Doubling times are a measure of the rate of spread of disease and also indicate the magnitude of control efforts required to curtail spread. Because doubling times change substantially over the course of an epidemic, current estimates should not be used to extrapolate into the future.

CFRs of SARS have typically been estimated by dividing the number of deaths by the total number of cases. This method is sufficient for an advanced epidemic. However, the method is not accurate at an early stage of an epidemic, particularly when the time from infection to recovery or death is not brief, relative to the duration of the epidemic, as is currently true with SARS. The method underestimates the CFR because it does not account for a proportion of currently infected persons' dying from the disease. A more accurate method would be to divide the number of deaths by the total number of deaths plus the number of persons who recovered. By applying this method to publicly available WHO data (1), the cumulative CFR estimates appear more stable, relatively constant within a country (aside from Taiwan, which is in the earliest stage of its epidemic) but varying considerably among countries (Figure 1).

Average overall CFR for all countries increased from 10.4% on April 21 to 14.7% on May 12 (largely attributable to the sudden rise in CFRs in China and Taiwan). In countries with few deaths, this estimate of CFR may be a slight overestimate if the time from infection to death tends to be shorter than time to recovery. However, recent cohort data from Hong Kong (2) suggest the opposite, implying that our crude estimates of CFR may still be underestimates. Such inaccuracies are, however, unlikely to modify the results of a general comparison of CFRs across countries. Nevertheless, caution is warranted in comparing CFRs across countries since differences may exist in the various surveillance systems that report cases and the number of persons who recovered.

Figure 1 does not directly provide information on whether the CFR shows temporal trends in any country as it plots the average CFR since the beginning of the epidemic. Unfortunately, the publicly available WHO data do not permit a CFR to be estimated over time since cases reported in one period are not linked to recoveries at the same or future time. The determination of factors, including date of infection, that influence death rates awaits detailed analyses of cohort data on infected persons.

We identify an inverse relationship between the average CFR and the average doubling time for different countries (Figure 2). (The average doubling time is a cumulative

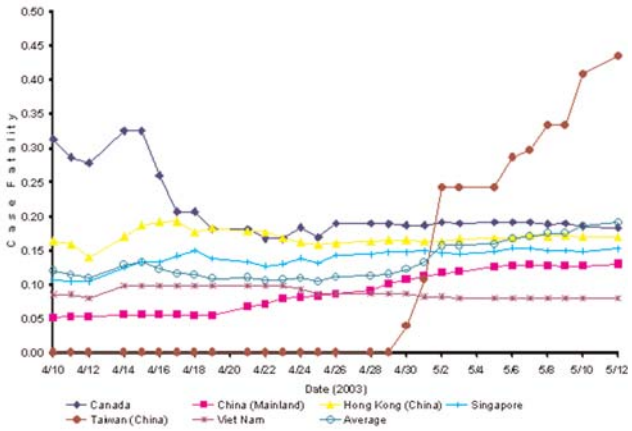

Figure 1. Cumulative case-fatality rates for severe acute respiratory syndrome over time.

*University of California, Berkeley, California, USA

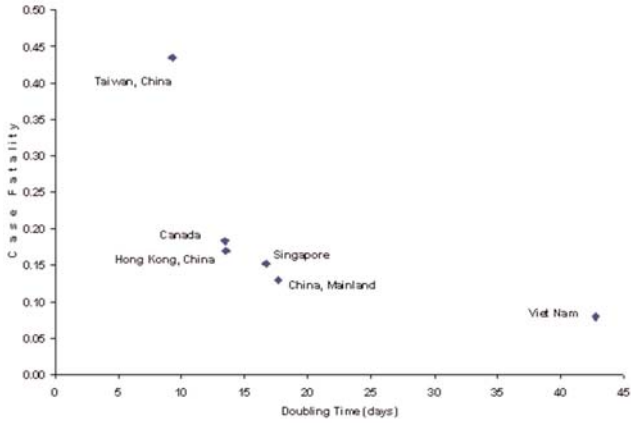

Figure 2. Cumulative case-fatality rate for severe acute respiratory syndrome compared with average doubling time as of May 12, 2003.

measure reflecting average growth from the beginning of reported data and is estimated by the length of a time period divided by the $\log_2$ of the relative growth in numbers of reported cases during the same period; see Appendix.) This relationship is probably generated by the influence of the efficacy of control policy affecting both parameters, rather than a reflection of different characteristics of viral infectiousness and virulence across epidemics. The rapid hospitalization of infectious persons is likely to reduce the CFR and increase the doubling time (by reducing the spread of SARS). Consistent with this explanation is the successful containment of a sizable epidemic in Viet Nam and the relatively low CFR and long doubling time there. In contrast, Canada has the highest CFR and shortest doubling time (except for Taiwan, where the CFR has yet to reach a steady state). Stochasticity in personal contacts plays a key role during the invasion phase of the epidemic. In Toronto, the stochasticity of social contacts resulted in a second outbreak after public health officials thought that SARS had been controlled. Transmission also occurred in Toronto before public awareness of SARS was widespread, resulting in delayed hospitalization of the first few patients; this scenario, in turn, facilitated transmission and may have elevated death rates (3). Thus, the means of disease introduction may be important in determining early doubling times and CFR values. These factors, combined with small sample sizes, may have been the cause of particularly high CFRs from April 10 to April 15 in Canada.

Variation in CFRs among countries will arise from differences in intensity and speed of medical care, age structure of the population (older infected patients are more likely to die [2]), and factors such as coinfection. For example, the high prevalence of coinfection with other respiratory diseases, such as infections caused by *Chlamydia pneumoniae* (4), *C. psittaci,* and paramyxoviruses in China, could increase the CFR there. Likewise, should SARS spread in Africa, the disease could have a devastating effect, given its high prevalence of tuberculosis and HIV/AIDS.

Estimates of CFR may change as polymerase chain reaction (PCR) assays become more widely used in diagnosis (5). Diagnostic tests could identify mild cases that currently are not reported. Our estimate of the size of the epidemic would then increase in terms of number of cases, but the estimates of CFR would decrease. Conversely, PCR tests might eliminate the diagnosis of SARS in some suspected cases. Ultimately, accurate estimates of population distributions of parameters reflecting the clinical course of disease will be best provided by follow-up of clearly defined cohorts of infected persons identified by appropriate diagnostic procedures.

As an epidemic declines, the doubling time increases. Variation in doubling time among countries probably arises from variation in both transmission rates and control efforts (Figure 3). Transmission rate (with units of time^{-1}) is determined by the expected number of susceptible persons with whom each infectious person comes into contact during a time unit in their infectious periods and by the probability of disease transmission per contact. High-density population centers, crowded public transportation systems, and hospital waiting rooms increase the number of contacts, while personal hygiene affects the probability that transmission will occur with each contact. In all countries, seasonal effects may also play a substantial role with the virus spreading faster in winter.

In Viet Nam, the doubling time increased over the period that the epidemic was being controled (Figure 3). The dramatic drop in doubling time in China in early April corresponds to a change in reporting practices (Figure 3). Similarly, in the United States, a shift in the definition of SARS to correspond to that recommended by WHO complicates estimation of doubling time. However, the doubling time appears to be relatively long in the United States

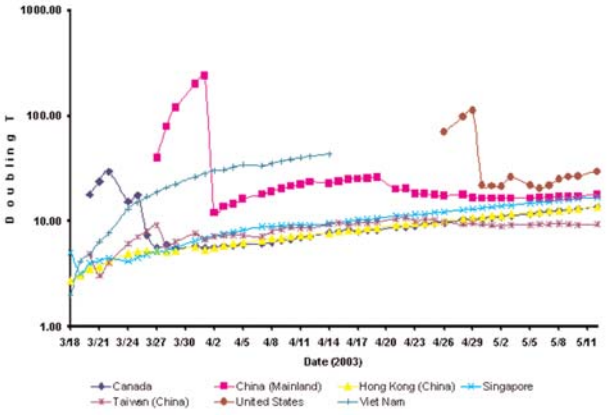

Figure 3. Time series of log of average doubling time for severe acute respiratory syndrome.

because most cases are caused by seeding from travel to Asia, with few cases occurring from local transmission.

Epidemic models may provide a framework for evaluating alternative control measures. Central to the accurate parameterization of epidemic models is the reproductive ratio, R_0, which is the average number of secondary cases generated by one initial infection in a susceptible population in the absence of control measures (6). R_0 defines a threshold that determines whether an infection is likely to spread. If R_0 is <1, each infection will not replace itself, on average, and the disease will likely die out, although in such cases spatial dynamics, latency, and stochastic variation may contribute to localized flare-ups of the disease that may persist for a long time. Thus, R_0 also defines the level of intervention required to contain an epidemic. The doubling rate can be used to calculate R_0, given that, where γ is the duration of the incubating period, α is the duration of the symptomatic period, and τ is the doubling time (7). Accurate characterization of the incubation and symptomatic periods is essential to the translation of doubling times to R_0. Typical estimates of the incubation period for SARS range from approximately 2 to 10 days (median and mean 5 days) (4,8), whereas the symptomatic period has a mean (± standard deviation) of 16±8 days (4,8). Recent data from Hong Kong (2) suggest somewhat longer incubation on average. However, severe infections may be overrepresented in current estimates, which have been based largely on persons who have received intensive medical treatment, another factor that may affect the symptomatic period. At this point in any of the epidemics, we are reluctant to use this approach for calculating R_0 from doubling times since the latter is confounded by evolving control policies (e.g., Hong Kong, Toronto); the most natural epidemic (in Guangdong Province) offers the least complete data.

Figure 4 plots the reported case counts in China, together with an exponential curve fitted to a smooth version of the counts (to allow for the discreteness in reports in early April). The estimated doubling time from this curve is 16.2 (which closely matches the May 3 value for China in Figure 3 of 16.3; the May 12 doubling time is now 17.7, since the growth in counts has declined in the 9 days after May 3). The curve suggests that 502 cases existed in China on March 17 (with a 95% confidence interval of 468 to 538) (Appendix), which is consistent with underreporting at that time. Control measures, evolving contact patterns, stochastic effects, and potential acquired immunity will all impact this doubling time (equivalently, the growth in case counts) and ultimately lead to a flattening of the growth observed to date. This decline in the rate of growth can be seen already in the last week of data from China, although whether this decline is real or due to delayed reporting is not yet known.

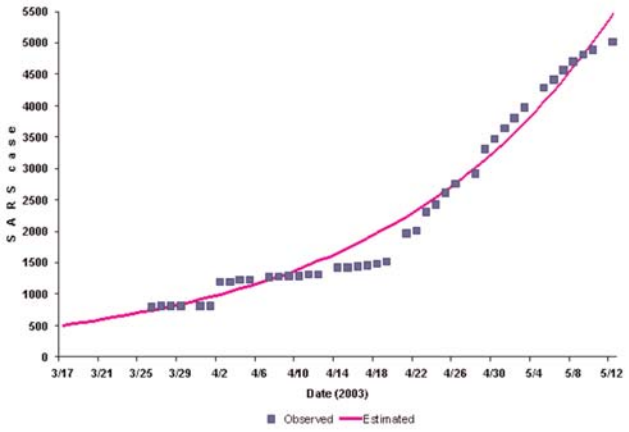

Figure 4. Observed and expected cumulative number of cases of severe acute respiratory syndrome in China.

The rapid increase in the number of cases in China suggests an urgent need to control the epidemic in Asia before it gains further momentum. Containment of an outbreak at an early stage affords a greater chance of success than does a later response and clearly puts less strain on the healthcare system. Isolation of cases, infection-control measures in hospitals, and vigilant surveillance at community and population levels are imperative. Failing this, SARS could become endemic in China, particularly if it evolves antigenically to evade pre-existing immunity, such that recovered patients could be reinfected, as is the case for influenza (9). In this eventuality, international travel would continually seed new cases in other parts of the world. SARS reaffirms what we have previously learned from other infectious diseases, namely that epidemic control is a global concern and not the problem of one or a few nations.

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Dr. Galvani is a Miller Research Fellow at University of California, Berkeley. Her primary research interests are focused on the evolution and epidemiology of infectious diseases, including influenza.

Appendix

Definition of Doubling Time

The average doubling time from time t_0 to time t_1 is simply

$$\frac{t_1 - t_0}{\log_2\left(N_1 / N_0\right)}$$

where N_1 and N_0 are the number of cases at times t_1 and t_0, respectively. The units correspond to those used to measure the interval length t_1-t_0. For example, if N_1=2N_0, then the average doubling time is exactly t_1-t_0. In Figures 2 and 3, the time t_0 (in days) is always taken to be the earliest time when case counts are available in WHO data.

Analysis of SARS Data from China

Figure 2 was obtained by (i) first applying a lowess smooth function (9) to the observed case counts, (ii) applying least squares linear fit of the logarithm of the smoothed case counts against time, and (iii) transforming the fitted line back to the original scale. In particular, in (ii) the estimated line is

$\log(\text{case}) = 6.2 + 0.04t$

(where t=time in days, ranging inclusively from the value 1 [March 17] to value 57 [May 12])

Prediction of Cases with 95% Confidence Interval for March 17, 2003

The predicted confidence interval for $Y_{(new)}$ is the following:

$$\frac{Y_{(new)} - \hat{Y}}{\sqrt{Var(pred)}} \rightarrow t_{(n-2)}, \quad Var(pred) = MSE * \left[1 + \frac{1}{n} + \frac{(X_{new} - \bar{X})^2}{\sum (X_i - \bar{X})^2}\right]$$

The estimates and confidence intervals are then transformed back to the original scale.

On March 17, 2003, the estimated case is 502, with a 95% confidence interval (468 to 538); note that this confidence interval is for the actual number of cases on March 17.

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Address for correspondence: Alison Galvani, Department of Integrative Biology, University of California, Berkeley, CA 94720, USA; fax: 510-643-6264; email: agalvani@nature.berkeley.edu

Emerging Pathogen of Wild Amphibians in Frogs (*Rana catesbeiana*) Farmed for International Trade

Rolando Mazzoni,* Andrew A. Cunningham,† Peter Daszak,‡ Ada Apolo,* Eugenio Perdomo,* and Gustavo Speranza*

Chytridiomycosis is an emerging disease responsible for a series of global declines and extinctions of amphibians. We report the causative agent, *Batrachochytrium dendrobatidis*, in North American bullfrogs (*Rana catesbeiana*) farmed for the international restaurant trade. Our findings suggest that international trade may play a key role in the global dissemination of this and other emerging infectious diseases in wildlife.

Cutaneous chytridiomycosis is an emerging fungal disease of amphibians responsible for a series of mass die-offs, population declines, and extinctions of amphibians on a global scale (1,2). In wild, susceptible species, chytridiomycosis may be able to cause catastrophic population loss, sometimes completely removing local populations (2). This disease is a serious threat to the conservation of wild amphibians, and policy measures to control amphibian movements have been established by at least one authority (Parks and Wildlife Commission of the Northern Territory, Australia; Bill Freeland, pers. comm.). Chytridiomycosis is caused by a zoosporic fungus, *Batrachochytrium dendrobatidis*, which develops solely within keratinized cells (3), causing extensive hyperkeratosis and death by an, as yet, unknown mechanism (1,4,5).

Chytridiomycosis is a key example of an emerging infectious disease in wildlife (6,7). The most important factor driving the emergence of such wildlife diseases is the anthropogenic introduction of pathogens into new geographic areas (pathogen pollution) (6–9). We report on *B. dendrobatidis* in captive bullfrogs, which was identified during an episode of unusually high death rates of unknown cause and which implicates a relatively new food animal trade in the spread of this disease.

*Instituto de Investigaciones Pesqueras, Montevideo, Uruguay; †Institute of Zoology, London, United Kingdom; and ‡Consortium for Conservation Medicine, Palisades, New York, USA

The Study

Mass deaths occurred at a large rearing facility for North American bullfrogs (*Rana catesbeiana*), 46 km from Montevideo, Uruguay. The farm is a commercially managed operation, with trained employees under permanent, technical supervision. The farm produces approximately 150,000 tadpoles each summer and 30,000 metamorphs. Mortality rates during the days before the outbreak were approximately 0.5% per week, which is considered normal for this type of farm. Frog growth rates were also within normal parameters for these conditions (10).

At this farm, spawning occurs in 50-L tanks from October to February (spring and summer), tadpoles grow until March, and metamorphosis occurs from March to May (fall season). Newly metamorphosed frogs (5–8 g) are harvested from tadpole ponds and stocked in tanks with circulating water until resorption of the tail is complete. Frogs are then transferred to large growing tanks, in which the frogs are reared at a density of 500 per square meter while standing in shallow water with only their bodies immersed (10). Permanently running water is piped into the system from a well on the farm. Water temperature ranges from 17°C to 19°C, and pH is stable at 7.1. Frogs are fed with trout chow (Purina Aquamax Carnivorous) and earthworms (*Eisenia foetida*) cultured at the farm. Tanks are cleaned every day to eliminate excess feed and feces, and animals are sorted to maintain homogeneous sizes in each tank. Dead animals are removed twice a day.

The mass die-off began on May 1, 1999. Just before the mass die-off, a tank containing 5,000 recently metamorphosed frogs had been emptied, and the population placed in three other tanks along with other frogs that had metamorphosed during the same week. The following day, frogs started to die in unusually high numbers (300 deaths on day 1) in these three tanks. To reduce the risk of bacterial infections, poly-hexamethylene-biguanide-hydrochloride (Vantocil IB, Zeneca Biocides, Blackley, Manchester, UK) was added to the tanks to give a final concentration of 500 ppm. The deaths continued and spread to other tanks; 15,000 frogs died during the first week. An antifungal therapy (benzalkonium chloride 2 ppm baths, "Farmazul" 1% solution, Lab. Farmaco-Uruguayo, Acuña de Figueroa, Montevideo) (11) was used, but this treatment was also ineffective and almost 95% of the farm's total population of recently metamorphosed frogs died within 30 days. Mortality rates in individual tanks were extremely high, with affected tanks losing 10% of the population on day 1 and usually >90% of the population by day 3 or 4. By day 10, all frogs in the three index tanks were dead, and the disease had spread to metamorphs in other tanks on the farm. The epidemic continued for 26 days (until May 27), when only 2,000 of the original 30,000 metamorphs remained. None of the broodstock was affected, even

though these animals were in close spatial association with the affected tanks.

Affected frogs became lethargic with visible accumulations of sloughed skin. Although nonspecific, these signs, the high mortality rate, and the age of affected frogs are consistent with those reported for chytridiomycosis (2). Many of the frogs had agonal convulsions and extension of hind limbs; death occurred 12–24 h later. Reddening of ventral skin was found in some cases a few hours before death, usually in the pelvic and femoral areas. Tadpoles showed no signs of disease or increased death rates.

Routine bacteriologic tests (culture on blood agar for 24 h and 48 h at 37°C) were performed on liver, kidney, and heart blood collected from a sample of euthanized frogs, but these tests did not implicate causative pathogens. Necropsies were performed systematically on 30 frogs, but no gross abnormalities of the viscera were found. Internal organs (lung, liver, kidney, heart, and spleen) from 10 of these frogs were examined by histologic testing, but no lesions were detected. Examination of skin smears (wet-mounted, stained with a 1:1 mixture of cotton blue [parker ink] and 10% aq. KOH), and histologic and ultrastructural analyses of fixed skin indicated subspherical developing sporangia and flask-shaped mature sporangia within the superficial layer of the epidermis (Figure). Septa, characteristic of *B. dendrobatidis*, were observed in many developing sporangia (Figure). Slight hyperplasia of the keratinized cells of the stratum corneum and stratum granulosum was observed in some areas where chytrid developmental stages were present. Specific examinations for viruses were not performed, as facilities to test for viruses were unavailable.

In a basic transmission experiment, two groups of five apparently healthy frogs from affected tanks were placed in separate aquaria, one contained five frogs from an unaffected farm in Uruguay and the other empty. Deaths began on day 4, with death occurring 24–48 h after first signs of disease. Within 8 days, all 15 frogs were dead. A group of five control frogs from the unaffected farm remained healthy.

After the incident, death rates returned to levels before the event. As a result of this event, monthly surveillance for cutaneous chytrid infection was instigated; ventral hind limbs, feet, and digits are examined from five healthy frogs and from any sick or dead frogs. *B. dendrobatidis* has been repeatedly observed in skin smears of healthy frogs from the farm at which the outbreak occurred. Similar results have since been obtained from six other bullfrog farms in Uruguay (R. Mazzoni, unpub. data). Histologic examination of infected frogs on which necropsies were performed since the 1999 epidemic shows focal mild hyperplasia of the superficial epidermis associated with sporangia of *B. dendrobatidis*. No unusual death rates have occurred at the index site since the 1999 incident.

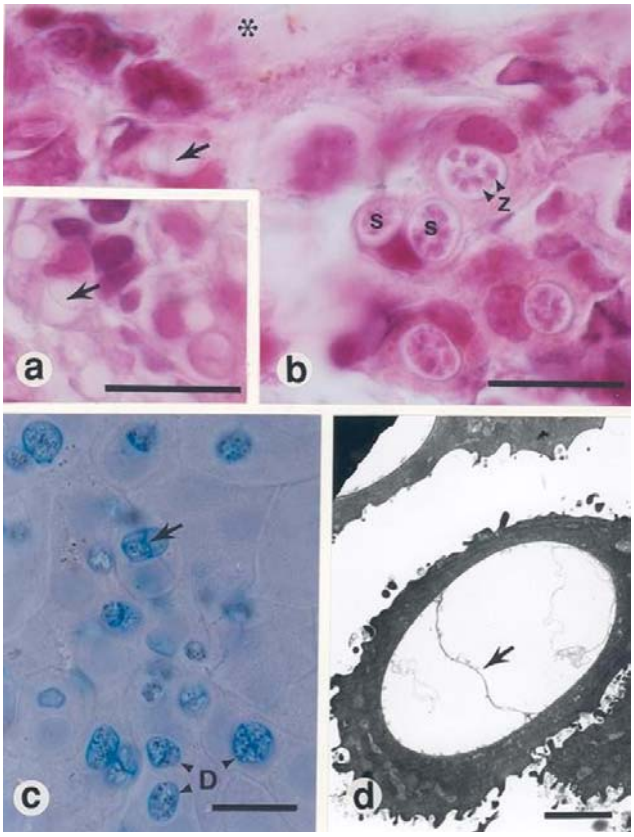

Figure. a and b, histopathologic findings from infected frogs. Characteristic sporangia (s) containing zoospores (z) are visible in the epidermis (asterisk, superficial epidermis; arrow, septum within an empty sporangium; bars, 10 mm. c, Skin smear from infected frog, stained with 1:1 cotton blue and 10% aqueous potassium hydroxide (aq KOH) (D, developing stages of *Batrachochytrium dendrobatidis*; arrow, septum within a sporangium; bar, 10 μm. d, Electron micrograph of an empty sporangium showing diagnostic septum (arrow) (bar, 2 μm).

Conclusions

Cutaneous chytrid infection was detected during an investigation into mass deaths in farmed North American bullfrogs. Although chytridiomycosis has been reported as an important cause of mass deaths in wild amphibians, whether chytrid infection was the cause of deaths in the disease outbreak described in this paper is unclear. First, while no examinations for *B. dendrobatidis* had been performed before the deaths, this organism has been repeatedly observed in skin examinations of healthy frogs in follow-up studies. Second, although not proved efficacious against cutaneous chytridiomycosis, the use of benzalkonium chloride, a recognized therapy for infections with nonhyphal fungi, failed to reduce mortality rates.

Possible causative agents include an infectious pathogen, which could not be detected by the examinations used, such as a ranavirus (2) or adverse environmental factors. Alternatively, chytridiomycosis could be the cause of

the outbreak if the current chytrid is a different strain from that causing the outbreak or became attenuated, if the farmed bullfrogs had immunity to the pathogen, or if a change in the environment before the die-off allowed a normally benign infection to become pathogenic. For example, increased temperatures reversibly inhibit both growth of *B. dendrobatidis* in culture (3) and the progress of disease outbreaks (D. Nichols, Smithsonian Institute, pers. commun.). Also, chytridiomycosis epizootics in wild U.S. amphibians often coincide with late-winter breeding (2). The deaths we describe occurred at the beginning of winter and may have been precipitated by lowered environmental temperatures.

The mortality rates reported here were dramatic and costly. The rearing of bullfrogs is a growth industry in South America, particularly Brazil, Uruguay, and Argentina. Anecdotal reports of similar loss of stock in a number of farms in Uruguay (R. Mazzoni, unpub. data) suggest that disease is a major economic threat to this industry in South America. This situation is exacerbated by a lack of veterinary diagnostic capacity, management protocols, and treatment or prophylaxis.

Regardless of the cause of the deaths, the North American bullfrog may be relatively resistant to chytridiomycosis, at least under the environmental conditions in the current study. Unpublished data suggest that metamorph bullfrogs can be experimentally infected by *B. dendrobatidis* without developing signs of disease (12), and others have noted differential susceptibility to this disease among amphibian species (1,2).

Nonclinical (silent) infections in farmed bullfrogs suggest that this species may act as a carrier of chytridiomycosis, which has serious implications for conservation of biodiversity. *B. dendrobatidis* has been implicated in the complete removal of multiple species amphibian populations over large geographic areas in the wild (1,2). Farmed bullfrogs originate in North America and have been introduced to South America during the last few decades for the lucrative restaurant trade. Live, farmed South American frogs are exported to other South American countries and to the United States. The trade in bullfrogs is large in scale and global in scope. According to the U.S. Fish and Wildlife Service, over 1 million bullfrogs are imported into the United States each year from South America alone (9), with others shipped in from Asia. In the United States, animals are usually imported live at U.S. ports of entry and undergo veterinary inspections that are inadequate for identifying chytrid infection.

Recently, guidelines for amphibian translocations have been published that include specific quarantine and testing recommendations for chytridiomycosis (13). These procedures, however, are intended for animal movements for International Union for Conservation of Nature and Natural Resources programs and are not legally binding. In the United States, the U.S. Department of Agriculture (USDA) may be the most suitable regulatory body for the bullfrog trade since these animals are a commercial agricultural product. No current USDA regulations exist for the identification of infectious agents in amphibians.

The farm we studied was opened in October 1998 with stock that originated from Brazil. The Brazilian farmers originally imported 300 pairs of frogs from Canada to set up the first South American farms approximately 30 years ago. The pathogen has been present in bullfrogs in North America since at least the 1970s (14). *B. dendrobatidis* in this outbreak may have been imported with breeding stock from North America. Alternatively, wild South American amphibians may be the source of infection. The Uruguay farm in our study is enclosed by concrete walls, but outdoor ponds have open access to wildlife, and wild amphibians (tree frogs) have been observed in the greenhouses where indoor rearing tanks are located. Whatever the origins of *B. dendrobatidis* in farmed bullfrogs in Uruguay, such open-plan farms are likely sources of infection and disease for wild amphibian species. In view of this risk, surveillance for chytrid infection and for unusual levels of deaths in endemic amphibian species should be established in the vicinity of frog farms in Uruguay and elsewhere in the world.

Global trade and commerce are regularly cited as drivers of disease emergence in humans, domestic animals, wildlife, and plants (6,15–18). For wildlife emerging infectious diseases, quantitative analyses demonstrate that this process is the most important driving factor (8). For example, the (unknown) causative agent of the bullfrog deaths we describe may be spread by such trade. Also, the identification of *B. dendrobatidis* in an international food animal trade has implications for amphibian conservation and for disease emergence in general. Our study adds to reports of chytridiomycosis in the international pet trade (19), amphibians for outdoor pond stocking in the United States (2,20), importation for zoo collections (5), trade in laboratory animals (21), and species known to have recently been introduced into new geographic regions (e.g., cane toads in Australia) (1).

With a continued rise in the international air transport volume (22), we predict a growing impact of trade and commerce on disease emergence within all populations, including wildlife. For this reason, we urge a revision of national and international veterinary guidelines for the inspection and quarantine of imported animals and animal products.

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Mr. Mazzoni is a candidate in the Ph.D. program at the Federal University of Goias-Brazil, where his project is the study and control of frog diseases. His main interests are the development and improvement of frog farming techniques, including farm design, husbandry protocols and disease control. Current projects include studying the effects of handling and storage techniques on meat quality and identifying the main productive parameters of economic significance in the frog farming industry.

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Address for correspondence: Andrew Cunningham, Institute of Zoology, Regent's Park, London NW1 4RY, UK; fax: +44 (0) 20 7586 1457; email: andrew.cunningham@ioz.ac.uk

NmcA Carbapenem-hydrolyzing Enzyme in *Enterobacter cloacae* in North America[1]

Sudha Pottumarthy,* Ellen Smith Moland,† Stefan Juretschko,* Susan R. Swanzy,* Kenneth S. Thomson,† and Thomas R. Fritsche*

An imipenem-resistant *Enterobacter cloacae* isolate was recovered from the blood of a patient with a hematologic malignancy. Analytical isoelectric focusing, inhibitor studies, hydrolysis, induction assays, and molecular sequencing methods confirmed the presence of a NmcA carbapenem-hydrolyzing enzyme. This first report of NmcA detected in North America warrants further investigation into its distribution and clinical impact.

Carbapenem resistance mediated by acquired carbapenemases is a growing concern worldwide (1–3). Carbapenems are the most potent of β-lactam antibiotics with a broad spectrum of activity against aerobic and anaerobic bacterial pathogens. These antibiotics are also the most reliable of the β-lactams for treatment of infections caused by organisms that produce extended-spectrum β-lactamases (ESBLs) or AmpC β-lactamases (4,5). Intrinsic resistance does occur, however, in a number of species, including *Bacillus cereus*, *Stenotrophomonas maltophilia*, *Flavobacterium* sp. and *Chryseobacterium* sp., *Aeromonas hydrophila*, *Legionella gormanii,* and *Janthinobacterium lividum,* because of the presence of metallo-β-lactamases (1). Acquired carbapenemases, on the other hand, are a heterogeneous mixture of enzymes that belong to Ambler class A, B, or D enzymes and may be plasmid or chromosomally encoded (1,2). Here we describe the characterization of imipenem resistance attributable to an acquired class A carbapenem-hydrolyzing enzyme, NmcA, in an *Enterobacter cloacae* isolate.

The Study

A 33-year-old man with acute myeloid leukemia was admitted to a hospital in Seattle, Washington, with neutropenic fever; he was initially treated with ceftazidime. His therapy was changed to acyclovir and imipenem because of unremitting fevers and oral lesions positive for herpes simplex virus type 1, whereupon his fever defervesced immediately. The fever recurred 2 weeks after treatment was begun, and imipenem-resistant *E. cloacae* was isolated from the blood. After the results of susceptibility testing were obtained, his therapy was changed to levofloxacin, and he exhibited a good clinical response.

The *E. cloacae* isolate was susceptible to piperacillin, piperacillin-tazobactam, ceftazidime, ceftriaxone, cefepime, ciprofloxacin, gentamicin, aztreonam, and trimethoprim-sulfamethoxazole and was resistant to ampicillin, amoxicillin-clavulanic acid, cefazolin, and cefoxitin. By both disk diffusion and E-test methods (AB Biodisk, Solna, Sweden), the zones of inhibition around the imipenem, meropenem, and ertapenem disks were indistinct, with inner colonies extending up to the disk or the highest concentration on the E-test strip (MIC ≥32 μg/mL) and were interpreted as resistant (Figure 1). Addition of 10 μL of 1,000 μg/mL clavulanic acid to the imipenem, meropenem, and ertapenem disks resulted in clearly defined and enlarged zones of inhibition and loss of the inner colonies (Figure 2). Using either cefoxitin or imipenem as inducers by the disk induction method resulted in the formation of blunted or D-shaped zones of inhibition to meropenem and ertapenem (6). We performed a carbapenem bioassay by incubating 100 μL of a 50 μg/mL solution of each carbapenem (imipenem, meropenem, or ertapenem) with 100 μL of either crude β-lactamase extract or phosphate-buffered saline for 90 min at room temperature. The inactivating capacity of the enzyme was then tested by a disk diffusion assay that demonstrated hydrolysis of imipenem, meropenem, and ertapenem (7).

Isoelectric focusing (IEF) to determine isoelectric points (pIs) and general inhibitor characteristics with an ampholine polyacrylamide gel (pH range 3.5–9.5) on a flatbed apparatus (Multiphor LKB, Bromma, Stockholm, Sweden) indicated that this organism produced a β-lactamase enzyme with a pI of 6.9 that was inhibited by clavulanic acid and not by cloxacillin (8,9). This enzyme hydrolyzed 1 μg/mL imipenem on the IEF gel overlay. Inducibility of the enzyme by imipenem (4 mg/L) and cefoxitin (8 mg/L) was confirmed by the broth induction method and IEF (10). After the broth induction procedure, the uninduced and induced preparations were serially diluted in 0.1 M potassium phosphate buffer, pH 7.0, permitting both qualitative and semiquantitative assessment of enzyme activity among the various β-lactamases in the

*University of Washington School of Medicine, Seattle, Washington, USA: and †Creighton University Medical Center, Omaha, Nebraska, USA

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Figure 1. Susceptibility testing of *Enterobacter cloacae* to carbapenems. Both methods had ill-defined zones of inhibition with inner colonies growing up to the disks or the E-test strips, respectively.

isolate, as determined by IEF. The broth induction enzyme preparation from a 100-mL, 3.5-h Mueller-Hinton broth produced a visible but light AmpC band, pI >9.0. The induction with cefoxitin and imipenem increased the levels of both the AmpC and the pI 6.9 carbapenem-hydrolyzing enzyme, as visualized in Figure 3. A cefoxitin-induced preparation of *E. cloacae* NOR-1 (that produces NmcA) was included as a control strain for IEF (11).

Chromosomal DNA was extracted from overnight cultures with the Qiagen DNeasy Tissue kit (QIAGEN Inc., Valencia, CA). Polymerase chain reaction (PCR)-amplification with a thermal cycler (MJ Research PTC - 200-DNA Engine, San Francisco, CA) was used to obtain a 2,122 base pair gene fragment consisting of the carbapenemase structural gene *nmcA* and its regulatory gene *nmcR* (12,13). The nucleotide sequences of the primers were 5'-TGC CAG CTT AAT TAT TTT CAG ATT AG -3' (*nmcR* positions 32–56) and 5'- ATT TTT TTC ATG ATG AAG TTA AGC C -3' (*nmcA* positions 2,129–2,154). PCR amplification of the *nmcR/nmcA* genes showed positive results with both NOR-1 and the strain under study, suggesting that the latter strain harbored a carbapenem-hydrolyzing enzyme similar to that of NOR-1 (11). Subsequent sequence analysis of the amplified DNA-region showed a similarity of 100% (2,122 base pairs) between the sequence of our strain and the carbapenemase genes, regulatory (*nmcR*) and structural (*nmcA*) genes of the *E. cloacae* strain NOR-1 (13).

Conclusions

Ambler class A carbapenem-hydrolyzing enzymes are relatively rare and have been reported to date in only a handful of isolates of *Enterobacteriaceae* worldwide (1,2,14–16). Sme-1 was identified from two *Serratia marcescens* strains collected in London in 1982, and IMI-1 was found in two strains of *E. cloacae* isolated in southern California in 1984. Both enzymes were retrospectively characterized from clinical isolates recovered before carbapenems were marketed (17,18). Subsequently, imipenem-resistant *S. marcescens* strains were identified from Minnesota, California, and Boston during a 15-year period, 1985–1999. The Minnesota isolate produced an enzyme identical to the London Sme-1 enzyme, but the Boston and California strains produced a related Sme-1 enzyme with a single amino-acid variation, named Sme-2. More recently, a fourth class A β-lactamase, KPC-1, has been described in a strain of *Klebsiella pneumoniae* collected in North Carolina (15).

NmcA was the first class A carbapenemase identified in a clinical isolate of *E. cloacae*, NOR-1, in 1990, following the introduction of carbapenems (11). This strain was recovered from the pus of a fistulized subcutaneous abscess of a hospitalized French patient who had received one intravenous bolus (500 mg) of imipenem before the strain isolation. The present strain was also isolated from the patient during therapy with imipenem. Similar to the NOR-1 isolate, this strain displayed decreased susceptibility to carbapenems (imipenem and meropenem) while remaining fully susceptible to extended-spectrum cephalosporins (1). The strain also had decreased susceptibility to ertapenem. Although the zones of inhibition around the carbapenem disks were poorly defined, leading us to question the purity of the isolate, repeated testing confirmed our initial observations. The carbapenem-hydrolyzing enzyme was inhibited by clavulanic acid, as shown by the disk diffusion method and confirmed by the IEF overlay method, similar to the other class A carbapenem-hydrolyzing enzymes (1,2). We found that adding of

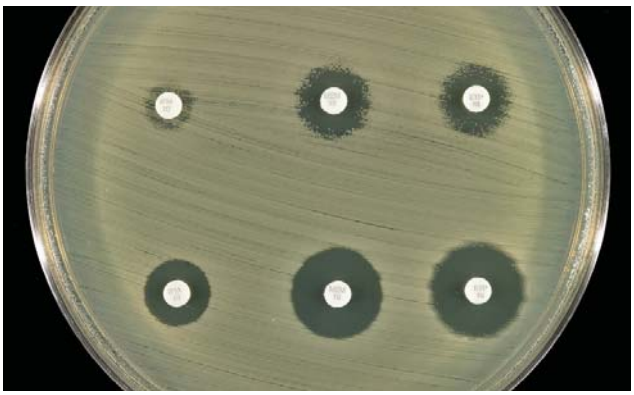

Figure 2. Effect of addition of clavulanic acid (10 μL of 1,000 mg/mL) to the zones of inhibition of the three carbapenem disks. Top row (left to right): imipenem, meropenem, and ertapenem disks without clavulanic acid. Bottom row (left to right): imipenem, meropenem, and ertapenem disks with clavulanic acid.

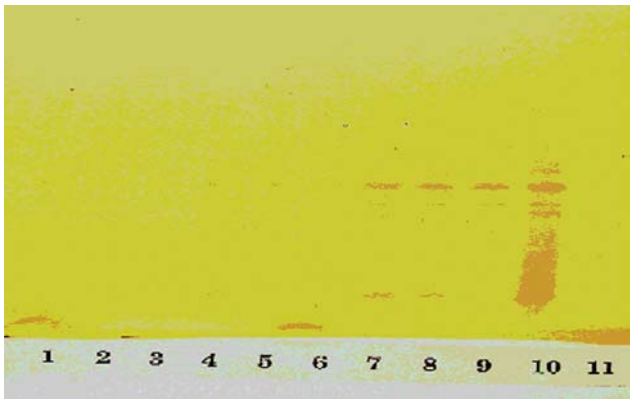

Figure 3. Analytical isoelectric focusing (IEF) of serially diluted broth induced enzymes. The isoelectric points (pIs) of the two prominent bands midway and at the bottom of the gel are 6.9 and >9.0. Lanes 1–3: uninduced enzyme serially diluted 1:1, 1:2, and 1:4. Lanes 4–6: enzyme induced with 8 mg/mL of cefoxitin serially diluted 1:1, 1:2, and 1:4. Lanes 7–9: enzyme induced with 4 mg/mL of imipenem serially diluted 1:1, 1:2 and 1:4. Lane 10: NOR-1 control, induced with 16 mg/mL of cefoxitin.

clavulanic acid to the carbapenem disks, similar to the method used for National Committee for Clinical Laboratory Standards ESBL confirmatory testing, was a simple and helpful diagnostic approach. Because other investigators have defined the hydrolytic spectrum of the NmcA enzyme, we have not established whether the NmcA enzyme was solely responsible for the carbapenem resistance in our isolate (11,13).

Class A carbapenem-hydrolyzing β-lactamases are usually coproduced with other β-lactamases, conferring distinct biochemical advantages to the parent strain (3). The carbapenem-hydrolyzing enzyme IMI-1 was produced along with AmpC and TEM-type enzymes; Sme-1 was coproduced along with a chromosomal AmpC (3,18). Our strain was similar to NOR-1 and produced an AmpC enzyme of pI >9.0 along with the carbapenem-hydrolyzing enzyme of pI 6.9. The inducibility of NmcA in this strain of *E. cloacae* by cefoxitin and imipenem was evident by disk induction and confirmed by broth induction with IEF, similar to the NOR-1 strain. Expression of NmcA has been shown to be inducible because of the presence of a LysR-type regulatory gene, Nmc-R, which precedes the bla_{NMC-A} gene (1). NmcR mediates biosynthesis of the carbapenemase enzyme at the basal state, which is further increased when β-lactam-mediated induction occurs (13). Naas et al. have shown that NmcR acts as a positive regulator for carbapenemase biosynthesis even in the absence of an inducer (unlike Amp-R, which is a negative regulator of the cephalosporinase expression in the AmpC-AmpR system) and deletion of NmcR results in a sharp decrease in carbapenem MIC and loss of β-lactamase inducibility (13). A recent study has shown that AmpD from *E. cloacae* NOR-1 is involved in the regulation of expression of both b-lactamases (NmcA and AmpC) (19). These findings suggest that different structural genes may be under the control of identical regulatory systems and that nucleotide substitutions in AmpD could lead to the stable co-expression of the NmcA together with overexpression of the AmpC enzyme in *E. cloacae* (19).

This is the first report of the isolation of the NmcA carbapenem-hydrolyzing enzyme from a clinical isolate of *E. cloacae* in North America. The importance of this finding for clinicians and laboratorians is illustrated by the following: the NmcA enzyme is able to hydrolyze carbapenems; the NmcA enzyme occurred in *E. cloacae* (a common nosocomial pathogen); the NmcA enzyme was difficult to detect; the NmcA enzyme is inducible; and the expression of NmcA is coregulated with AmpC. Accurately identifying the different mechanisms of carbapenem resistance will help determine the epidemiology, risk factors, and therapeutic options in each case. The continued emergence of novel resistance mechanisms to carbapenems worldwide reemphasizes the need not only for prudent carbapenem use but also for prudent antibiotic use in general.

Dr. Pottumarthy, a fellow of the Royal College of Pathologists of Australasia, is currently a senior fellow in laboratory medicine at the University of Washington Medical Center, Seattle. Her research interests include antimicrobial susceptibility testing, approaches, and methods; antimicrobial resistance detection; and β-lactamases.

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Address for correspondence: Thomas R. Fritsche, The JONES Group/JMI Laboratories, 345 Beaver Kreek Centre, Suite A, North Liberty, IA 52317-9258, USA; fax: 319/665-3371; email: thomas-fritsche@jmilabs.com

Fluid Intake and Decreased Risk for Hospitalization for Dengue Fever, Nicaragua

Eva Harris,* Leonel Pérez,† Christina R. Phares,* Maria de los Angeles Pérez,‡ Wendy Idiaquez,‡ Julio Rocha,§ Ricardo Cuadra,§ Emelina Hernandez,¶ Luisa Amanda Campos,† Alcides Gonzalez,† Juan Jose Amador,† and Angel Balmaseda†

In a hospital and health center–based study in Nicaragua, fluid intake during the 24 hours before being seen by a clinician was statistically associated with decreased risk for hospitalization of dengue fever patients. Similar results were obtained for children <15 years of age and older adolescents and adults in independent analyses.

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are major public health problems worldwide (1). In addition to causing considerable illness and death, dengue epidemics also have a major economic impact, attributable primarily to the cost of medical care, plus vector control programs and lost productivity (2,3). DF and DHF/DSS are indistinguishable in the initial febrile phase, and few reliable clinical prognostic indicators of DHF/DSS exist (4). Near the time of defervescence, the plasma leakage syndrome of DHF/DSS can develop suddenly and be fatal if not managed properly. As a result, in areas relatively new to dengue, such as Latin America where DF and DHF/DSS have been spreading over the last 2 decades, high hospitalization rates for DF can occur because of the fear of discharging a patient whose case may progress to DHF/DSS. As DHF/DSS becomes hyperendemic, as in Southeast Asia, and extensive experience is garnered in case management, most hospitalizations for dengue are due to frank DHF/DSS. We conducted a study to assess the extent of hospitalization attributable to dengue in Nicaragua and to examine the role of factors such as prior fluid intake on the risk for hospitalization.

*University of California, Berkeley, California, USA; †Ministerio de Salud, Managua, Nicaragua; ‡Hospital Infantil Manuel de Jesús Rivera, Managua, Nicaragua; §Hospital Escuela Oscar Danilo Rosales Argüello, Leon, Nicaragua; and ¶Centro de Salud Francisco Buitrago, Managua, Nicaragua

The Study

Participating hospitals and health centers in urban centers of the Pacific region of Nicaragua included the Hospital Escuela Oscar Danilo Rosales Argüello in León, the pediatric reference Hospital Infantil Manuel de Jesus Rivera in Managua, and four health centers in Managua, which serve a combined population of 1.95 million people. A cross-sectional study of 2,820 suspected dengue cases was conducted from January 1 to December 31, 1999. Enrollment criteria consisted of acute febrile illness accompanied by two or more of the following symptoms of DF: fever, severe headache, retroorbital pain, myalgias, arthralgias, and rash (4,5). A standardized questionnaire was used to collect demographic and clinical information, including ingestion of fluids in the 24 hours before first being seen by a clinician, as reported by the patient or parent. Of the 1,312 patients who tested positive for DENV infection, the mean age was 15.3 years (range 0–85), and 557 (42%) were male (Table 1). Venous blood was drawn at the first visit, and convalescent-phase serum specimens were obtained when possible (19% of patients). Clinical evolution of the illness among hospitalized patients was documented by chart review with standardized data-entry forms. This study was approved by the Institutional Review Boards of both University of California, Berkeley (#99-4-38) and the Centro Nacional de Diagnóstico y Referencia of the Nicaraguan Ministry of Health (#99-04).

Dengue virus (DENV) infection was identified in 1,312 patients by serologic methods (immunoglobulin M-capture enzyme-linked immunosorbent assay [ELISA] or inhibition ELISA for total anti-DENV antibodies), virologic means (virus isolation, reverse transcriptase-polymerase chain reaction), or both (5). DENV2, DENV4, and DENV3 accounted for 79%, 17%, and 5% of the typed viruses, respectively. Dengue fever was divided into classic DF and DF with hemorrhagic manifestations (DFHem); severe dengue was defined as DHF (World Health Organization classification DHF grades I and II), DSS (World Health Organization DHF grades III and IV) (1), or an additional classification designated "dengue with signs associated with shock" (DSAS). DSAS was designated when either hypotension for age or narrow pulse pressure plus clinical signs of shock were present and serial hematocrit and platelet counts failed to document thrombocytopenia and hemoconcentration, potentially attributable to fluid replacement therapy (5). The relationship between hospitalization and fluid intake was assessed by unconditional logistic regression. Because virtually every patient with severe dengue was hospitalized, such risk factor analysis was not suitable for these patients as a separate group. Thus, the analysis was restricted to DF and DFHem patients. Univariate and multivariate analyses were performed separately on children (<15 years of age) and older

Table 1. Characteristics of all hospitalized and unhospitalized dengue patients

	Hospitalized cases		Unhospitalized cases		Total	
Data	n	Mean (sd) or % of group	n	Mean (sd) or % of group	n	Mean (sd) or % of group
Demographic data						
Age (y)	478	9.4 (8.9)	834	18.7 (15.5)	1,312	15.3 (14.2)
Male	229	47.8%	328	39.2%	557	42.3%
Distance (km) to health facility	464	10.0 (25.0)	831	2.7 (6.1)	1,295	5.3 (16.1)
Disease classification[a]						
Classic DF	113	23.5%	706	84.4%	819	62.1%
DFHem	240	50.0%	129	15.4%	369	28.0%
DHF	67	14.0%	0	0%	67	5.1%
DSS	16	3.3%	1	0.1%	17	1.3%
DSAS	30	6.3%	0	0%	30	2.3%
No classification	14	2.9%	1	0.1%	15	1.1%
Duration of hospitalization (days)[b]						
Classic DF	52	5.7 (1.7)				
DFHem	95	5.1 (1.7)				
DHF	56	6.1[c] (1.5)				
DSS	15	6.9[c,d] (1.9)				
DSAS	28	6.1[c] (1.8)				
No classification	11	6.2 (1.9)				
Clinical data at presentation						
No. of glasses of fluid ingested during previous 24 h[e]	331	2.9 (2.3)	757	5.6 (3.9)	1,088	4.8 (3.7)
Thrombocytopenia	289	60.2%	58	6.9%	347	26.4%
Anorexia	239	50.9%	422	53.3 %	661	52.4%
Stomach pain	271	58.4%	404	51.5%	675	54.4%
Days since onset of symptoms	476	5.5 (5.6)	826	5.2 (5.7)	1,302	5.3 (5.7)

[a]DF, dengue fever; DFHem, DF with hemorrhagic manifestations; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; DSAS, dengue with signs associated with shock.

[b]Information on the duration of hospitalization was available from 52 (46%) of hospitalized classic DF patients; 95 (40%) of hospitalized DFHem patients; 56 (84%) of hospitalized DHF patients; 15 (94%) of hospitalized DSS patients; and 28 (93%) of hospitalized DSAS patients. Outliers (≥12 days) were removed before analysis.

[c]The mean duration of hospitalization was significantly longer for DHF cases, DSS cases, and DSAS cases as compared with duration for DFHem cases ($p<0.05$ for each, Student t test).

[d]The mean duration of hospitalization was significantly longer for DSS cases compared with duration for classic DF cases ($p<0.05$, Student t test).

[e]The average glass contains approximately 8 oz.

adolescents and adults (≥15 years of age). Covariates found to be significant in univariate analyses were included in the initial multivariate models (Table 2).

Although a much higher proportion of severe dengue patients were hospitalized compared with DF and DFHem patients, larger absolute numbers of hospitalizations were due to classic DF and DFHem because many more cases of DF/DFHem than of severe dengue occur (Table 1). Thus, of all the laboratory-confirmed DENV-positive hospitalized patients, classic DF (23.5%) and DFHem (50.0%) cases comprised three times as many as DHF, DSS, and DSAS combined (23.6%). These profiles have been maintained from year to year, with 28% and 20% of hospitalized cases due to classic DF and 51% and 58% due to DFHem in 1998 and 2000, respectively (5). Furthermore, the mean duration of hospitalization was similar for patients with DF and those with severe dengue (Table 1).

Conclusions

Among children with DF or DFHem, ingestion of fluid in the 24 hours before being seen by a clinician was found to be protective against hospitalization after adjusting for distance from health facility, date of symptom onset, and thrombocytopenia (odds ratio [OR]=0.74 per each additional glass consumed, 95% confidence intervals [CI] 0.66 to 0.83, $p<0.01$) (Table 2). Similar results were obtained for older adolescents and adults after adjusting for date of symptom onset and thrombocytopenia, with an OR of 0.71 (95% CI 0.59 to 0.85, $p<0.01$). In analyses with a statistical model that did not assume a linear relationship between number of glasses ingested and hospitalization, additional benefit was noted for each glass up to five, after which the incremental benefit remained constant. Thus, in a model that compared the fluid intake of five glasses or less than five glasses with ingestion of more than five glasses, the adjusted OR for hospitalization was 0.19 (95% CI 0.09 to 0.39, $p<0.01$) among DF and DFHem cases in children and 0.20 (95% CI 0.07 to 0.57, $p<0.01$) in those ≥15 years of age (Table 2). We also performed the analysis with all disease states, including severe dengue cases, and the results were virtually identical to those obtained with only DF/DFHem cases (data not shown).

Table 2. Crude and adjusted odds ratios and 95% confidence intervals for factors potentially associated with hospitalization for classic dengue fever or dengue fever with hemorrhagic manifestations

	Children (<15 years of age)[a]			Older adolescents and adults (≥15 years of age)[a]		
Characteristic	No. of patients[b]	OR (95% CI)[c] Crude	OR (95% CI)[c] Adjusted[d]	No. of patients[b]	OR (95% CI)[c] Crude	OR (95% CI)[c] Adjusted[f]
Fluid intake during 24-h period before presentation	587			405		
For each additional glass		0.68 (0.62 to 0.75)	0.74 (0.66 to 0.83)		0.67 (0.5 to 0.79)	0.71 (0.59 to 0.85)
>5 glasses		0.14 (0.08 to 0.25)	0.19 (0.09 to 0.39)[e]		0.16 (0.06 to 0.43)	0.20 (0.07 to 0.57)[g]
Age	719		—[h]	464		—
For each additional year		0.93 (0.8 to 0.97)			0.98 (0.96 to 1.00)	
Sex	718			464		
Male	395	1.43 (1.06 to 1.94)		291	1.27 (0.74 to 2.17)	
Female	323			173		
Distance from healthcare facility	701			460		
For each additional 5 km		2.13 (1.68 to 2.69)	1.46 (1.12 to 1.91)		1.16 (0.92 to 1.46)	—
Date of onset of symptoms	709			455		
For each additional month		1.26 (1.16 to 1.37)	1.51 (1.26 to 1.81)		1.87 (1.53 to 2.29)	2.08 (1.53 to 2.83)
Days between onset of symptoms and being seen at facility	713			457		
For each additional day		1.04 (1.0 to 1.07)	—		0.98 (0.93 to 1.03)	—
Thrombocytopenia	499			227		
Yes	189	6.5 (4.25 to 9.96)	6.16 (3.57 to 10.64)	33	3.31 (1.53 to 7.15)	3.62 (1.24 to 10.52)
No	310			194		
Stomach pain	681			439		
Yes	370	0.94 (0.69 to 1.28)	—	216	1.50 (0.89 to 2.56)	—
No	311			223		

[a]The age distribution of children <15 years of age was 7.2 (SD 3.9) with a range from 0 to 14 years and that of older adolescents and adults was 30.6 (SD 13.9) with a range of 15 to 85 years.
[b]The numbers differ based on completeness of information for each variable.
[c]OR, odds ratios; CI, 95% confidence intervals.
[d]Adjusted for glasses of liquid consumed (continuous variable), distance from healthcare facility, date of onset of symptoms, and thrombocytopenia.
[e]The adjusted OR and 95% CI for glasses of liquid consumed (dichotomous variable) were obtained from a separate model that adjusted for the same factors as footnote d.
[f]Adjusted for glasses of liquid consumed (continuous variable), date of onset of symptoms, and thrombocytopenia.
[g]The adjusted OR and 95% CI for glasses of liquid consumed (dichotomous variable) were obtained from a separate model that adjusted for the same factors as footnote f.
[h] Dash indicates that this variable did not significantly contribute to the multivariate model.

The most common liquid ingested was water (70%), followed by fruit juice (42%), lemonade (27%), milk (25%), coffee (14%), oral dehydration serum (6%), and tea (2%). The mean number of glasses ingested by nonhospitalized DF/DFHem case-patients was 5.2, whereas the mean number glasses ingested by hospitalized DF/DFHem patients was 2.8, similar to the mean of 2.9 glasses ingested by hospitalized severe patients (DHF/DSS/DSAS). These findings suggest that maintaining hydration may lead to reduced hospitalizations of patients with DF/DFHem. Other independent risk factors for hospitalization included in the final multivariate model were increasing distance from the healthcare facility, later date of symptom onset, and the presence of thrombocytopenia. The risk conferred by later date of symptom onset reflects increased awareness of dengue by medical staff as the annual epidemic intensified. Because thrombocytopenia is an indication for hospitalization (especially for pediatric dengue patients), its emergence as a risk factor is not surprising.

Because inpatient medical care of DF and DFHem patients can contribute significantly to the economic impact of dengue, we sought to define outpatient measures that could decrease DF hospitalization rates. Dengue patients are likely to be susceptible to dehydration because of high fever and concomitant anorexia. While oral rehydration therapy is listed as standard outpatient management for DHF (1), little discussion exists on the use of such therapy for DF and DFHem cases (4), and no published studies have examined the effect of fluid intake at home. Our results show that this simple measure may have a significant protective effect against hospitalization and potentially on the severity of DF/DFHem, although causality cannot be confirmed because of the observational nature of this study. To definitively demonstrate this effect, this question must be investigated by prospective intervention studies. However, our data do suggest that promoting a high fluid intake at home could help reduce the need for hospitalization and thus attenuate economic impact of dengue in countries experiencing epidemics of dengue fever.

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Dr. Harris is an assistant professor in the Division of Infectious Diseases in the School of Public Health at the University of California, Berkeley, where her research program focuses on the pathogenesis and epidemiology of dengue virus. Over the past 8 years, she has collaborated with scientists at the Nicaraguan Ministry of Health to strengthen dengue surveillance and investigate the epidemiology of dengue in Nicaragua.

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Address for correspondence: Eva Harris, Division of Infectious Diseases, School of Public Health, University of California, Berkeley, 140 Warren Hall, Berkeley, CA 94720-6350, USA; Fax: (510) 642-6350; email: eharris@socrates.berkeley.edu

From Pig to Pacifier: Chitterling-Associated Yersiniosis Outbreak among Black Infants

Timothy F. Jones,* Steven C. Buckingham,†‡ Cheryl A. Bopp,§ Efrain Ribot,§ and William Schaffner¶

In this case-control study of *Yersinia enterocolitica* infections among black infants, chitterling preparation was significantly associated with illness (p<0.001). Of 13 samples of chitterlings tested, 2 were positive for *Yersinia intermedia* and 5 for *Salmonella*. Decontamination of chitterlings before sale with methods such as irradiation should be strongly considered.

Yersinia enterocolitica is an uncommon cause of illness outbreaks in the United States. Of 7,390 foodborne disease outbreaks reported to the Centers for Disease Control and Prevention (CDC) from 1990 through 1999, 5 (<0.1%) were reported to be caused by *Yersinia* (1). It is also a relatively uncommon cause of sporadic disease, accounting for <0.3% of all foodborne illness in the United States (2). *Y. enterocolitica* has been isolated from a variety of animal reservoirs, and outbreaks have been attributed to contaminated water, milk, bean sprouts, and pork intestines (3). We investigated an outbreak of gastrointestinal illness in black infants in Tennessee.

The Study

Case-patients were defined as residents of Tennessee <1 year of age with culture-confirmed *Y. enterocolitica* infection occurring from November 15, 2001 to February 15, 2002. A case-control study was performed to define risk factors for infection. Controls were randomly selected from a list of black patients <1 year of age who were evaluated in the emergency department of the large urban children's hospital where the outbreak was initially identified, with any diagnoses other than *Yersinia* gastroenteritis during the outbreak period. A structured questionnaire was administered to patients and controls by telephone. Both patients and controls were asked about exposures from November 1, 2001 to January 31, 2002.

Isolates of *Y. enterocolitica* from cases were confirmed, biotyped, and serotyped at CDC (4). Isolates were also tested for several biochemical markers of pathogenicity (5). All available isolates were subtyped by pulsed-field gel electrophoresis (PFGE) after restriction of the genomic DNA with *Bln*1 or *Not*1 (6). Samples of chitterlings purchased 2 months after the outbreak from grocery stores in two large urban areas of the state, including the city where most case-patients lived, were cultured for *Yersinia* and *Salmonella* (4).

Twelve cases of *Yersinia* infection in infants <1 year of age were identified in Tennessee with onset from November 15, 2001 to February 15, 2002 (Figure 1). All cases were identified by stool culture. Six cases occurred in December, and 10 were medically evaluated in the same city. All case-patients were black. In comparison, 49% of the population of the urban county in which the outbreak was identified is black.

Ten patients or caregivers and 51 controls were interviewed in the case-control study. Two infants with *Y. enterocolitica* infection could not be located. Of the 10 cases interviewed, 8 had received medical care in the same city, and 7 of those were seen at the same pediatric medical center. Median age of cases was 165 days (range 46–275 days), and median age of controls was 181 days. Four (40%) patients were hospitalized for their illness for a mean of 4 days. All patients had diarrhea, 7 (70%) reported bloody stools, 7 (70%) had vomiting, and 8 (80%) reported fever. Case-patients did not share apparent common exposures, such as childcare, social gatherings, grocery stores, or foods.

Chitterlings had been prepared in the homes of all case-patients from November 1, 2001, to January 31, 2002, compared with 35% of controls (p<0.001). The specific brand of chitterlings purchased could not be identified by

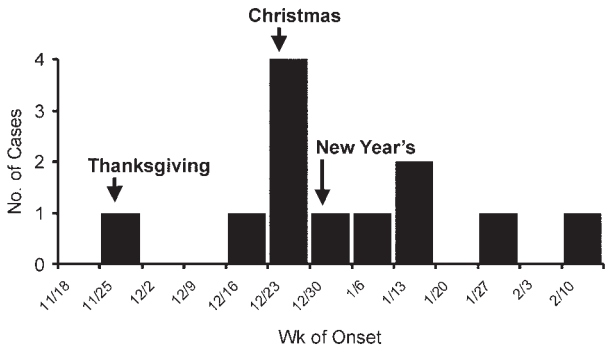

Figure 1. Epidemiologic curve of *Yersinia enterocolitica* outbreak in Tennessee.

*Tennessee Department of Health, Nashville, Tennessee, USA; †University of Tennessee Health Science Center, Memphis, Tennessee, USA; ‡Le Bonheur Children's Medical Center, Memphis, Tennessee, USA; §Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and ¶Vanderbilt University School of Medicine, Nashville, Tennessee, USA

50% of infants' caretakers. At least four different brands of chitterlings were purchased from at least five different grocery store chains by the families. The median time from preparation of chitterlings to onset of symptoms was 4 days (range 0–43). From 10 to 80 pounds of chitterlings were prepared at a time; chitterlings were thawed and cleaned in the kitchen sink over several hours. Parents of seven case-patients acknowledged exposures that could have led to infection of the infant. These exposures included the infant roaming freely in a walker in the room where chitterlings were being cleaned, chitterling juices splashing on clean dishes or a baby's bottle, washing bottles in a sink that was not thoroughly cleaned, and feeding or handing a pacifier to an infant during cleaning or preparation of chitterlings.

Nine *Yersinia* isolates from stool specimens of ill persons were available for further testing; all were identified as serotype O:3, biotype 4. Ten clinical isolates from nine case-patients were available for PFGE testing. Seven similar but distinct PFGE patterns differing by at least one band were noted (Figure 2). Three infants shared an indistinguishable pattern, and two other infants had another indistinguishable pattern. One case-patient had two isolates with distinct PFGE patterns. Of 13 samples of frozen chitterlings purchased in Tennessee in February, 2 were positive for *Y. intermedia*; 5 were positive for *Salmonella* (2 contained *S. Derby*, 1 *S.* Minnesota, and 2 *S.* Typhimurium var. Copenhagen).

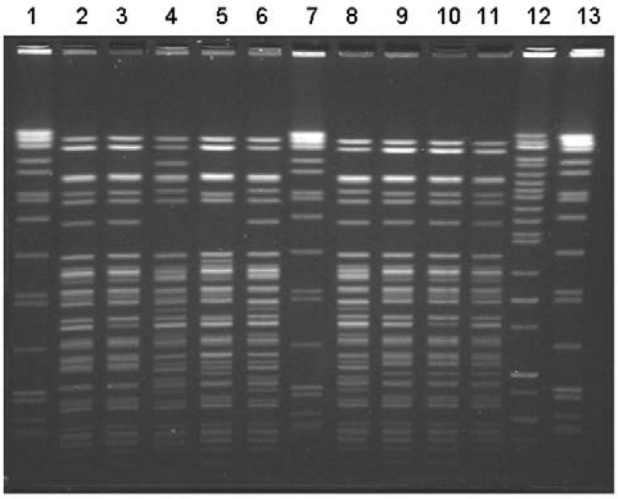

Figure 2. Pulsed-field gel electrophoresis (PFGE) patterns of *Yersinia enterocolitica* isolates from patients in this outbreak associated with chitterlings. Ten isolates from nine patients were available for typing. Seven distinct BlnI PFGE patterns were noted. Three infants had pattern #1 (lanes 9–11). Two infants shared pattern #2 (lanes 2 and 3). One patient had two distinct isolates (lanes 6 and 12). The molecular size standard is located in lanes 1, 7, and 13.

Conclusions

This outbreak of *Y. enterocolitica* infections affected black infants <1 year of age. Exposure to the preparation of chitterlings was a substantial risk factor for disease, though none of the infants directly ate chitterlings. No particular brand of chitterlings or specific preparation practice was implicated as the cause. The traditional holiday preparation of chitterlings involves a lengthy and messy process of cleaning and cooking raw pork intestines that may be fecally contaminated. Attempts to educate the public and change traditional methods of preparation (7,8) have been unsuccessful in preventing chitterling-associated outbreaks, and vulnerable "innocent bystanders" continue to be affected by the disease. Measures to eliminate contamination of chitterlings before sale should be developed to prevent disease among this high-risk population.

In many countries, *Y. enterocolitica* is a common cause of acute bacterial gastroenteritis, rivaling *Campylobacter* and *Shigella* in frequency (9). In the United States, *Y. enterocolitica* has been an uncommonly reported pathogen, although in recent years it has emerged as an occasional cause of sporadic illness and foodborne disease outbreaks. Outbreaks have been associated with water, contaminated milk, bean sprouts, tofu, and chitterlings (8–10). During a 7-year period at a hospital in Michigan, *Yersinia* accounted for 12.6% of bacterial intestinal pathogens isolated, with a rate comparable to *Campylobacter*. In that study, 99% of patients with *Yersinia* were black and 85% were infants; most illnesses occurred between November and January (11). At a large hospital in Georgia, *Y. enterocolitica* was isolated from 1% of rectal swabs submitted for culture. Among infants, *Y. enterocolitica* was second in frequency only to *Salmonella*, with a predominance in winter months (12).

Active laboratory-based surveillance for *Y. enterocolitica* infections in five sites in the United States demonstrated that the incidence in infants <1 year of age was more than 40-fold higher than the incidence in older age groups (SM Ray et. al, unpub. data). Furthermore, the incidence of *Yersinia* infection in blacks was more than seven times the incidence rate in non-blacks. A seasonal variation in incidence, with a marked peak in December, was noted only among blacks.

Since the 1980s, serogroup O:3 has replaced O:8 as the predominant serotype of *Y. enterocolitica* reported to CDC (10). Swine are the major reservoir of this serogroup (3,13), and the emergence of *Y. enterocolitica* infections in the United States has been attributed to the establishment of a widely distributed swine reservoir here (8).

This outbreak did not appear to be caused by a single brand or distributor of chitterlings. No apparent epidemiologic links existed between cases. The hospital at which the outbreak was recognized is the primary hospital for

children in the area. In many black households, chitterlings are prepared annually by traditional methods. We were unable to identify any recent change in stool-testing procedures, products, or handling that would have explained an outbreak this year, in comparison with prior years, when similar methods were presumably used. The outbreak was recognized during active surveillance for foodborne pathogens at a hospital laboratory, as part of a program begun approximately 2 years ago. Earlier seasonal increases of *Y. enterocolitica* in this population may have gone unrecognized or unreported.

Chitterlings, prepared by boiling the large intestines of pigs after the removal of fat and fecal material, are a traditional winter-holiday food in many black families. In this outbreak, all affected persons were black infants. While none of the infants ate chitterlings, all were potentially exposed in homes where cleaning and preparation of chitterlings occurred. Because chitterlings are traditionally thoroughly boiled, the final cooked product is likely not to be bacterially contaminated. The preparation process, however, involves substantial handling of large amounts of potentially contaminated product, and the risk for exposure of infants to this process is high. This outbreak underscores the importance of rigid adherence to strict hygiene measures during handling of potentially contaminated foods. While we isolated a nonpathogenic *Yersinia* species from chitterling samples, this and other studies (7,10) demonstrate that chitterlings are not infrequently contaminated with enteric bacteria when purchased. Unfortunately, attempts to disseminate education regarding the safe preparation of chitterlings (7,8,14) have not prevented continued outbreaks among this high-risk population.

Irradiation of ground beef and other products can markedly reduce contamination with bacterial pathogens and improve food safety (15). Additional research is necessary to better understand barriers to acceptance of food irradiation among consumers and producers. Chitterlings are often purchased frozen in large containers and may be amenable to irradiation before sale. Irradiation should be studied as a potential means of preventing recurrent outbreaks among a vulnerable population of infants.

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Dr. Jones is deputy state epidemiologist at the Tennessee Department of Health and director of the Tennessee FoodNet Program. His research interests include foodborne disease epidemiology and the investigation of communicable disease outbreaks.

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Address for correspondence: Timothy Jones, Communicable and Environmental Disease Services, Tennessee Department of Health, 4th Floor, Cordell Hull Bldg., 425 5th Ave. N., Nashville, TN 37247, USA; fax: (615) 741-3857; email: tim.f.jones@state.tn.us

Acute Hemorrhagic Conjunctivitis Caused by Coxsackievirus A24 Variant, South Korea, 2002

Myoung-don Oh,* Sangwon Park,* Youngju Choi,* Hongbin Kim,* Kiduk Lee,* Wanbum Park,* Youngae Yoo,† Eui-Chong Kim,*‡ and Kangwon Choe*‡

In summer 2002, a nationwide outbreak of acute hemorrhagic conjunctivitis occurred in South Korea. The etiologic agent was confirmed as coxsackievirus A24 variant (CA24v) by virus isolation and sequencing of a part of the VP1 gene. Phylogentic analysis, based on the protease 3C sequences, showed that the Korean isolates were clustered into a lineage distinct from the CA24v isolates reported in previous outbreaks in Asia.

Acute hemorrhagic conjunctivitis (AHC) is characterized by sudden onset of painful, swollen, red eyes with subconjunctival hemorrhages and excessive tearing. Most cases are self-limited but highly contagious, with the potential for causing considerable illness. Adenoviruses and picornaviruses can cause AHC outbreaks (1). Among picornaviruses, enterovirus 70 and coxsackievirus A24 variant (CA24v) have caused large outbreaks of AHC (2).

During the summer of 2002, a nationwide outbreak of AHC occurred in South Korea, which affected more than one million people. The epidemic started in late August and reached its peak in mid-September, when 1,100 schools were closed. The epidemic ended in early October. We report the investigation into the viral etiology of this outbreak.

The Study

Conjunctival swabs were taken from AHC patients at a community-based ophthalmology clinic in Seoul on September 3–16, 2002. In the initial stages of the epidemic, attempts were made to determine whether the causative agent belonged to the adenovirus or picornavirus family. To attempt to answer this question, we performed a polymerase chain reaction (PCR) assay on conjunctival specimens without pretreatment.

Conjunctival specimens from 10 AHC patients were subjected to nested PCR that used primers to common adenoviruses (AD1F, AD2F, AD2R, and AD1R) (3). The specimens were also subjected to reverse transcription (RT)–PCR with primers common to enteroviruses (EV1, EV2) (4). Of the 10 specimens, 2 yielded a 114-bp amplification product, consistent with enteroviruses. To determine if this amplification product belonged to enterovirus 70 or CA24v, PCR was performed with primers specific to each virus (5), which yielded a 171-bp amplification product, consistent with CA 24v. From these preliminary results, we tried to isolate the virus.

The conjunctival specimens were added into 24-well culture plates, containing HeLa cells, and incubated at 37°C. The cells were observed daily for cytopathic effects and were harvested when the cytopathic effects involved >80% of the cells. To confirm that the isolates belonged to picornaviruses, RT-PCR was carried out with PCR primers that would anneal at conserved sites in the 5′ nontranslated region of all the enteroviruses (including coxsackieviruses) (4). The serotypes of the isolates were determined by partial sequencing of the VP1 region, as described previously (4). In brief, viral RNA was extracted from an infected cell culture supernatant, by using the QIAamp Viral RNA kit (QIAGEN, Inc., Valencia, CA). The VP1 region was amplified by RT-PCR with the 188/222 primer pairs, and the nucleotide sequence was determined by the cycle-sequencing and dye terminator methods, using an automated DNA sequencer (MJ Research Gradient Cycler, Applied Biosystems, Inc., Foster City, CA). The serotype was determined by comparing the sequence of the VP1 amplicon with those contained within the database of human enterovirus and picornavirus sequences available in GenBank.

For the molecular epidemiologic study, the protease 3C region of CA24v was amplified by PCR with the D1/D2 primer pairs and sequenced as described previously (6). The nucleotide sequences of this region of the 14 Korean isolates from this study were compared with those of the strains reported from the Asian countries where previous outbreaks had occurred (6–8). A phylogenetic tree was constructed by the unweighted pairwise grouping method of the arithmetic average.

Conjunctival swabs were taken from 88 AHC patients. Clinical features of the patients were consistent with the findings reported from previous outbreaks. All the patients recovered without sequelae.

Of the 88 specimens added into the HeLa cell culture, 39 (44%) showed extensive cytopathic effect on the HeLa cells. The viral RNA was extracted from the infected cell culture supernatants, and the VP1-specific fragment was

*Seoul National University College of Medicine, Seoul, Republic of Korea; †Hamchun Eye Clinic, Seoul, Republic of Korea; and ‡Seoul National University Hospital, Seoul, Republic of Korea

amplified by RT-PCR with the 188/222 primer pairs. All 39 isolates were positive for the VP1 fragment, consistent with enterovirus. Nine of the 39 isolates were randomly selected for PCR, performed with the S3/AS3 primer pairs, which are specific to CA24v. All nine isolates yielded the 171-bp amplification product, consistent with CA24v.

The nucleotide sequences of the protease 3C region among the 14 Korean isolates of CA24v were 98.1% to 100% homologous (data not shown). A phylogenetic analysis showed that the Korean isolates were closely related and clustered to a lineage distinct from those isolates reported from the previous outbreaks in other Asian countries which had been attributed to CA24v (Figure).

The 14 sequences reported here were deposited in the GenBank sequence database under the accession nos. AY216777 to AY216790. We also sequenced capsid protein VP1 region of CA24v isolates from two of our patients. GenBank accession nos. of these sequences are AF545847–AF545848.

Conclusions

Our study demonstrated that CA24v was the causative agent of the South Korean outbreak of AHC in the summer of 2002. During this outbreak, CA24v was also isolated from patients with AHC in Busan, the second largest city in South Korea, and other provinces (9). During the last 3 decades, several large outbreaks of AHC have occurred in South Korea. However, in the 1970s, the causative agents of the outbreaks were rarely determined. Ishii et al. tried to isolate the virus from patients with acute conjunctivitis in Busan between April and August 1983. Of 123 patients with acute conjunctivitis, 74 (60.2%) were infected with adenoviruses (type 8 was the most prevalent, with 57 cases) and 5 (4.0%) with enterovirus 70 (10). Kim also reported the isolation of adenoviruses and enterovirus 70 from patients with acute conjunctivitis from Seoul between 1987 and 1990 (11). In 2002, CA24v was determined to be the cause of an AHC outbreak in South Korea for the first time.

An outbreak of AHC attributable to CA24v was first recognized in Singapore in 1970; outbreaks have occurred periodically there since (12,13). CA24v had not been reported outside of Southeast Asia and the Indian subcontinent until 1986, when it appeared in the Western Hemisphere in an outbreak in the Caribbean (14). A phylogenetic analysis, based on the proteinase 3C region of the CA24v isolates collected from the Eastern Hemisphere in 1970 through 1989, demonstrated that most of the lineages of the tree contained Singapore strains (6). Based on these findings, Lin et al. suggested that CA24v has been circulating endemically in Singapore and the surrounding areas and has occasionally spread to other countries (8). Whether CA24v was newly introduced or had already been introduced and was circulating in South Korea before the AHC outbreak in 2002 is not known. In September 2002, an outbreak of AHC was reported in southern China (15). However, further information on the cause of that outbreak is not available.

Although using sequences from the capsid regions of enteroviruses for molecular epidemiologic studies would have been more informative (because of discontinuities from recombination outside the capsid region), we used 3C regions for the phylogenetic analysis because sequence data for capsid regions of the CA24v isolates from previous outbreaks in Asian countries were not available (16). The nucleotide sequence homologies of the protease 3C region among the 14 Korean isolates of CA24v were high (98.1% to 100%), consistent with a common source outbreak. The phylogenetic analysis showed that the Korean isolates of CA24v clustered together into a distinct lineage

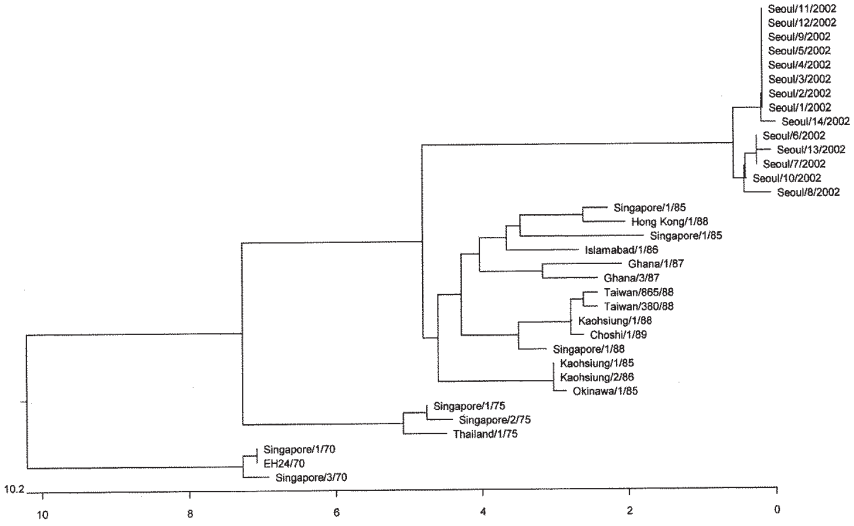

Figure. Phylogenetic analyses of the 14 isolates of coxsackievirus A24 variant (CA24v) from the South Korean outbreak of acute hemorrhagic conjunctivitis, summer 2002. The entire protease 3C region (549 nucleotides) of CA24v was amplified by polymerase chain reaction and sequenced. The nucleotide sequences of this region of the 14 Korean isolates were compared with those of the strains reported from other Asian countries in previous outbreaks. The phylogenetic tree was constructed by the unweighted pairwise grouping method of the arithmetic average. (GenBank accession nos. for the 14 Korean isolates are AY216777–AY216790.)

(Figure). Our data suggests that CA24v evolved from the previous Asian isolates and caused the South Korean outbreak of AHC during the summer of 2002.

Dr. Oh is an associate professor in the Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea. His major research interests include *Staphylococcus aureus*, HIV, and opportunistic infections in immunocompromised hosts.

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Address for correspondence: Eui-Chong Kim, Department of Laboratory Medicine, Seoul National University College of Medicine, 28 Yeongun-dong, Chongro-gu, Seoul, 110-744, Republic of Korea; fax: 82-2-764-3698; email: euichong@plaza.snu.ac.kr

Nonhemolytic, Nonmotile Gram-Positive Rods Indicative of *Bacillus anthracis*

Elie G. Dib,* Samar A. Dib,* Dany A. Korkmaz,* Neville K. Mobarakai,* and Jordan B. Glaser*

We report a 40-year-old female patient who was admitted to the hospital because of a left ovarian mass torsion. A nonhemolytic, nonmotile *Bacillus*, suspicious of *Bacillus anthracis*, was isolated from a blood culture. We discuss the evaluation that led to the final identification of the bacterium as *B. megaterium*.

Bacillus represents a genus of ubiquitous gram-positive bacteria. The species are used in many medical, pharmaceutical, agricultural, and industrial processes, including those for making antibiotics and insecticides (1–4). Even the anthrax toxin is being evaluated as a choice for tumor cell surface targeting in chemoresistant neoplasms (5).

Although most species are harmless, two are medically significant: *Bacillus anthracis* and *B. cereus*. *B. anthracis* causes anthrax in its cutaneous, pulmonary (inhalational), and intestinal forms. *B. cereus* causes two distinct food poisoning syndromes, a rapid-onset emetic syndrome characterized by nausea and vomiting and a slower onset diarrheal syndrome.

Bacillus are often isolated on blood culture and usually represent blood culture contamination. For example, *Bacillus* species pseudobacteremia has been traced to contaminated gloves used in collection of blood from patients (6). In immunocompromised hosts, a blood culture growing *Bacillus* species should be evaluated carefully. Rarely, these species cause important clinical diseases such as bacteremia, sepsis, meningitis, pneumonia, empyema, ophthalmitis, osteomyelitis, endocarditis, soft tissue infection, and intravascular catheter-acquired sepsis.

Pseudotumour of the lung has been reported as the cause of infection with *B. sphaericus* (7). Endocarditis has been reported to be caused by *B. subtilis* (8). An outbreak of *Bacillus* species in a cancer hospital in Brazil was reported (9) and was strongly associated with use of calcium gluconate solution and central venous lines. The outbreak was controlled by stopping use of the implicated calcium gluconate vials.

*Staten Island University Hospital, Staten Island, New York, USA

Case Study

A 40-year-old woman, with no significant medical history, was seen at the emergency room because of worsening left lower quadrant abdominal pain. The pain, which had started a few days previously, was constant, localized to the left lower quadrant of the abdomen, and described as dull and moderately to severely intense. The pain was not related to meals or bowel movements and was not accentuated or relieved by any specific position. The patient noted constipation but had no nausea or vomiting. No rectal bleed or melena occurred, and she reported no urinary symptoms or vaginal discharge. Her last normal menstrual period was 8 days before. She felt warm but did not check her temperature and did not experience chills. Her primary medical physician prescribed ciprofloxacin 500 mg orally twice a day for the presumptive diagnosis of colitis. She took the antibiotic for 2 days without improvement.

In the emergency room, the patient was afebrile and hemodynamically stable. The physical examination showed tenderness on palpation of the left lower quadrant of the abdomen with minimal rebound tenderness. The pelvic examination showed left adnexal tenderness with a possible mass. Results of urinalysis and a urine pregnancy test were negative. No leukocytosis was noted. A pelvic ultrasonograph showed a left ovarian complex mass measuring 14 cm x 9 cm x 6 cm as well as a moderate amount of free fluid in the cul-de-sac. The study suggested left ovarian mass torsion. A laparascopic resection was performed successfully. The patient received intravenous cefazolin perioperatively. The final pathology report showed a mature teratoma of the left ovary featuring dermoid cyst, respiratory anlaga, and struma ovarii. The patient improved and was discharged 2 days after surgery.

One day later, a blood culture, drawn in the emergency room, grew nonhemolytic, nonmotile gram-positive rods. *B. anthracis* was suspected. The blood culture was reported to the New York City Department of Health. The patient was called for reassessment at the hospital. She was afebrile, and her only complaint was mild low back pain. She had mild dry cough, but results of a chest roentgenogram were unremarkable. She was started on intravenous clindamycin, ciprofloxacin, and rifampin. Two days later, the New York City Department of Health reported the following: results of the direct fluorescent-antibody (DFA) assay, using fluorescein-labeled monoclonal antibodies specific to the *B. anthracis* capsule (CAP-DFA) antigens, were positive; results of the DFA assay, using fluorescein-labeled monoclonal antibodies specific to the *B. anthracis* cell wall (CW-DFA) were negative; and the *Bacillus* isolated was not lysed by the γphage.

The organism was confirmed to be *Bacillus* non-*anthracis*. Based on its characteristics, it was classified as

B. megaterium. The information was disclosed to the patient, and the intravenous antibiotic therapy was discontinued. The patient's initial dry cough had resolved, and she had no evidence of any cutaneous, respiratory, or neurologic sign of disease. She was counseled about any potential side effects of the antibiotic therapy she had received, otherwise reassured, and then discharged.

Conclusions

Presumptive identification of *B. anthracis* in a hospital laboratory is based on the direct Gram-stained smear of a skin lesion, cerebrospinal fluid, or blood showing encapsulated, broad, gram-positive bacilli. Indicators of growth apparent on cultures are also factors. *B. anthracis* is nonmotile and nonhemolytic on sheep's-blood agar. In vitro it grows as long chains, but in the host *B. anthracis* appears as single organisms or chains of two or three bacilli. The organism forms mucoid colonies and exhibits a prominent capsule when grown on nutrient agar containing 0.7% sodium bicarbonate in the presence of 5% to 20% carbon dioxide (10).

The only nonmotile *Bacillus* are *B. anthracis* and *B. cereus* subsp. *mycoides*. Some other *Bacillus* species show variable motility and may often be nonmotile. These species include *B. megaterium*, *B. firmus*, and *B. circulans*. At the community laboratory level, once the *Bacillus* colonies are identified as catalase-positive, nonhemolytic, nonmotile gram-positive rods, the organism should be packaged properly and transported to a state or county public health laboratory for confirmation (11).

Confirmatory diagnostic tests are performed at the Laboratory Response Network for Bioterrorism (LRN) (11), which consists of laboratories at four levels (12–14). Laboratories at the community level, considered level A, should recognize the clues to a suspicious agent and package the agent for transfer to the next higher level laboratory.

Level-B laboratories often include the state and county public health laboratories. Criteria for confirming *B. anthracis* at this level include susceptibility to lysis by γphage and a two-component DFA assay, using cell wall (CW-DFA) and capsule (CAP-DFA) antigens (11). The two-component DFA assay is a sensitive, specific, and rapid confirmatory test for *B. anthracis* in cultures (15,16). The lysis by γ phage (17) is highly specific for *B. anthracis,* and when demonstrated concomitantly with the presence of a capsule, confirms the identification. The New York City Department of Health protocol reports a sample as positive only if it has all the following phenotypes: nonmotile, penicillin sensitive, γ-phage positive, and positive by both cell wall and CAP-DFA assays (11).

The level-C laboratory has the capacity of the level-B laboratory, plus antimicrobial susceptibility testing and advanced detection methods. It also can help with surge capacity and has much greater biosafety-level working capacity. Advanced detection methods include time-resolved fluorescence and polymerase chain reaction (PCR) (14,18). These methods are employed to quickly yield preliminary data in advance of the classical microbiology final report (11).

The level-D laboratory has the highest level of containment (biosafety level) and expertise in diagnosis. Various tests to determine the molecular characteristics of isolates are conducted, including molecular subtyping with multilocus variable-number tandem repeat analysis and sequencing of genes coding for 16S ribosomal RNA (19,20). The analysis allows for identification of a particular pattern that can be associated with geographic, temporal, or other relevant epidemiologic designations. The Centers for Disease Control and the U. S. Army Medical Research Institute of Infectious Diseases maintain level-D laboratories.

Once the *Bacillus* colonies from our patient were identified as catalase positive, nonhemolytic, nonmotile gram-positive rods, the organism was transported to the New York City Department of Health laboratory for further testing, as mandated by LRN. Although the patient's symptoms did not correlate with classic anthrax disease, a fatal case of inhalational anthrax mimicking intraabdominal sepsis had been recently reported (21). The organism isolated in our patient was identified as *B. megaterium*, a frequent blood culture contaminant but rare cause of meningitis, brain abscess, and catheter-related bacteremia. The patient's strain showed a positive reaction to the CAP-DFA assay. A recent study (16) also reported one *B. megaterium* strain (out of 11 strains) with a positive reaction to the CAP-DFA assay. This study analyzed a total of 230 *B. anthracis* isolates; 228 and 229 were positive by CW-DFA and CAP-DFA assays, respectively. A total of 56 *B*. non-*anthracis* strains were also tested; 10 *B. cereus* and 2 *B. thuringiensis* were positive by the CW-DFA assay, and 1 *B. megaterium* strain was positive by CAP-DFA. Analysis of the combined DFA results identified 227 of 230 *B. anthracis* isolates; all 56 strains of the other *Bacillus* species were negative (16).

A nonhemolytic, nonmotile *Bacillus* should be highly suspicious for *B. anthracis*. However, species like *B. cereus* subsp. *mycoides, B. megaterium*, *B. firmus*, and *B. circulans* can also be nonhemolytic and nonmotile. The community laboratory is limited in differentiating these species, which can lead to delays in diagnosis and response to potential terrorist events. This case emphasizes the need for local (level A) laboratories to increase their potential to differentiate nonmotile, nonhemolytic *Bacillus* in order to secure a rapid preliminary diagnosis and avoid unnecessary costly treatment. The combined DFA assay would be

a potential solution. It provides sensitive and specific confirmation of *B. anthracis* cultures within 3 to 6 hours. The assay specificity is similar to the highest levels achieved by PCR assays, and its sensitivity is similar to that of culture or perhaps considerably greater if the patient is receiving antimicrobial agents (16).

Dr. Dib is a fellow in training in hematology/oncology at the University of Rochester-Strong Memorial Hospital in New York. His research interests include the relationship of infectious agents and carcinogenesis.

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Address for correspondence: Elie G. Dib, University of Rochester Medical Center, 601 Elmwood Avenue, Box 704, Rochester, NY 14642, USA; fax: 585-273-1051; email: Elie_Dib@urmc.rochester.edu

Eating Dirt

Gerald N. Callahan

> Please
> This earth is blessed
> Do not play in it
>
> Sign on the wall of El Santuario de Chimayo, New Mexico

This place feels old beyond human recollection. The carvings and paintings were surely done by human hands, but no one remembers whose hands those were. The work is striking, especially in the apse behind the altar. There, the colors of surrounding hills have been transferred onto nearly luminous wooden reredos full of Catholic symbolism. Above the altar hangs a most intricate ancient Christ crucified on a green cross. Overhead, the roof is held in place by massive carved wooden beams, big around as human bodies and blackened by nearly two centuries of incense and candle smoke. The air is rich with the memory of thousands of benedictions and baptisms. Threadbare trousers have polished the pews to a high varnish that this afternoon ripples with a low orange glow from dozens of votive candles burning purposefully in back of the church.

This is El Santuario de Chimayo, an old adobe-brick and stucco structure in the hills of northern New Mexico. This chapel was built in 1816, but a sanctuary has been at this site for much longer. The locals offer many legends about its origins, fanciful tales of miraculous crucifixes and Santo Niños. But the truth is buried beneath the murk of time. One thing is clear though, as beautiful as the sanctuary is and as striking as the crucifix (El Sefior de Esquipalas) above the altar is, nearly none of those in the pews today have come to see the sanctuary or the crucifix. Instead, they have come from all over the world to this place in New Mexico to eat the dirt that lies beneath the adobe floor.

According to legend, that dirt is sacred, consecrated by Christ himself. Crutches cast off by the newly healed fill the anteroom, and on some days, the line of pilgrims stretches for blocks. Some call this place the Lourdes of America, but in Chimayo the miracle can be seen each day by anyone who peers into a low-ceilinged room off the main entrance. There, a hole (the *posito*), half a meter across, pierces the floor. Beside it, someone has left a plastic spoon to aid the faithful. Beyond the spoon, beneath the opening, lies only dirt, only the deep-red dirt of Chimayo.

Most of the faithful here today have come to eat that dirt. This religious tradition is practiced, as far as I know, only at one other place—a Catholic shrine in Esquipalas, Guatemala. But pilgrims to these shrines are not the only humans who eat dirt. Nor are religious reasons the only reasons to imagine that dirt may have special powers.

Geophagy (Eating Dirt) and Its Reasons

Other than water, what little stuff we humans have inside us is largely dirt. Admittedly, this dirt is sometimes highly processed before we receive it, but most solids that make up humans and other creatures either are now or recently were dirt (the simple stuff that stripes the outer surface of our world, the thin paste that raises us above the rocks) transformed by sunlight into plants or animals. Most of us prefer the dirt we eat in the form of cows and sheep and carrots and squash and bison and sorghum. Other dirt we'd just as soon scrape from our feet and leave at the door.

But not everyone wishes to be so far removed from the stuff of mud pies and mucilage. On every continent (except, possibly, Antarctica), some of us intentionally eat dirt, and we are joined in this practice by a myriad of rats, mice, mule deer, birds, elephants, African buffalo, cattle, tapirs, pacas, and several species of primates (1). Most scientists consider animal geophagy "normal," probably because most soil consumption by animals has no obvious adverse effects and is sometimes beneficial (2); however, some of these same scientists consider most (or all) human geophagy "abnormal."

Abnormal Behavior

In the United States, many of us believe that humans should only eat food. We consider the consumption of nonfood items pathological, even though we know that what people define as "food" varies dramatically by region and ethnicity. We call the pathological act of eating nonfood items pica. Pica is a disease, but a disease different from polio or smallpox. No infectious agent is obviously associated with pica. Pica is a disease only because we believe normal "undiseased" persons would not eat anything but traditional human foods; some of those who do, some of the time, are at considerable risk because of their unusual appetites.

Pathological consumption of soil, "soil pica," is associated with several psychological abnormalities. But all ingestion of soil is not soil pica. How much soil a person has to eat to be considered ill is not known. One report

described soil pica in a developmentally disabled person who regularly consumed more than 50 g of soil per day (3). Most of us would consider that level of geophagy at least potentially pathological, although I am not sure why.

In June 2000, the U.S. Agency for Toxic Substances and Disease Registry appointed a committee to review soil pica. The committee settled on pathological levels as consumption of more than 500 mg of soil per day but conceded that the amount selected was arbitrary (3). Soil consumption is defined as pathological according to the amount eaten (no normal person could possibly eat that much dirt) and the severity of health consequences (lead poisoning, parasites). Because underlying psychological or biologic abnormalities are not easy to establish, I explore only what appears to be nonpathological dirt eating in pregnant women (especially in sub-Saharan Africa), migrants from sub-Saharan cultures to other parts of the world (notably the United States), and children worldwide.

Inadvertent Exposure

Why is it, that in spite of all the times we've been told not to, we still eat dirt? This is a very complex question with many possible answers. And while each proposed answer has its advocates, no single answer seems satisfactory to all—except one. Almost everyone agrees on one cause of geophagy, inadvertent consumption of air-, water-, and foodborne dirt. Contaminated food, soiled hands, and inhaled dust add soil to our diets. Children ingest considerable amounts of soil in these ways. My children did. Of course, my children also ate dirt on purpose. But child or adult, each of us inadvertently eats a little dirt every day. This dirt can pose a health threat, especially near sites of industrial contamination, but dirt we eat intentionally poses a greater challenge. Intention may indicate something biologic that drives some of us (sometimes regularly, sometimes religiously, sometimes ritually) to eat dirt.

Tradition and Culture

For centuries, indigenous peoples have routinely used clays (decomposed rock, silica and aluminum or magnesium salts, absorbed organic materials) in food preparation. The clays were used to remove toxins (e.g., in aboriginal acorn breads); as condiments or spices (in the Philippines, New Guinea, Costa Rica, Guatemala, the Amazon and Orinoco basins of South America); and as food during famine (4). Clays were also often used in medications (e.g., kaolin clay in Kaopectate). But the most common occasion for eating dirt in many societies (the only occasion in some societies) is pregnancy. When sperm and egg collide, the world changes. That is obvious. But why pregnant women eat dirt is not.

Wiley and Katz (5) have proposed that eating clay serves different purposes during different periods of pregnancy, soothing stomach upset during morning sickness in the first trimester and supplementing nutrients (especially calcium) during the second and third trimesters, when the fetal skeleton is forming. This type of geophagy occurs most commonly in cultures of sub-Saharan Africa and their descendants (5). The timing of dirt ingestion and amounts consumed vary with tribes and individual persons, but soil comes consistently from certain sites. In some cultures, well-established trade routes and clay traders make rural clays available for geophagy even in urban settings. Clays from termite mounds are especially popular among traded clays, perhaps because they are rich in calcium (5). Whatever the underlying reason, geophagy in Africa does not appear to be a recent cultural development; it may predate *Homo sapiens*.

Women eat dirt during the first, second, or third trimester or throughout pregnancy (5), often throughout the day, as a supplement rather than a meal. Most commonly consumed are subsurface clays, especially kaolin and montmorillonite (5), 30 g to 50 g a day (sometimes much more) (3). However, eating dirt is not always confined to pregnant women, even among the cultures of sub-Saharan Africa (4), nor is it limited to tribes with little or no access to dairy-derived calcium (5), so these hypotheses do not adequately explain local tastes for dirt.

Soil, including kaolinitic and montmorillonitic clays, contains considerable amounts of organic material, including many live microorganisms. The human gut is the largest area of direct contact between a person and the world. Gut-associated lymphoid tissue (GALT) is a major site of T-cell differentiation and selection in adults and of intense immunologic activity (including T lymphopoiesis) in children and adults (6–9). And while it is not entirely clear why some gut-introduced antigens promote tolerance of microorganisms and others immunize against them (10), it is clear that immunization via the gut is a major source of immunoglobulin (Ig)A, both locally and systemically (6–10).

Regular consumption of soil might boost the mother's secretory immune system. Monkeys that regularly eat dirt have lower parasite loads (1). In some cultures, clays are baked before they are eaten, which could boost immunity from previous exposures. For decades we have used aluminum salts, like those found in clays, as adjuvants in human and animal vaccines. Adjuvants are compounds that nonspecifically amplify immune response, probably because of their effects on innate defenses such as macrophages, dendritic cells, and the inflammatory response. Aluminum compounds make effective adjuvants because they are relatively nontoxic, the charged surfaces of aluminum salts absorb large numbers of organic molecules, and macrophages and dendritic cells readily phagocytose the particulates produced by the com-

bination of the adjuvants and the organic compounds (11). The clays that pregnant women and others consume, which are rich in aluminum compounds, likely make at least passable immunologic adjuvants. For all these reasons, clays might act as vaccines. And the IgA antibodies produced against the associated organic antigens may appear in breast milk and have a major role in mucosal protection of newborns.

In pregnant women, this type of gut immunization might produce high levels of IgA against endemic pathogens and other antigens. All this IgA would appear shortly before birth in the breast milk and would provide protection for infants against precisely the pathogens encountered immediately after birth. Furthermore, IgA antibodies prevent attachment of bacteria and some viruses at mucosal surfaces (12), the major contact between the infant and the infectious world. In humans, mucosal surfaces offer the only routes of natural immunization short of wounding, and dirt would seem to offer a potent vaccine containing many endemic pathogens—no needles, no sugar-cube, no gene gun.

Eating dirt, then, rather than being abnormal, may be an evolutionary adaptation acquired over millennia of productive and not-so-productive interactions with bacteria—an adaptation that enhances fetal immunity and increases calcium, eliminates gastric upset, detoxifies some plant and animal toxins, and perhaps boosts mothers' immunity at times when the hormones of pregnancy (13), factors produced by the fetus (14), changes in the complement system, replacement of MHC class I antigens in the trophoblast (15), and who knows what else suppress the mother's natural immunologic desire to destroy her fetus—a miracle, nearly.

Innate Tendency

My children ate dirt with surprising gusto, garden soil, road soil, leaf-mush soil, sod soil, bug-body soil—even gutter soil. As usual with my children, before I could talk them out of this behavior, they gave it up on their own—their behavior depending more on personal likes and dislikes than on my paternal concerns. I was pleased when they quit. Later I was reassured to discover from other parents that their children were just as taken with dirt as mine, some even more so. I felt less like the parent of a couple of dirt-eating, psychosis-ridden, nutritionally deprived children, even if my children were never quite "normal."

Eating dirt appears nearly universal among children under 2 years of age. When I asked my 2-year-old daughter why she ate dirt, she just stared at me, her eyes wide open, a thick moustache of loam limning her lips. She must have decided that either what I had asked was unfathomably abstract or her answer would be far beyond my comprehension.

Soil pica has been defined as eating 500 mg to >50 g of soil per day (3). But the general applicability of these numbers is widely disputed (pregnant women in Africa eat far more soil than this). By inference, however, normal soil consumption must fall into the range of 0 mg to 500 mg per day per small mouth. Soils consumed by children may differ from those consumed by adults. Generally, children consume topsoils and not the deep (60 cm- to 90-cm deep) clays adults regularly consume (5). And children are considerably less selective in the sites they choose for dirt to eat. But why children eat dirt remains largely obscure to all but children.

Children may eat soil for the same reasons pregnant women and some animals do (2,4,16–18). Because of their rapid growth, they have special nutritional needs and surface soils may serve as supplemental nutrients; detoxification of plant or animal toxins might be accelerated by geophagy— particularly in some parts of the world; or soil components, especially clays, may relieve gastric distress. But topsoils are probably not as effective as deep clays at gastric soothing.

Among children, too, it seems eating dirt might have immunologic consequences. Maternal immunoglobulins are secreted in breast milk shortly before birth and for 1 year or more afterwards. Children often begin eating dirt a year or two after birth. As maternal immunity wanes, eating dirt might "vaccinate" children who are losing their maternal IgA, which could stimulate production of nascent immunoglobulins, especially IgA. Eating dirt might also help populate intestinal flora.

But all of this remains speculative. No clear evidence supports a biologic benefit to geophagy among children. Its frequency and distribution, though, suggest a greater biologic involvement than the simple oral obsessions of children.

Risks of Eating Dirt

How dangerous is eating dirt? My mother was pretty certain about this—damn dangerous. Soils contaminated by industrial or human pollutants pose considerable threat to anyone who eats them. Reports abound of lead poisoning and other toxicities in children eating contaminated soils. Similarly, we do not have to look farther than the last refugee camp or the slums of Calcutta or Tijuana or Basra to find the dangers of soils contaminated with untreated human waste. But the inherent biologic danger of soil is difficult to assess. Soil unaffected by the pressures of overpopulation, industry, and agriculture may be vastly different from the soil most of us encounter routinely.

Using DNA-hybridization analyses, Torsvik et al. (19–20) found an estimated 4,600 species of prokaryotic microorganisms per gram of natural soil. Subsequent investigations, using more sophisticated techniques, found

even more species (20), 700–7,000 g of biomass per cubic meter of soil. Soil is a considerable biologic sink, and certainly some organisms found in it are pathogenic in humans. Yet evidence of soil as a major cause of disease in humans and other animals is limited. And many reported diseases are the result of an abnormal situation, e.g., industrial pollution or untreated sewage.

Most infectious diseases acquired through eating dirt are associated with childhood geophagy, which routinely involves topsoils rather than deep clays. One recent report describes infection of two children at separate sites with raccoon roundworm (*Baylisascaris procyonis*) (21). The infection resulted in severe neurologic damage to both children, and one died. The roundworm was ingested along with soil in both cases. Eating dirt can have dire consequences.

In the United States, the most common parasitic infection associated with geophagy is toxocariasis, most often caused by the worm *Toxocara canis.* Seroprevalence is 4% to 8% depending on the region, but incidence of antibodies to *T. canis* is as high as 16%–30% among blacks and Hispanics. The most common route of infection is ingestion of soil contaminated with dog or cat feces (22). Even though, humans are only paratenic hosts of *T. canis*, under some circumstances (though severe cases are rare), the worm can cause considerable damage (visceral larva migrans, ocular larva migrans, urticaria, pulmonary nodules, hepatic and lymphatic visceral larva migrans, arthralgias) (22–24). Toxocara eggs persist in soil for years. As with soils contaminated by human wastes, soil consumption itself does not cause toxocariasis. And studies of seroprevalence do not distinguish between infection and immunization.

Among children in Nigeria, the most common parasitic infection associated with eating dirt is ascariasis (25). Ascarid worms infect as many as 25% of the world's population (more than 1.25 billion). *Ascaris lumbricoides* is the most common worm. Asymptomatic in many adults, infection is much more serious in children; intestinal obstruction is the most common symptom. Because the worms do not replicate in humans, reexposure is required to maintain infection beyond 2 years.

The correlation between geophagy and helminth infection varies with different helminths. Geissler et al. reported correlation between geophagy and ascariasis (especially caused by *A. lumbricoides*) and possibly trichuriasis but none between geophagy and reinfection with *Schistosoma mansoni, Trichuris trichiura,* or hookworm (26). All parasites that infest soil do not uniformly infect people who consume dirt. Nor do all who eat dirt routinely contract disease.

Immunologic Development and Infectious Disease

Many nonhuman animals regularly eat dirt, generally without ill effects and in many cases with some benefits. Even in humans, there are few reports of infections routinely associated with geophagy by pregnant women in sub-Saharan Africa, probably because women take clays from 60 cm to 90 cm below the soil surface and, at least some of the time, they bake the clays. But these factors seem inadequate to fully account for the frequent absence of overt ill effects.

Helminth infections associated with geophagy appear to affect the frequency of inflammatory bowel diseases, which occur most often in industrialized nations. The underlying cause of these diseases may be abnormal immune response to the contents of the gut or perhaps to the gut itself (27). Inflammatory bowel diseases occur at much lower rates in regions where helminth infections are common. Development of normal gut-associated immune response may be aided by the presence of worms.

In studies of healthy mice, *Trichinella spiralis* prevented colitis induced with tri-nitrobenzene sulfonic acid by redirecting a primarily Th-1 response to a Th-2 response (28). Preliminary studies indicate that helminth infection may also alter the course of inflammatory bowel disease in humans (29). Soil is a rich source of parasitic worms. Studies using a number of other animals have also, at least indirectly, associated dirt and microorganisms with normal immunity.

The Environmental Protection Agency estimates that children in the United States consume, on average, 200–800 mg of dirt per day. Some children regularly consume more than their allotment. Still, that doesn't seem like a lot of dirt. We parents have tried for years to put a stop to it. I don't know of an instance in which anybody has succeeded in keeping children away from dirt. But animals have been successfully raised in absolutely sterile environments. Rabbits, mice, guinea pigs, and rats have been raised under such conditions (30,31). In each case, the immune system failed to develop normally. Lymph nodes and GALT did not achieve the right shape or composition and could not initiate normal immune response. Reexposure to infection later in life does not work, at least not fully. There is a window when infection drives the immune system toward its proper end. After that, mice, rats, rabbits, and guinea pigs are at the mercy of the microbial world.

Evidence suggests that the results would be the same in children. In large families, children with many older brothers and sisters are less likely to have asthma, hay fever, or eczema. West African children who have had measles are half as likely to have allergies as children who never had measles. Italian students who recovered from infection with hepatitis A had fewer and less severe allergies than fellow students who were never infected. Children with Type I diabetes (an autoimmune disease) are less likely to have had infections before their fifth birthdays than

healthy children of the same age. Children raised in rural areas, especially on farms, have fewer allergies and autoimmune diseases than children raised in cities. All of these notions have been referred to as the "hygiene hypothesis" (32).

Children exposed a little more to the infectious face of this world seem to fare better as adults. I do not mean to say that vaccination is inappropriate. Vaccination *is,* most often, infection, and vaccinations have done more to improve childhood survival rates that any other single bit of modern medicine. Nor are water purification and sewage treatment inappropriate. Water and sewage treatment have done even more than vaccination to eliminate disease in areas where human populations have exceeded the ability of the local environment to deal with human waste and the pathogens associated with it. But, evidence indicates that infection early in life is critical for the development of normal immune systems.

Exposure-dependent development is not limited to the immune system. Animals, humans included, must be exposed to the sights, sounds, feels tastes, and smells of this septic world. When we are not, our nervous systems do not develop normally, do not rewire, expand, and contract as they must to survive (33). For humans, as for rabbits, there is a window in childhood when our experiences, our infections, change everything, once and for all. Inside that window, infection causes lymph nodes and GALT to enlarge and reorganize, to separate into cortices and medullae, into primary lymphoid follicles, and develop T- and B-lymphocyte–rich regions of immune competence destined to someday be germinal centers, where our defenses will muster and the real battle will be fought. This window is a defining moment, when the simplest and lowest forms of life—the dirty, the infectious, the parasitic, and the septic—alter who we are.

We do not know which childhood infections are most important, but several studies implicate mycobacterial infections. A large group of bacteria, most of which cause no apparent disease, the mycobacteria, have strains that cause serious diseases (e.g., tuberculosis, leprosy). Mice injected with ovalbumin (the major protein in egg white) become allergic to ovalbumin. But mice first infected with mycobacteria and then injected with ovalbumin do not become allergic (34).

Early infection of children with some mycobacteria may promote strong immune systems, a normal sense of self, and a normal defense of that self. Mycobacteria are found in large numbers in dirt. And animals (probably including humans) kept from this dirt may lose the ability to recognize certain dangerous organisms as a threat, lose the ability to discriminate between self and not self, and lose the ability to distinguish the fatal from the innocuous.

The "Age of Bacteria"

For more than 3 billion years, microorganisms, especially bacteria, have ruled earth. As Stephen Jay Gould said, "We live now in the 'Age of Bacteria.' Our planet has always been in the 'Age of Bacteria' ever since the first fossils, bacteria of course, were entombed in rocks more than three and a half billion years ago" (35). And bacteria have done more than any other living group to alter the character of this earth (36). It has been estimated that more than 10^{29} bacteria live on this planet and as many as 10^{14} live on each one of us. Through all of history, we humans have waltzed with bacteria and the rest of the microscopic world. We had no choice. Bacteria outnumber, outweigh, out-travel, and outevolve us.

That bacteria cause so many human diseases is not astounding. It is astounding that so few bacteria cause human disease. Pathogenic bacteria are merely the microscopic tip of the largest of all biologic icebergs. How fortunate, we imagine. But fortune may have little or nothing to do with our survival. Billions of years of confrontation rather than luck were likely our benefactor. Through those confrontations and those eons, nearly all of us learned to coexist peacefully. Neither humans nor microorganisms benefit from fully destroying the other. Fatal infections seem, biologically at least, shortsighted. And even a brief course of antibiotics is enough to remind us that a world without bacteria would be a poorer world. This is not a war, as it has often been described, even though we have mustered an impressive array of weapons—bactericidal cribs and mattresses, toilet cleaners and counter tops, blankets, deodorants, shampoos, hand soaps, mouthwashes, toothpastes. This is not a war at all. If it were, we would have lost long ago, overpowered by sheer numbers and evolutionary speed. This is something else, something like a lichen, something like a waltz. This waltz will last for all of human history. We must hold our partners carefully and dance well.

Chimayo

Here beneath the old wood crucifix, as I watch the faithful leave the little chapel in Chimayo, I marvel with them at the miracle beneath this adobe floor, the same miracle buried beneath most every place human feet have trod.

Dr. Callahan is associate professor of immunology/public understanding of science in the Department of Microbiology, Immunology, and Pathology at Colorado State University in Fort Collins, Colorado. His research on immunity, infectious diseases, and self-perception has been published in poetry and two books of creative nonfiction in addition to scientific reports.

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Gerald N. Callahan, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80523, USA: fax: 970 491 0603; email: gnc@lamar.colostate.edu

Carbapenem-Resistant *Pseudomonas aeruginosa*–Carrying VIM-2 Metallo-β-Lactamase Determinants, Croatia

To the Editor: Carbapenem-hydrolyzing enzymes of the VIM-type (six different variants are known: VIM-1, VIM-2, VIM-3, VIM-4, VIM-5, and VIM-6) are new molecular class B metallo-β-lactamases. These enzymes have recently been identified in carbapenem-resistant isolates of *Pseudomonas aeruginosa* and other gram-negative nonfermenters from European countries in the Mediterranean basin (Italy, France, Greece, Spain, Portugal, and Turkey), as well as in Far East countries (Korea, Taiwan, and Singapore) and the United States (1–3, Midilli et al., GenBank accession no. AY144612, Koh et al., GenBank accession no. AY165025). Similar to bla_{IMP}, bla_{VIM} genes are located on mobile gene cassettes inserted in the variable regions of integrons (1), a condition that provides a wide potential for expression and dissemination in gram-negative pathogens. VIM enzymes possess the broadest range of substrate hydrolysis and can degrade virtually all β-lactams, except monobactams (4).

According to a recent report, the overall resistance rate to imipenem in *P. aeruginosa* isolated from 17 representative laboratories in Croatia was 11% (range 0%–20%) (5). However, molecular basis of carbapenem resistance was not investigated.

In October 2000, two *P. aeruginosa* isolates with an unusual resistance profile were isolated from two Croatian patients (66 and 74 years of age, respectively) who underwent hysterectomies at the Split University Hospital. Both isolates were cultured from urine a week after surgery; a urinary catheter had been used for both patients who had become febrile and had signs and symptoms of urinary tract infection. Analysis of the macrorestriction profiles of chromosomal DNA of the two isolates by pulsed-field gel electrophoresis, carried out as described previously (6), indicated that the two isolates were clonally related (the two profiles were apparently identical). In routine antibiotic susceptibility testing, done by disk diffusion, both isolates showed a multidrug-resistant phenotype, including ureidopenicillins, piperacillin, piperacillin-tazobactam, ceftazidime, cefoperazone, cefepime, aztreonam, ciprofloxacin, gentamicin, netilmicin, imipenem, and meropenem.

MICs to imipenem and meropenem were high (>128 μg/mL). These findings suggested production of an acquired carbapenemase. In fact, crude extracts of the two isolates exhibited carbapenemase activity in a spectrophotometric assay (7) (imipenem hydrolyzing–specific activity was, in either case, >170 nmol/min/mg protein).

A colony blot hybridization, carried out as described with a bla_{IMP} and a bla_{VIM} probe (6), yielded a positive result with the latter probe. Polymerase chain reaction (PCR) amplification of the variable region of class 1 integrons, carried out as described previously by using primers designed on the 5′- and 3′-conserved segments of the integron (8), yielded a 4-kb amplification product from either isolate. Direct sequencing of these amplification products showed, in both cases, the presence of a bla_{VIM-2} allele located in a gene cassette inserted in the *attI* site of a class 1 integron.

The metallo-β-lactamase determinant was not transferred to *Escherichia coli* MKD135 or *P. aeruginosa* 10145/3 (9) in diparental mating experiments conducted on solid medium (the sensitivity of the assay was $\geq 1 \times 10^{-8}$ transconjugants per donor). Plasmid extraction was performed with several techniques, including lysis with sodium dodecyl sulfate (10) and alkaline lysis conducted with a conventional method (10) or with the Nucleobond BAC100 system (Macherey-Nagel, Duren, Germany). Extraction of whole genomic DNA was also performed, as described (8). Plasmid DNA was not detected in any of these preparations, either when analyzed by agarose gel electrophoresis or after Southern blot hybridization analysis with a bla_{VIM-2} probe generated with PCR amplification of the entire bla_{VIM-2} gene. In Southern blots, a hybridization signal was only detectable in correspondence of the band of chromosomal DNA.

To our knowledge, this isolation is the first one of clinical strains producing acquired metallo-β-lactamase in Croatia. A similar finding underscores the progressive emergence of these determinants in different geographic areas and emphasizes the need for an early recognition of these strains. In fact, monitoring dissemination of new antibiotic resistance determinants is essential to enforce adequate control measures and adjust guidelines for antimicrobial chemotherapy in different hospital settings.

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Sanda Sardelic,* Lucia Pallecchi,† Volga Punda-Polic,* and Gian Maria Rossolini†

*University Hospital and School of Medicine Split, Split, Croatia; and †University of Siena, Siena, Italy

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Address for correspondence: Gian Maria Rossolini, Dipartimento di Biologia Molecolare, Sez. Microbiologia, Università di Siena, Policlinico Le Scotte, 53100, Siena, Italy; fax: 39 0577 233325; email: rossolini@unisi.it

Rickettsia felis in the United Kingdom

To the Editor: *Rickettsia felis* is a bacterium transmitted by the cat flea (*Ctenocephalides felis*), which also acts as a reservoir by means of transovarial transmission (1–3). The distribution of *R. felis* is potentially as wide as that of its insect host, and to date, its presence has been confirmed in cat flea populations in North and South America and southern Europe (4,5). *R. felis* was first identified as a human pathogen in 1994 (6), and cases of "flea-borne spotted fever," which have signs and symptoms of febrile exanthema, have now been reported in the United States, Mexico, Brazil, France, and Germany (7,8). To our knowledge, reports on the presence of *R. felis*, or indeed any other spotted fever group rickettsia, in the United Kingdom have not been published.

To determine whether *R. felis* is present in the United Kingdom, we surveyed cat fleas collected from dogs and cats seen at veterinary practices in southern England and Northern Ireland. A total of 31 dogs and 79 cats from veterinary practices in Bristol, Dorset, London, Devon, Gloucestershire, Hampshire, and Antrim were included in our study. Fleas were collected by combing these animals for 10 minutes. All fleas from each animal were pooled in 70% ethanol. A total of 316 *Ct. felis* (Bouché, 1835), identified by using accepted morphologic criteria, were obtained, with each animal yielding one to five fleas. DNA was extracted from each of the 110 flea pools by using a standard silica cartridge method (QiaAmp DNA mini kit, QIAGEN Ltd., Crawley, West Sussex, U.K.) using the manufacturer's instructions for tissue DNA extraction. The presence of rickettsial DNA was determined by using the polymerase chain reaction (PCR) with oligonucleotide primers that target rickettesial *ompB* (5) or *gltA* (2) genes. Positive control material was cultured *R. felis*. Rigorous controls to limit contamination were carried out, including the use of separate, dedicated rooms for DNA extraction, PCR setup, and gel analysis. Amplification products obtained from *ompB* and *gltA* PCRs were analyzed by using DNA sequencing. Sequences obtained were edited by using BioEdit (available from: URL: http://www.mbio.mncsu.edu/BioEdit/bioedit.html). Similarity to published sequences was determined with the BLAST program (available from: URL: http://www.ncbi.nlm.nih.gov) hosted by the National Centre for Biotechnology Information.

Eighteen flea DNA pools were positive for spotted fever group rickettsia. All 18 yielded PCR products with both *ompB* and *gltA*-targeting PCRs. The *ompB* and *gltA* DNA sequences of all PCR products were 100% identical to those published for *R. felis*, thereby providing evidence for the presence of *R. felis* in fleas collected from >16% of the animals surveyed. PCR-positive fleas were collected from 4 dogs and 14 cats from Bristol, Hampshire, Dorset, and Northern Ireland. Taking into account the number of fleas in each pool, we estimate that 6% to 12% of the fleas collected were infected with *R. felis*.

This study represents the first description of a spotted fever group rickettsia endemic to the United Kingdom. The species detected, *R. felis,* has clear public health implications. The bacterium appears to be widely distributed within the country, infecting a geographically dispersed population of *Ct. felis*. Up to 12% of *Ct. felis* may be infected with *R. felis,* a flea that is by far the most common species of ectoparasite encountered on cats and dogs in the U.K. main-

land. Furthermore, *Ct. felis* often feeds on humans.

Clinicians encountering patients with fever or rash (or both) and a history of cat contact or flea bites should consider a diagnosis of *R. felis*. Laboratory confirmation of infection is not easy, but in vitro culture of *R. felis,* and hence material for a serologic assay for the diagnosis of human *R. felis* infections, has recently been described, and serology appears to be an accurate indicator of exposure (9). As with other spotted fever group rickettsial infections, molecular diagnostics may provide a useful alternative approach to detecting and identifying *R. felis* in infected tissues. In culture, *R. felis* has been shown to be resistant to erythromycin (unlike other rickettsia), gentamicin, amoxicillin, and trimethoprim-sulfamethoxazole. Thus, infection with this bacterium should be considered in cases of antibiotic-insensitive fever with a rash, especially in young, old, and immunosuppressed persons. The organism is sensitive to doxycycline, rifampicin, thiamphenicol, and fluoroquinolones (10)

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Martin J. Kenny,*
Richard J. Birtles,†
Michael J. Day,*
and Susan E. Shaw*

*University of Bristol, Langford, Somerset, United Kingdom; and †University of Liverpool, Leahurst, Neston, Cheshire, United Kingdom

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Address for correspondence: Susan E. Shaw, Department of Clinical Veterinary Sciences, University of Bristol, Langford, Somerset, BS40 5DU, United Kingdom; fax: +44 (0)117 9289505; email: susan.e.shaw@bris.ac.uk

Community Transmission of Extended-Spectrum β-Lactamase

To the Editor: The spread of multiresistant gram-negative bacteria in the general population is a problem of paramount importance, but the responsible mechanisms are poorly understood. Several studies have focused on β-lactam resistance in *Enterobacteriaceae* isolated from stools in healthy people, but they did not specifically investigate the extended-spectrum β-lactamases (ESBL). Furthermore, none of these studies detected ESBL in the evaluated population (1,2). We performed three survey studies to determine the incidence of *Enterobacteriaceae* strains producing ESBLs in the stools of outpatients attending our hospital. The first study was performed during a 4-month period (February–May 2001), the second during a 3 month-period (April–June 2002), and the third during 1 month (October 2002).

Stool samples were spread onto plates of MacConkey agar containing 2 mg/L of cefotaxime. A colony of each distinct morphotype was analyzed further. Species were identified according to conventional methods (3). The susceptibility to β-lactam antibiotics was determined by the disk-diffusion test, following recommendations of the National Committee for Clinical Laboratory Standards (4,5). The interpretative reading of the antibiogram was performed according to standard guidelines (4–6). The MICs of cefotaxime and ceftazidime, with and without clavulanic acid, were later determined by Etest (AB Biodisk, Solna, Sweden). Strains producing ESBL were defined as strains showing synergism between amoxicillin-clavulanic acid and cefotaxime, ceftazidime, cefepime, or aztreonam (4,5).

All strains suspected of carrying a resistance pattern compatible with

hyperproduction of the chromosomal enzymes, as well as resistant strains without synergy, were disregarded. During the first period, 15 (2.1%) of 707 outpatients were carriers of *Escherichia coli* (14 patients) or *Proteus mirabilis* (1 patient) with ESBL. This percentage increased during the second period, when 17 (3.8%) of 454 outpatients were carriers of *E. coli* with ESBL, and again in the third period, when 12 (7.5%) of 160 were carriers of *E. coli* (11 patients) or *Enterobacter cloacae* (1 patient) with ESBL. Characterization of the different ESBL isolated during the three study periods is in process. Although *Klebsiella pneumoniae* carrying ESBL has been detected in our hospital (7), as well as in other hospitals in Barcelona (8), no ESBL-producing *K. pneumoniae* strains were identified in this survey.

Although we did not disregard either the patients' previous treatment with antibiotics or previous hospitalization, these patients came to the hospital from the community carrying strains that express ESBL. Moreover, during these three periods we observed a significant increase in the frequency of ESBL carriers (from 2.1% to 7.5%; $p<0.005$). These data suggest that the community could be a reservoir for these enzymes, as occurs with other microorganisms (9–11). Many questions remain unanswered regarding the diffusion mechanisms of this resistance in the community. Confirmation of community-based transmission of ESBL would indicate a need for heightened vigilance and further studies to determine the reservoirs and vehicles for dissemination of ESBL within the community.

Beatriz Mirelis,*† Ferran Navarro,*† Elisenda Miró,* Raul Jesús Mesa,* Pere Coll,*† and Guillem Prats*†

*Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; and †Universitat Autònoma de Barcelona, Barcelona, Spain

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Address for correspondence: Beatriz Mirelis, Departament de Microbiologia, Hospital de la Santa Creu i Sant Pau, Av. Sant Antoni Mª Claret, 167, 08025 Barcelona, Spain; fax: 34 93 2919070; email: bmirelis@hsp.santpau.es

Polymyxin-Resistant *Acinetobacter* spp. Isolates: What Is Next?

To the Editor: In Brazilian hospitals, *Acinetobacter* spp. has been an important etiologic agent of nosocomial infections, mainly pneumonia (1–3). In general, ampicillin/sulbactam and carbapenems remain the last therapeutic options for treatment of such infections (3,4). However, resistance rates to carbapenems have increased, reaching rates approximately 12% or higher in some Brazilian hospitals (1,3,4). Thus, more toxic agents such as polymyxins have been used as alternative therapeutic drugs against multidrug-resistant *Acinetobacter* infections (5,6). The clinical use of polymyxins has been based on antimicrobial susceptibility results and previous clinical experience. However, the National Committee for Clinical Laboratory Standards (NCCLS) documents do not currently provide interpretative criteria for the testing of polymyxins (7). In addition, the disk diffusion technique was reported to be an unreliable method for evaluating the susceptibility to polymyxins (8). Since *Acinetobacter* clinical specimens exhibiting high MICs for polymyxins (MIC, 8–32 μg/mL) were recently detected, we searched for the frequency of occurrence of *Acinetobacter* spp. strains exhibiting reduced susceptibility to polymyxin B among 100 bloodstream isolates of *Acinetobacter* spp. (8). The bacterial isolates were consecutively collected between September 1999 and December 2000 from a tertiary Brazilian hospital,

where *Acinetobacter* spp. infections have reached endemic levels and polymyxins have been frequently used. Only one isolate per patient was included in the study.

The isolates were identified to the species level using the BBL Crystal System (Becton Dickinson, Sparks, MD). The susceptibility to polymyxin B and meropenem were tested by disk diffusion and agar dilution techniques according to NCCLS recommendations (9,10). The susceptibility interpretative criteria for meropenem and polymyxin B were based on the current and former NCCLS documents, respectively (7,11). The MIC was defined as the lowest antimicrobial concentration that inhibited bacterial growth. *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922 were used as quality control strains. Testing errors and agreements were determined by comparing the results of the disk diffusion with the standard criterion agar dilution method. Categorical agreement was obtained when the isolates were classified within the same susceptibility category. The very major and major errors were related to false susceptibility and false resistance results, respectively. To evaluate whether the polymyxin B-resistant strains isolates were epidemiologically related, these isolates were molecularly typed by pulsed-field gel electrophoresis (PFGE) as previously described (12). PFGE patterns were considered identical if they shared every band, similar if they differed from one to three bands, and distinct if they differed by four or more bands (12).

Despite the limitation of commercial systems for identifying the genus *Acinetobacter* at species level, *Acinetobacter baumannii* (80.0%) was the most commonly identified species, followed by *A. lwoffi* (4.0%). Sixteen percent of the *Acinetobacter* isolates were not identified to species level by the BBL Crystal System. Meropenem (MIC50, 1 μg/mL) and polymyxin B (MIC50, 1 μg/mL) showed similar in vitro potency. However, meropenem exhibited the highest susceptibility rate (99.0% susceptible). In contrast to previous studies, only one strain was resistant to meropenem (1,2,3,8), which indicates that the carbapenem-susceptibility rates among *Acinetobacter* spp. isolates may vary according to the period evaluated even in the same institution. By using the polymyxin B resistance breakpoint (MIC ≥4 μg/mL) presented by the former NCCLS document, which was recently validated, we found that five *Acinetobacter* spp. isolates were considered resistant to polymyxin B (MICs, 8–32 μg/mL) (8,11). All isolates were susceptible to meropenem and belonged to *A. baumannnii* (4) and *A. lwoffi* (1) species. The polymyxin B–resistant isolates were categorized as susceptible by disk diffusion (100%, very major error). The disk diffusion method is widely used in Brazil and worldwide. However, disk diffusion was confirmed to be an unreliable test for detecting *Acinetobacter* spp. isolates with reduced susceptibility to polymyxins. These results are in agreement with those previously reported (8).

Among the five polymyxin B–resistant *Acinetobacter* spp., four distinct patterns were characterized by PFGE. Two polymyxin B–resistant strains, which were isolated from different units of the São Paulo Hospital complex, shared an identical PFGE pattern. The PFGE results suggest that the polymyxin B use may have played a role in the selection of resistant strains. On the other hand, two isolates shared an identical PFGE pattern, which raises the possibility of patient-to-patient transmission of epidemic strains. Intra- and interhospital dissemination of multidrug-resistant *Acinetobacter* spp. clones has already been reported in Brazilian hospitals (13).

Our findings suggest that the polymyxin B–resistant strains have emerged because of antimicrobial selective pressure and dissemination of clonal strains. Further epidemiologic studies are necessary to correlate the emergence of polymyxin-resistant *Acinetobacter* spp. isolates to the clinical response with polymyxin B therapy. Since the emergence of polymyxin B resistance may leave no efficacious drugs for the treatment of infections caused by multidrug-resistant *Acinetobacter* spp. isolates, strict infection control measures must be adopted to avoid the emergence and spread of such isolates. The low accuracy of routine susceptibility tests, especially disk diffusion, may jeopardize rapid implementation of such measures.

Adriana O. Reis,* Deise A.M. Luz,*
Maria C.B. Tognim,*
Hélio S. Sader,* and Ana C. Gales*
*Universidade Federal de São Paulo, São Paulo, Brazil

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Address for correspondence: Ana Cristina Gales, Laboratório Especial de Microbiologia Clínica, Division of Infectious Diseases, Universidade Federal de São Paulo, Rua Leandro Dupret, 188 São Paulo, SP, Brazil, CEP 04025-010; fax: (55-11) 5081-2819/5571-5180/5081-2965; email: galesac@aol.com

Multidrug-Resistant Shiga Toxin–Producing *Escherichia coli* O118:H16 in Latin America

To the Editor: We report the first isolation of a multiple antimicrobial drug–resistant strain of Shiga toxin–producing *Escherichia coli* (STEC) O118:H16 from cattle in Latin America. The strain was isolated during a study of fecal STEC in 205 healthy and 139 diarrheic cattle on 12 beef farms in the state of São Paulo, Brazil, in February 2000; one case of STEC was found in a 1-month-old calf with diarrhea. This bovine STEC O118:H16 strain showed resistance to eight antimicrobial substances; the following resistance (R)-genes were detected: ampicillin ($bla_{\text{TEM1-like}}$), kanamycin and neomycin (*aphA1*), streptomycin (*strA/B*), sulphametoxazol (*sul2*), tetracyclin (*tet*[A]), trimethoprim (no *dfrA1, A5*, *A7*, *A12*, *A14*, or *A17*), and trimethoprim/sulphamethoxazol. The STEC O118:H16 strain from Brazil was found to be similar for virulence genes (Shiga toxin 1 [*stx*1], intimin beta 1 [*eae* β1], and EHEC-hemolysin [E-*hly*A]) and for antimicrobial drug resistance to STEC O118:H16 strains, which were isolated in different countries of Europe (1). Beginning in 1986, STEC O118:H16 was identified as an emerging pathogen for calves and humans in Belgium and Germany (2–4). Cattle and human STEC O118:H16 isolates were similar in virulence attributes and antimicrobial drug resistance and belonged to a distinct genetic clone (1). Transmission of these pathogens from cattle to humans on farms was observed (5).

Beginning in 1996, STEC O118:H16 has become important as an emerging pathogen in humans and has been associated with bloody diarrhea and hemolytic uremic syndrome (2). Analysis of the antimicrobial resistance profiles showed that >96% of the European STEC O118:H16 strains showed resistance to one or more antimicrobial drugs in contrast to the 10% to 15% drug-resistant strains that were detected among STEC belonging to other serotypes (1,6,7). STEC O118:H16 showing multiresistance in up to eight different antimicrobial drugs predominated among younger isolates, indicating that drug resistance genes have accumulated over time in STEC O118:H16 strains. The frequency of antimicrobial drug resistance in STEC and Stx-negative *E. coli* in humans and animals was compared in a study by Schroeder et al. (8). Among human clinical *E. coli* isolates, antimicrobial resistance was less frequently observed in STEC than in Stx-negative strains, whereas in cattle, antibiotic-resistant strains were found at similar frequencies in both groups of *E. coli*. The relatively higher frequency of antimicrobial-resistant STEC in cattle was explained by the use of antimicrobial drugs in cattle production, whereas human infections with STEC are generally not treated with antibiotics (8). Cattle could thus be an important source of new emerging antibiotic-resistant STEC strains such as O118:H16.

The genetic basis of antimicrobial resistance in STEC O118:H16 is broad, including R-plasmids, integrons, transposons, and chromosomally inherited drug-resistance genes. Fluoroquinolone resistance has also been acquired by some STEC O118:H16 strains (1). The heterogenicity of antimicrobial drug–resistance patterns, the increase of multidrug-resistant strains over time

of isolation, and the evidence for multiple aquisition and genetic location of R-determinants indicate that strains belonging to the STEC O118:H16 clone have a propensity to acquire and accumulate R-genes. The finding that multidrug-resistant STEC O118:H16 is isolated from cattle in South America indicates the global spread of this new emerging EHEC type.

Antonio Fernando Pestana de Castro,* Beatriz Guerra,† Luciana Leomil,* Leila Aidar-Ugrinovitch,‡ and Lothar Beutin§

*Universidade de São Paulo, São Paulo, Brazil; †Federal Institute for Risk Assessment, Berlin, Germany; ‡Universidade de Campinas, Campinas, Brazil; and §Robert Koch-Institute, Berlin, Germany

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Address for correspondence: Lothar Beutin, Division of Microbial Toxins, Department of Biological Safety, Robert Koch-Institute, Nordufer 20, D-13353 Berlin, Germany; fax: +49 30 4547 2673; email: beutinl@rki.de

Correction, Vol. 9, No. 6

In "Serogroup W-135 Meningococcal Disease during the Hajj, 2000," p. 665, author Sahar Makki was inadvertently not included. The correct author list should read as follows:

Jairam R. Lingappa,* Abdullah M. Al-Rabeah,† Rana Hajjeh,* Tajammal Mustafa,† Adel Fatani,† Tami Al-Bassam,† Amira Badukhan,† Abdulhafiz Turkistani,† Sahar Makki,† Nassen Al-Hamdan,† Mohamed Al-Jeffri,† Yaqoub Al Mazrou,† Bradley A. Perkins,* Tonja Popovic,* Leonard W. Mayer,* and Nancy E. Rosenstein*

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Saudi Arabian Ministry of Health, Riyadh, Kingdom of Saudi Arabia

Emerging Infectious Diseases Policy on Corrections

The Emerging Infectious Diseases journal wishes error-free articles. To that end, we

1) Make corrections as quickly as we become aware of errors

2) Publish corrections online and in print. Online, we correct the error in the article it occurred with a note that the article was corrected and the date of correction. In print, we prominently publish a full correction, printing all needed information, and provide the URL of the corrected online article for reprints.

For additional information on corrections, send e-mail to eideditor@cdc.gov.

Conference Summary

Emergence and Control of Zoonotic Viral Encephalitis

More than 50 researchers and administrators from over a dozen countries attended a symposium on the emergence and control of zoonotic viral encephilitis. Held April 6–8, 2003, in a convivial setting at Les Pensières, Veyrier du Lac, near Annecy in the French Alps, this meeting was one of a series on the emergence and control of infectious diseases, sponsored and organized by the Mérieux Foundation. The general objectives were to review the biology of viral encephalitis, the virulence and genetic evolution of encephalitis viruses, and the factors involved in emergence of these diseases.

Emergence or reemergence of viruses may be due to virus evolution, to the impact and influence of human populations on previously undisturbed ecosystems, or to better recognition. Clearly, we must understand the basic mechanisms by which these viruses emerge or reemerge and cause illnesses. Methods for detecting infections caused by neurotropic viruses and for detecting viruses or their genome sequences are available and improving. Methods for detecting antibodies also have improved.

Examples of recently recognized viruses causing encephalitis in humans, livestock, or wildlife include Hendra and Nipah viruses (henipaviruses; family *Paramyxoviridae*, genus *Henipavirus*), both of which are neurotropic, and Australian bat lyssavirus (family *Rhabdoviridae*, genus *Lyssavirus*), also a neurotrope. All three viruses, and others related to them, have been shown to have fruit bats (*Pteropus* spp.) as their natural hosts. Progress is being made in understanding transcription regulation and cell fusion by henipaviruses. In addition, basic epidemiologic procedures and classical prevention strategies have been put into place to prevent infections caused by these viruses.

Since 1988, a worldwide effort has been under way to eradicate the nonzoonotic but encephalitogenic poliomyelitis viruses. The number of cases has been reduced by 99%, and the natural occurrence of these viruses now is limited to seven countries. The system established to conduct surveillance and response may provide a model for use in tracking and controlling other viruses causing encephalitis. Long-term studies of ecologic parameters, seasonality, and changing virus and vector prevalence rates are being used to determine risk factors in various arbovirus infections, including Japanese encephalitis virus in Thailand, and are being applied for prevention and control.

In Russia, where West Nile virus has long been recognized but has not caused any major diseases, recent detection of various virus genotypes suggests a mélange of genotypes circulating in various areas and transported between areas by birds. Generation and maintenance of continuous genetic variation may lead to partial protection and escape mutants, which could provide a "pump" that generates more variants and "new" viruses. When these genotypes adapt to naïve populations of birds, horses, and humans, in the presence of competent arthropod vectors, epidemics may arise and new opportunities for these viruses and virus variants may occur, perhaps including West Nile virus into the New World in 1999. Evidence presented suggests that little genomic variation in New World West Nile virus has occurred since its 1999 recognition there; this situation is likely to change.

Continuing to make the classical epidemiologic observations that have characterized disease investigations in the previous half-century is important. However, to understand the overall effects of virus outbreaks, denominators are needed. Numerous presentations demonstrated that we are beginning to understand the molecular mechanisms leading to pathogenetic events. Further studies may provide information useful for the development of antiviral compounds and candidate vaccines.

Attendees were provided with an overview of various transmission cycles of arboviruses, which are concomitantly diverse in regards to their hosts and vectors. Viral neuroinvasiveness appears to depend on the uniqueness of phylogenetically diverse hosts, their ages, genetic predispositions, immune status, virus origin, passage level, dose, and other factors—a complex situation to investigate and comprehend. Critical factors impacting neuroinvasiveness and neuronotropism must be coupled to cause encephalitis. Viral mutations may affect the ability of the virus to replicate in cells, altering viral virulence; however, specific genomic and polyprotein sequence changes may account for the high viremias and replication in neurons that are central to emergence. The extent of the roles of various proteins in virus infections, neuronal involvement, and apoptosis are being recognized. Now we are beginning to understand complex signaling mechanisms, antibody-producing cell types, cytokines, and the cellular responses and pathways leading to both disease and protection from disease.

Considerable progress has been made in understanding the relationships between genetic and functional diversities, neuronal receptors, transport, and cellular protein-virus interactions. Such understanding is critical to further insights to neurotropism, pathogenesis, pathogenetic mechanisms, and immunogenicity.

Phylogenetic trees were used to describe the evolution of encephalitic flaviviruses, geographic exclusion,

virus persistence, and flaviviral recombination as a mechanism of flaviviral evolution. In addition, data were presented that illustrated the persistence of, and immune modulation by, alphaviruses, which, in concert, allow the virus to replicate while preventing the host from responding to its benefit.

Other than the classical techniques of preventing infection, little was mentioned about disease control during this symposium. Control must be based on rapid recognition of early cases, subsequent immunization of persons or animals at risk, or immunization of persons or animals with the potential to be at risk, such as travelers, laboratory personnel, and attending clinicians. Attendees learned about diverse methods being used to develop vaccines. Representatives from the World Health Organization explained that organization's plans for responding to disease emergence and for preventing zoonotic diseases from reaching human populations.

New paradigms for field studies of zoonotic diseases are necessary. These approaches must include longitudinal and in-depth investigations of agent, host, habitat, and environment if we are to predict risk and respond in an appropriate manner. At this time, zoonotic disease control comprises prevention and public education and not much more. Progress is being made in rapid diagnosis, production of sophisticated vaccines, and understanding of the molecular mechanisms by which zoonotic viruses persist and cause disease.

Most of the papers presented will be published in a special issue of Archives of Virology.

Charles H. Calisher,* Betty Dodet,† and Diane Griffin‡

*Colorado State University, Fort Collins, Colorado, USA: †Fondation Mérieux, Lyon Cedex, France; and ‡Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

Address for correspondence: Charles H. Calisher, Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Ft. Collins, CO 80523, USA; fax: (970) 491-8323; email: calisher@cybersafe.net

Conference Summary

New and Reemerging Infectious Diseases

The Sixth Annual Conference on New and Reemerging Infectious Diseases was hosted April 24–25, 2003, by the Center for Zoonoses Research and the College of Veterinary Medicine, University of Illinois at Urbana-Champaign (UIUC). The conference featured seven speakers and 27 poster presentations.

Smallpox

Bertram L. Jacobs (Arizona State University, Tempe, AZ) opened the conference with a presentation on smallpox, one of the most devastating diseases known to humankind. Smallpox was eradicated from the wild in the 1970s, although the potential use of Variola virus as a bioterrorism agent makes it still of great concern. Dr. Jacobs described Vaccinia viruses deficient in E3L, a regulator of the cellular antiviral response and noted their potential for the production of improved vaccines. He also showed that double-stranded (ds)RNA- and ZDNA binding proteins had a role in poxvirus pathogenesis. In the poster section, Joanna Shisler (University of Illinois at Urbana-Champaign [UIUC], Urbana) reported that the modified virus, Ankara, activates nuclear factor κB through the mitogen-activated protein kinase, extracellular signal–regulated kinase (MEK)/ERK extracellular signal–regulated kinase (ERK) pathway, possibly facilitating the host immune response. This virus was used to vaccinate 100,000 people, with no reported complications, at the end of the global smallpox vaccination campaign led by the World Health Organization in the 1970s.

West Nile Virus and Geographic Information Systems

Since it was first detected in New York City in 1999, West Nile virus (WNV) has spread from coast to coast and has been found in 43 states from Maine to California. Stephen C. Guptill (U.S. Geological Survey, Reston, VA) reported that the U.S. Geological Survey is working with the Centers for Disease Control and Prevention (CDC) to learn the current geographic extent of WNV. This will allow us to understand how it moves between birds, mosquitoes, and humans and to better predict future outbreaks. A collaborative 3-year research project is being conducted on lands administered by the U.S. Fish and Wildlife Service, the National Park Service, and other federal lands, and on state, local, and private lands along the Atlantic and Mississippi flyways. This study tests sampled migratory and local wild birds to detect WNV and identify possible avian carriers. Over 10,000 birds of more than 150 species have been captured, sampled, and released at 20 federal sites and 3 other sites in 12 states during the spring and fall bird migration seasons of 2001 and 2002. A parallel study, conducted with CDC, is examining the distribution and number of mosquito species in relation to land cover, weather conditions, and avian deaths. Systematic mosquito surveillance (weekly collections at seven

sites) is being conducted year-round in St. Tammany Parish in Louisiana, complementing avian collections at the Bogue Chitto and Big Branch National Wildlife Refuges in the parish. Finally, WNV surveillance data from CDC is being studied to determine the spatial and temporal relationships between disease outbreaks in birds and animals and human illness. Information from these analyses will guide the creation of predictive models of disease risk. These surveillance systems provide the basic information on the "geography" of the virus. Combining these data with information about avian migratory patterns, landscape characteristics, and weather conditions, over space and time, will provide the foundation for developing spatial analytical and forecasting models to assess the risk for human illness. In related work, presented at the poster session, Marylin Ruiz (UIUC, Urbana) reported the efforts of the College of Veterinary Medicine Geographic Information System and Spatial Analysis Laboratory, in collaboration with the Illinois Department of Public Health, and the Illinois Department of Agriculture in the mapping and analysis of the WNV outbreak in Illinois. (Illinois was the state hit the hardest by the epidemic in 2002.) Geographic information systems in conjunction with fine resolution satellite data and spatial statistics are also useful to investigate the distribution of other diseases, for example, schistosomiasis (Julie A. Clennon, UIUC, Urbana).

Animal Models of Infectious Diseases

Streptococcal pathogens continue to evade concerted efforts to decipher clear-cut virulence mechanisms, although numerous genes have been implicated in pathogenesis. Melody N. Neely (Wayne State University, Detroit, MI) reported the development of a unique animal model, the zebrafish (*Danio rerio*), to characterize specific virulence mechanisms used within various tissues in vivo. Her group is using this model host to study infection by two streptococcal species that represent two forms of streptococcal disease: a natural pathogen of fish and humans, *Streptococcus initiae,* and a human-specific pathogen, *S. pyogenes*. *S. initiae* primarily causes a fatal systemic disease in the zebrafish after intramuscular injection, with pathologic changes similar to those seen in human infections caused by *S. agalactiae* and *S. pneumoniae*. The fatal infection by *S. pyogenes* causes a locally spreading necrotic disease confined to the muscle with pathologic features similar to those observed in a human infection of necrotizing fasciitis. By studying pathogens that are virulent for both fish and humans and that mediate disease states in the zebrafish identical to those found in human streptococcal infections, common virulence strategies shared by a number of gram-positive pathogens can be identified. Using several genetic strategies with the two streptococcal strains, Dr. Neely's group is currently conducting specific screens in the zebrafish to 1) identify and characterize cell membrane proteins that interact with the host in vivo to cause specific disease states; 2) identify genes required for growth in vivo, as well as progressive stages of infection; 3) identify genes that are only expressed while in the host along with tissue specificity of the encoded proteins; and 4) analyze responses of the host that affect progression of disease.

Foodborne Diseases

Shigella boydii is a food pathogen that was implicated in a 1999 foodborne outbreak involving contaminated bean salad that contained fresh parsley and cilantro. Hans Blaschek and collaborators (UIUC, Urbana) reported the high tolerance of this bacterium to acidic pH, and the presence and formation of biofilms in cilantro and parsley samples treated with produce wash and water. Cells in biofilms are known to be more resistant to antibiotics and disinfectants, and biofilm formation may explain the decreased efficacy of produce wash on parsley and cilantro samples.

Intracellular Parasites

The trypanosomatid protozoan *Leishmania* synthesizes a variety of glycosylphosphatidylinositol (GPI GPI)-anchored molecules on its surface. Sorting out their individual functions has been difficult and in some cases controversial as they share structural domains and generally have been tested outside the context of key components such as the major glycolipid lipohosphoglycan (LPG) and phosphoglycans (PGs); ether lipids, which constitute approximately 20% of the membrane lipids and most GPI anchors; and others. Stephen M. Beverley and his collaborators (Washington University, St. Louis, MO) have studied their role in the context of parasite knockouts and "add-back" controls, probing their role in diverse aspects of parasitism such as entry, inhibition of phagolysosomal fusion and host-signal transduction, and pathogenesis. All of these molecules play critical roles in virulence, although sometimes in unanticipated ways. PGs in particular are required for parasite persistence but not acute pathology, therefore defining a new class of parasite genes important to transmission. Ted Hackstadt (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT) reported that, unlike the majority of most intracellular parasites, which block maturation of endosomes to lysosomes at discrete stages and then replicate within those vacuoles, chlamydiae appear to dissociate themselves from the endocytic pathway shortly after internalization by actively modifying the vacuole to become fusogenic with sphin-

gomyelin-containing exocytic vesicles. Interaction with a secretory pathway appears to provide a pathogenic mechanism that allows chlamydiae to establish themselves in a site not destined to fuse with lysosomes. Fusion with Golgi-derived vesicles provides a likely source of cellular lipids for the growth of the inclusion membrane as it expands to accommodate the multiplying parasites.

Kasturi Haldar (Northwestern University, Chicago, IL) presented studies on the malaria parasite, focusing on protein trafficking, gene expression, and drug development. Her group has studied vacuolar trafficking of host raft proteins and parasite virulence determinants (by tagging genes with GFP and expressing them by transfection) for their consequences on malarial entry into the red blood cell, virulence secretion systems, and apicoplast biogenesis. The apicoplast is a newly identified residual plastid acquired by secondary endosymbiosis that has attracted attention for its evolutionary novelty and its potential as a drug target. Temporal regulation of plasmodial genes may be important for protein-targeting in cells. In this context, Dr. Haldar's group is examining the role of unique promoter elements and chromatin in regulating expression of secretory determinants such as the histidine-rich proteins and adherence antigens. Whole genome scanning approaches (microarrays and other functional approaches) are being used in combination with informatics to develop novel lipid-linked targets for drug development. Studies on *Salmonella* are currently examining effectors of the SPI-2 system (*Salmonella* Pathogenicity Island–2 system) for their effects on sterol recruitment, metabolism, and bacterial virulence in the mouse model. The requirement for nonsterol precursors in protecting infected cells from death by apoptotis, necrosis, or both, is also being investigated. Finally, microarrays are used to identify subsets of *S. typhimurium* virulence determinants required for lipid-linked intracellular bacterial replication.

Toxoplasma gondii is a major cause of birth defects and infections in immunocompromised persons. Like all members of the phylum Apicomplexa, *T. gondii* is an obligate intracellular organism. Micronemes and rhoptries are specialized secretory organelles of the Apicomplexa, whose contents are thought to be essential for successful invasion of host cells. Kami Kim (Albert Einstein College of Medicine, Bronx, NY) reported identifying two subtilisin-like serine proteinases from *T. gondii*, TgSUB1 and TgSUB2, which are necessary for successful invasion. Serine proteinase inhibitors have been reported to block host cell invasion by both *T. gondii* and the related apicomplexan parasite, *P. falciparum*. Disruption of *TgSUB2* was unsuccessful, which implies that *TgSUB2* is an essential gene. Both TgSUB1 and TgSUB2 undergo autocatalytic processing as they traffic through the secretory pathway. TgSUB1 is a microneme protein, whereas TgSUB2 localizes to rhoptries and associates with rhoptry protein ROP1, a potential substrate. Mutational analysis suggests that TgSUB2 is a rhoptry protein maturase. Processing of secretory organelle contents appears to be ubiquitous among the Apicomplexa. Since subtilases are found in genomes of all the Apicomplexa sequenced to date, they may represent a novel chemotherapeutic target. Transcriptional regulatory pathways in apicomplexan parasites are understudied and may contain novel drug targets. William Sullivan and his collaborators (Indiana University School of Medicine, Indianapolis, IN) identified and mapped a gene in *T. gondii* that encodes a homologue of chromatin remodeling factors that uses ATP to promote a more favorable environment for transcription. They also described a novel histone acetyltransferase, which contains a unique 820-amino acid *N*-terminal extension of unknown function.

Parasite Organelles

Acidocalcisomes are novel calcium-containing acidic organelles present in unicellular eukaryotes. Several posters from researchers in the groups of Silvia Moreno and Roberto Docampo (UIUC, Urbana) highlighted recent work on these organelles. Andrea Montalvetti described a functional aquaporin (water channel) found in the organelles of *T. cruzi*, the etiologic agent of Chagas disease, and Peter Rohloff showed that this protein is translocated to the contractile vacuole upon hypo-osmotic stress. Joanna Cox and Shuhong Luo demonstrated that a Ca^{2+}-ATPase is localized to acidocalcisomes and the plasma membrane of *Trypanosoma brucei*, the etiologic agent of African sleeping sickness, and is essential for cell growth, as demonstrated by RNA interference experiments. Manfredo Seufferheld presented evidence of the presence acidocalcisomes in the bacterium *Rodospirillum rubrum*. Felix Ruiz showed that acidocalcisomes of *Plasmodium falciparum*, one of the etiologic agents of malaria, do not differ significantly from the organelles in other apicomplexan parasites, whereas the platelet-dense granules possess several characteristics common to acidocalcisomes. *T. brucei* possesses a P-type proton ATPase with homology to fungal and plant H^{+}-ATPases but absent in mammalian cells; this proton constitutes a formidable target for chemotherapy (Shuhong Luo, UIUC, Urbana).

Chemotherapeutic Targets

A number of poster presentations identified novel chemotherapeutic targets for infectious diseases. Steve Grimme (UIUC, Urbana), who received an award for the best poster presentation, demonstrated that a GPI

mannosyltransferase (CaSMP3) was essential for the human pathogenic fungus *Candida albicans* and therefore was validated as a potential target for chemotherapy. M. Laura Salto (UIUC, Urbana) showed that inositolphosphoceramide, a lipid used in anchoring several proteins to the plasma membrane of *T. cruzi* and absent in the host, is remodeled during the differentiation of different stages of the parasite. Novel lipid modified phosphoinositide phospholipases C were reported in *T. cruzi* (Michael Okura, UIUC, Urbana) and *T. brucei* (Jianmin Fang, UIUC, Urbana). The enzymes are apparently involved in differentiation of the parasites and are different from their host counterparts. Recently, bisphosphonates have been proposed as novel antiparasitic drugs, and Yan Ling (UIUC, Urbana) reported the cloning and characterization of *Toxoplasma gondii* farnesyl pyrophosphate synthase, the target of these compounds. Tryptophan metabolism in mosquitoes and the separation and identification of mosquito chrorion proteins through two-dimensional electrophoresis and mass spectrometry will provide useful targets against these vectors of several infectious diseases, as reported by Jianyong Li and his collaborators (UIUC, Urbana).

Roberto Docampo*

*University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

Address for correspondence: Roberto Docampo, Laboratory of Molecular Parasitology, Department of Pathobiology and Center for Zoonoses Research, University of Illinois at Urbana-Champaign, 2001 South Lincoln Avenue, Urbana, IL 61802, USA; email: rodoc@uiuc.edu

The Yellow Book

The Division of Global Migration and Quarantine, National Center for Infectious Diseases, Centers for Disease Control and Prevention, announces the availability of the 2003-2004 edition of Health Information for International Travel (The Yellow Book). Features of this edition include new recommendations for malaria chemoprophylaxis; updated, expanded sections on injury during travel, motion sickness, altitude sickness, and travelers with disabilities; revised vaccine recommendations; changes in recommendations for insect repellents; a new chapter focusing on recommendations for children; new text on scuba diving safety and high-risk travelers; and improved maps and indexing.

The Yellow Book will also be available on CD-ROM later in the year. To order the book or the CD-ROM, contact the Public Health Foundation at 1-877-252-1200 or http://bookstore.phf.org.

Upcoming Infectious Disease Conferences

August 11–22, 2003
8th International Course: Dengue and Dengue Hemorrhagic Fever
Havana, Cuba
Contact: Prof. Maria Guzman
Phone: 53-7-202 0450
Fax: 53-7-202 0633
email: lupe@ipk.sld.cu
Web site: http://www.ipk.sld.cu/eventosipk/curso-dengue.htm

August 27–30, 2003
6th International Meeting on Microbial Epidemiological Markers
Les Diablerets, Switzerland
Contact: Administrative Secretariat
+41-61-686-77 11
email: info@akm.ch
Web site: www.akm.ch/immem6

September 9–12, 2003
ASTHO-NACCHO 2003 Joint Conference
Association of State and Territorial Health Officials
National Association of County and City Health Officials
Phoenix, Arizona
Web site: http://www.astho.org

September 14–17, 2003
43rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
Chicago, IL
Web site: ww.ICAAC.org/ICAAC.asp

September 15–18, 2003
Fourth International Conference on Tularemia
Bath, United Kingdom
email: tularemia@indexcommunications.com
Web site: http://www.tularemiaconf.co.uk

Jan Steen (c. 1625–1679). Beware of Luxury (c. 1665)
Oil on canvas 105 cm x 145 cm. Kunsthistorisches Museum, Vienna, Austria

"Life is a stage; we play our part and receive our reward," wrote Vondel, the great poet of the Netherlands, expressing the moral preoccupation of 17th-century Dutch culture (1). The 17th century was a time of geographic exploration and economic prosperity for the famed lowlands. Laboriously claimed from the sea, criss-crossed by canals and spotted with windmills, the tiny country produced a vibrant bourgeois society with an astonishing artistic legacy, in what has been called the Dutch golden age (2). Jan Steen, along with Rembrandt, Vermeer, Frans Hals, and many others who populated the local guilds, brilliantly chronicled the emergence of the distinct civilization that Oliver Cromwell said preferred "gain to Godliness" (3).

Jan Steen, born in Leiden the first son of successful brewers, recorded the travails and preoccupations of his contemporaries: women in their households, cavaliers on the town, village doctors, peasants in the countryside, children skating on the frozen canals, markets overflowing with goods, and the living quarters of average people. Domesticity and family harmony ruled, along with order and grace. Lavender-scented linens, Delft tiles, tended gardens, well-swept doorsteps featured in the work of Pieter de Hooch and others. But Steen, a Catholic painter in a Calvinist country, injected comic relief into the idyllic homes of the nouveau riche, by skillfully subverting the natural and social order (2). Genteel gatherings degenerated into drunken brawls, so much so that "Jan Steen household" became synonymous with "slovenly household" (1). But Steen did not condone chaos. His message, sometimes scribbled on the painting, was always clear: Memento mori (remember death).

A gifted storyteller in the tradition of Pieter Bruegel the Elder, Steen composed and embroidered with illustrated detail accounts of human behavior (4). Under realistic and didactic light, the shimmering garments of rich merchants and the gleaming shadows of refined interiors reflected the other side of tidy, devout Holland: crowded taverns, unruly children, untrained pets, rowdy village festivals, rampant sensuality, intemperance, and general unraveling of the domestic fabric (2).

Beware of Luxury, on this month's cover of Emerging Infectious Diseases, is a humorous allegory of everyday merrymaking—celebration gone out of hand. The mother at the table near the window, in drunken stupor, is oblivious of the surroundings. The dog eats from her plate, and the children are misbehaving (smoking a pipe, raiding the cupboard, toying with mother's pearls). A pig makes off with the spigot of the wine barrel; a monkey tinkers with the clock; a duck roosts on a guest (likely a religious quack thumbing the Bible in this ungodly context); and the man of the house succumbs to the guiles of a younger woman.

The ease and spontaneity of the painting is rivaled only by its harmonious completeness. In dead center hangs a basket prophetically filled with crutches, a bell (like the ones lepers had to carry), and a sword. Prosperity has been abused—pearls before swine. Signs of impending doom find the guilty asleep at the table. Inscribed on the right hand corner of the painting is a Dutch proverb: "In good times, beware."

Jan Steen's painting mirrors Dutch society of his day, which turns out not much different from our own—not only in its lack of decorum and temperance but also in its lax domesticity. In the 17th century and far before, humans cohabited with animals, on amicable terms and in close proximity. The ancient practice of keeping pets was at the heart of domestication. Exotic pets signaled power and wealth. Steen's household would not have been complete without animals, which like children, follow in the footsteps of derelict adults—because as Steen explained in another famous painting, "You sing what you hear."

Animals, pets and not, nest in our homes and often eat from our plates and sleep in our beds. In turn, we delight in them as companions, are awed by them in the wild, and raise them for food. This cohabitation, along with undisputed benefits, has created an evolutionary conundrum, closely involved in the emergence of infectious diseases. In some of these diseases (Ebola, hantavirus, avian flu), animals outside their natural habitat may spread viruses to humans and other species. In others (AIDS, porcine reproductive and respiratory syndrome), viruses may adapt to new hosts after unintended trans-species transmission. In yet others (severe acute respiratory syndrome, monkeypox), microorganisms, "singing what they hear," travel from furry creatures to humans, in a complex zoonotic cycle.

Polyxeni Potter

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.9, No.9, September, 2003

Upcoming Issue

For a complete list of articles included in the September issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming.htm

Look in the September issue for the following topics:

Role of China in the Quest to Define and Control Severe Acute Respiratory Syndrome

Lessons from Severe Acute Respiratory Syndrome Outbreak in Hong Kong

Automated, Laboratory-based System Using the Internet for Disease Outbreak Detection, the Netherlands

Hantavirus Infection in Humans and Rodents, Northwestern Argentina

Aggregated Antibiograms and Monitoring of Drug-Resistant *Streptococcus pneumoniae*

Antibiotic Use in Hispanic Households, New York City

Dyspepsia Symptoms and *Helicobacter pylori* Infection, Nakuru, Kenya

Epidemic and Non-Epidemic Multidrug-Resistant *Enterococcus faecium*

Reemergence of Epidemic *Vibrio cholerae* O139, Bangladesh

Skunk and Raccoon Rabies, Eastern United States: Temporal and Spatial Analysis

Early Identification of Common-Source Foodborne Virus Outbreaks in Europe

Fluoroquinolone and Macrolide Treatment Failure in Pneumococcal Pneumonia and Selection of Multidrug-Resistant Isolates

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and *West Nile virus* infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: 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.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES

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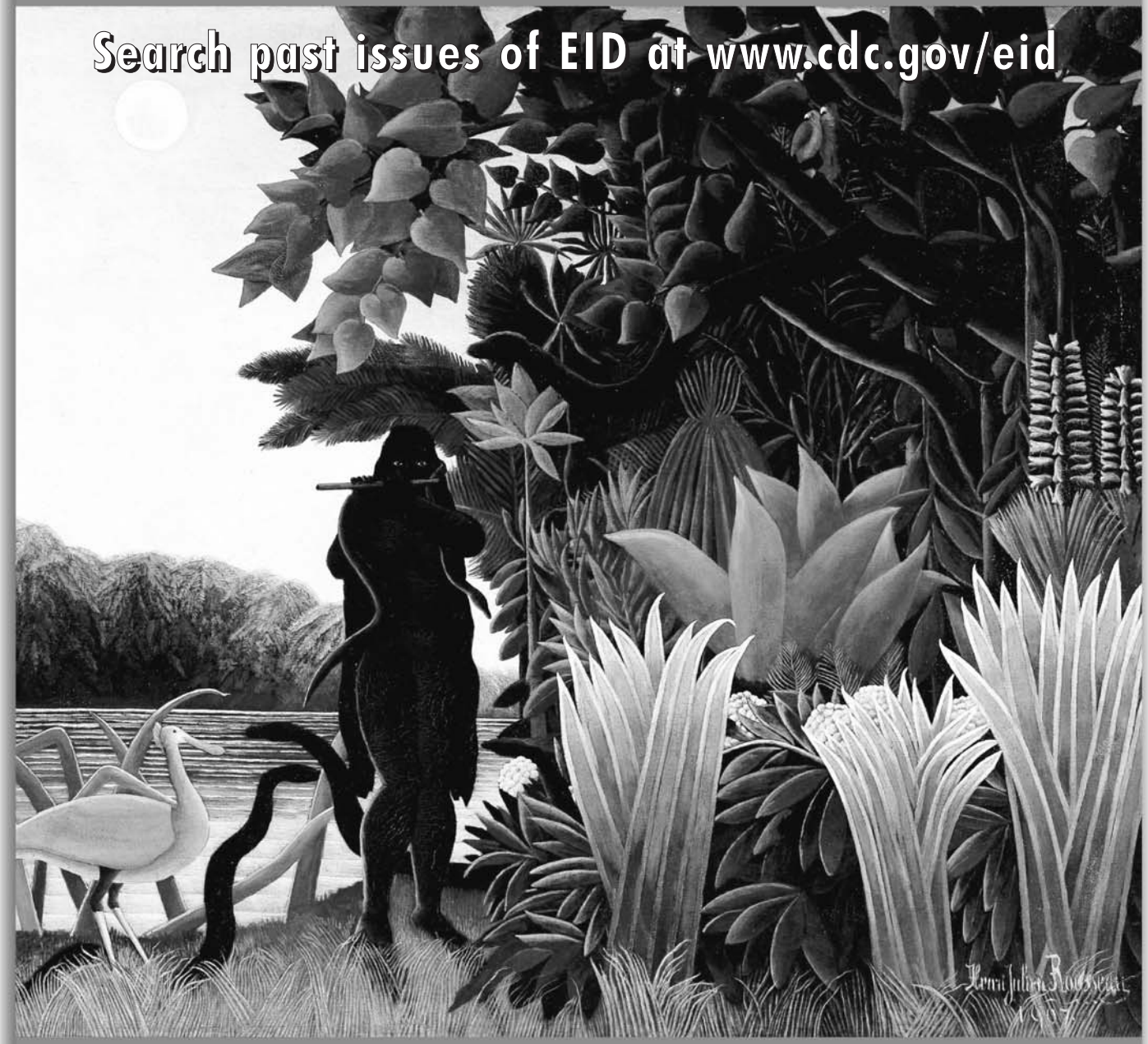

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West Nile virus

Editorial Policy and Call for Articles

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

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

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

Chinese, French, and Spanish translations of some articles can be accessed through the journal's home page at http://www.cdc.gov/eid.

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and e-mail address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (www.cdc.gov/eid).

Manuscript Types

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

Another Dimension. 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 evoke 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.

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

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. (500–1,000 words) of emerging infectious disease conferences may provide references to a full report of conference activities and should focus on the meeting's content rather than on individual conference participants.