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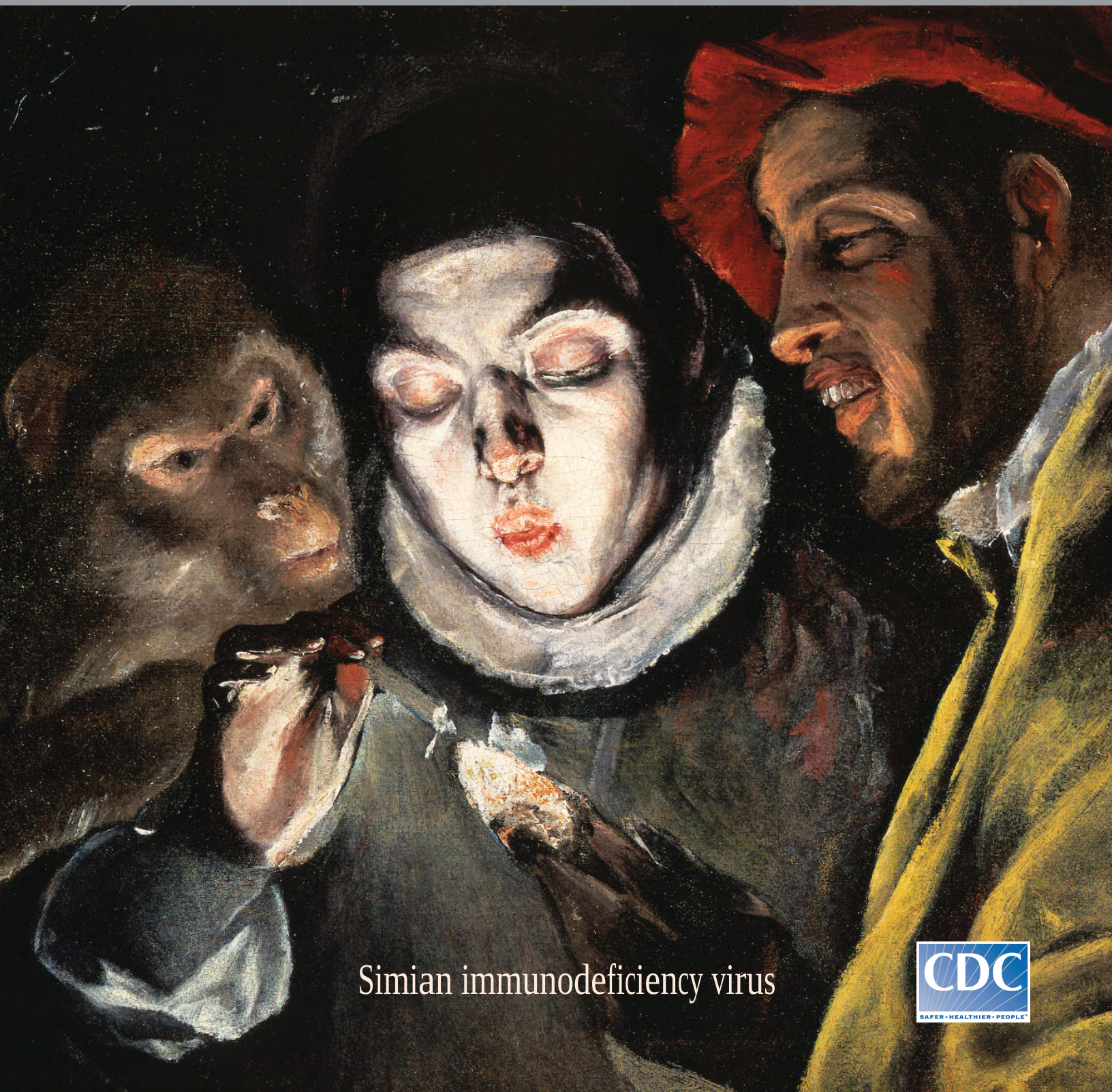

Simian immunodeficiency virus

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On the Cover: Domenikos Theodokopoulos (known as El Greco, 1541–1614); Fable, c. 1600 (oil on canvas, 50 cm x 64 cm). Courtesy of The Prado, Madrid, Spain.
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EMERGING INFECTIOUS DISEASES

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News & Notes

Risk to Human Health from a Plethora of Simian Immunodeficiency Viruses in Primate Bushmeat

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To assess human exposure to *Simian immunodeficiency virus* (SIV) in west central Africa, we looked for SIV infection in 788 monkeys that were hunted in the rainforests of Cameroon for bushmeat or kept as pets. Serologic reactivity suggesting SIV infection was found in 13 of 16 primate species, including 4 not previously known to harbor SIV. Overall, 131 sera (16.6%) reacted strongly and an additional 34 (4.3%) reacted weakly with HIV antigens. Molecular analysis identified five new phylogenetic SIV lineages. These data document for the first time that a substantial proportion of wild monkeys in Cameroon are SIV infected and that humans who hunt and handle bushmeat are exposed to a plethora of genetically highly divergent viruses.

First recognized in the early 1980s, AIDS represents the endstage of infection with one of two lentiviruses, termed *Human immunodeficiency virus type 1* (HIV-1) or *type 2* (HIV-2) (1,2). HIV-1 has spread to most parts of the world, while HIV-2 has remained largely restricted to West Africa (3,4). More than 40 million persons are estimated to have HIV infection or AIDS (4).

Both HIV-1 and HIV-2 are of zoonotic origin (5). The closest simian relatives of HIV-1 and HIV-2 have been found in the common chimpanzee (*Pan troglodytes)* and the sooty mangabey (*Cercocebus atys*), respectively (6-8), and phylogenetic evidence indicates that lentiviruses from these species (SIVcpz and SIVsm, respectively) have been transmitted to humans on at least eight occasions (5,9). Serologic evidence of SIV infection has so far been documented in 26 primate species, and 20 of these viruses have been at least partially molecularly characterized (5,10,11). Because humans come in frequent contact with primates in many parts of sub-Saharan Africa, additional zoonotic transfers of primate lentiviruses from species other than chimpanzees and sooty mangabeys are possible. The risk for acquiring SIV infection would be expected to be highest in persons who hunt primates and prepare their meat for consumption, as well as in persons who keep primates as pets. However, this risk cannot be assessed since the prevalence, diversity, and geographic distribution of SIV infections in wild primate populations are unknown. We report the first comprehensive survey of wild-caught primates in Cameroon, home to diverse primate species that are extensively hunted for food and trade (12). Much of the primate meat sold for consumption derives from infected monkeys, and a comparable number of pet monkeys also carry SIV. These data thus provide a first approximation of the magnitude and variety of SIVs to which humans are exposed through contact with nonhuman primates.

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Materials and Methods

Collection of Primate Tissue and Blood Samples

Blood was obtained from 788 monkeys wild-caught in Cameroon from January 1999 to April 2001. Species were determined by visual inspection according to the Kingdon Field Guide to African Mammals (13) and the taxonomy described by Colin Groves (14). We sampled 573 animals as bushmeat at markets in Yaounde (n=157), surrounding villages (n=111), or logging concessions in southeastern Cameroon (n=305), as well as 215 pet animals from these same areas (Table 1). All primate samples were obtained with government approval from the Cameroonian Ministry of Environment and Forestry. Bushmeat samples were obtained through a strategy specifically designed not to increase demand: women preparing and preserving the meat for subsequent sale and hunters already involved in the trade were asked for permission to sample blood and tissues from carcasses, which were then returned.

For the bushmeat animals, blood was collected by cardiac puncture, and lymph node and spleen tissues were collected whenever possible. The owners indicated that most of the animals had died 12 to 72 hours before sampling. For pet monkeys, blood was drawn by peripheral venipuncture after the animals were tranquilized with ketamine (10 mg/kg). Plasma and cells were separated on site by Ficoll gradient centrifugation. All samples, including peripheral blood mononuclear

Table 1. Wild-born primates surveyed, by species, age, and status, Cameroon

			Pet animals		Primate bushmeat		
Genus	Species	Common name	Adults	Juveniles/infants	Adults	Juveniles/infants	Total
Cercocebus	*agilis*	Agile mangabey	4	15	30	3	52
	torquatus	Red-capped mangabey	1	–	–	1	2
Lophocebus	*albigena*	Grey-cheeked mangabey	3	3	12	3	21
Cercopithecus	*cephus*	Mustached guenon	3	26	217	56	302
	mona	Mona monkey	–	7	1	1	9
	neglectus	De Brazza's monkey	2	6	21	5	34
	nictitans	Greater spot-nosed monkey	8	36	110	12	166
	pogonias	Crested mona	1	5	57	10	73
	preussi	Preuss's monkey	–	1	–	–	1
Chlorocebus	*tantalus*	Tantalus monkey	7	11	–	–	18
Miopithecus	*ogouensis*	Gabon talapoin	5	6	8	–	19
Erytrocebus	*patas*	Patas monkey	5	14	–	–	19
Colobus	*guereza*	Mantled guereza	–	2	24	–	26
Mandrillus	*leucophaeus*	Drill	–	2	–	–	2
	sphinx	Mandrill	5	15	–	2	22
Papio	*anubis*	Olive baboon	11	11	–	–	22
Total			55	160	480	93	788

cells (PBMCs), plasma, whole blood, and other tissues, were stored at –20°C.

Serologic Testing

Plasma samples were tested for HIV/SIV antibodies by the INNO-LIA HIV Confirmation test (Innogenetics, Ghent, Belgium), which includes HIV-1 and HIV-2 recombinant proteins and synthetic peptides that are coated as discrete lines on a nylon strip. Five HIV-1 antigens include synthetic peptides for the exterior envelope glycoprotein (sgp120), as well as recombinant proteins for the transmembrane envelope glycoprotein (gp41), integrase (p31), core (p24), and matrix (p17) proteins. HIV-1 group O envelope peptides are included in the HIV-1 sgp120 band. The HIV-2 antigens include synthetic peptides for sgp120, as well as recombinant gp36 protein. In addition to these HIV antigens, each strip has control lines: one sample addition line (3+) containing anti-human immunoglobulin (Ig) and two test performance lines (1+ and +/-) containing human IgG. All assays were performed according to manufacturer's instructions, with alkaline phosphatase-labeled goat anti-human IgG as the secondary antibody. We used the following working definition for SIV seropositivity: plasma samples were scored as INNO-LIA positive when they reacted with at least one HIV antigen and had a band intensity equal to or greater than the assay cutoff (+/-) lane; samples that reacted less strongly but still visibly with two or more HIV antigens were classified as indeterminant; and samples reacting with no bands or only one band with less than +/- intensity were classified as negative.

Polymerase Chain Reaction (PCR)

DNA was isolated from whole blood or PBMCs by using Qiagen DNA extraction kits (Qiagen, Courtaboeuf, France), and PCR was done with the Expand High Fidelity PCR kit (Roche Molecular Biochemicals, Mannheim, Germany). For amplification of SIV sequences, previously described degenerate consensus *pol* primers DR1, Polis4, UNIPOL2, and PolOR (15-17) were used in various combinations under previously described PCR conditions (16). PCR products were sequenced by cycle sequencing and dye terminator methods (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq FS DNA polymerase [PE Biosystems, Warrington, England]) on an automated sequencer (ABI 373, Stretch model; Applied Biosystems, Courtaboeuf, France) either directly or after cloning into the pGEM-T vector (Promega, Charbonnieres, France).

To test for DNA degradation, a 1,151-bp region of the glucose-6–phosphate dehydrogenase (G6PD) gene was amplified with the primers GPD-F1 5'-CATTACCAGCTCCATGACCAGGAC-3' and GPD-R1 5'-GTGTTCCCAGGTGACCCTCTGGC-3' in a single-round PCR reaction under the following conditions: 94°C for 2 min, then 35 cycles at 94°C for 20 sec; 58°C for 30 sec, and 72°C for 1 min (18).

Phylogenetic Analyses

Newly derived SIV nucleotide sequences were aligned with reference sequences from the Los Alamos HIV/SIV Sequence database by using CLUSTAL W (19) with minor adjustments for protein sequences. A phylogenetic tree was

constructed by the neighbor-joining method (20), and the reliability of branching orders was tested by the bootstrap approach (21). Sequence distances were calculated by Kimura's two-parameter method (22). SIV lineages were defined as clusters of SIV sequences from the same primate species that grouped together with significant (>80%) bootstrap values.

GenBank Accession Numbers

The new sequences have been deposited in GenBank under the following accession numbers: SIVgsn-99CM-CN71 (AF478588), SIVgsn-99CM-CN7 (AF478589), SIVgsn-99CM-CN166 (AF478590), SIVmon-99CM-CML1 (AF478591), SIVmus-01CM-S1239 (AF478592), SIVmus-01CM-S1085 (AF478593), SIVtal-00CM-271 (AF478594), SIVtal-00CM-266 (AF478595), SIVmnd2-99CM-54 (AF478596), SIVmnd2-01CM-S109 (AF478597), SIVmnd2-00CM-S46 (AF478598), SIVmnd2-00CM-S6 (AF478599), SIVdeb-01CM-1083 (AF478600), SIVdeb-99CM-CN40 (AF478601), SIVdeb-01CM-S1014 (AF478602), SIVdeb-99CM-CNE5 (AF478603), SIVdeb-01CM-1161 (AF478604), SIVdeb-99CM-CNE1 (AF478605), SIVcol-00CM-247 (AF478606), SIVcol-00CM-243 (AF478607), and SIVcol-99CM-11 (AF478608).

Results

Prevalence Estimates of SIV Infection in Bushmeat and Pet Monkey Samples

Previous studies of SIV infection have relied almost exclusively on surveys of captive monkeys or apes that were either kept as pets or housed at zoos, sanctuaries, or primate centers. While this approach has led to the discovery of novel SIVs (23-29), it has not provided information concerning SIV prevalence rates in the wild. Most pet monkeys are acquired at a very young age, often when their parents are killed by hunters. Two field studies of wild African green monkeys have shown that seroprevalence rates correlated with sexual maturity, suggesting transmission predominantly by sexual routes (30,31). SIV infection rates of captive monkeys may thus not accurately reflect SIV prevalence rates in the wild.

To ensure systematic sampling, we therefore collected blood from 573 monkeys sold as bushmeat and 215 pet monkeys (Table 1). Most of the bushmeat animals were adults, while most of the pets were still infants or juveniles at the time of sampling. Most primates came from the southern part of the country. All major SIV lineages known to date were initially discovered because their primate hosts had antibodies that cross-reacted with HIV-1 or HIV-2 antigens (23-29). Although the extent of this cross-reactivity has not been defined, we used a similar approach to examine the primate blood samples obtained in Cameroon. Since commercially available HIV screening assays (e.g., enzyme-linked immunosorbent assay or rapid tests) contain only a limited number of antigens, we used an HIV confirmatory assay (INNO-LIA), comprising a recombinant and synthetic peptide-based line immunoassay (Figure 1). One hundred thirty-one (16.6%) of 788 plasma samples reacted strongly with one or more HIV antigens, while an additional 34 samples (4.3%) reacted less strongly but visibly with two or more HIV antigens (Figure 1; Table 2). Of 13 primate species that had HIV cross-reactive antibodies, the prevalence of seroreactivity (positive plus indeterminant) ranged from 5% to 40%. Prevalences were lower in pet animals than in bushmeat primates, 11.6% versus 18.4%, respectively. Sera from only three species failed to react completely (*Cercopithecus preussi*, *Mandrillus leucophaeus*, *Cercocebus torquatus*), but these three species accounted for only 5 of the 788 samples tested.

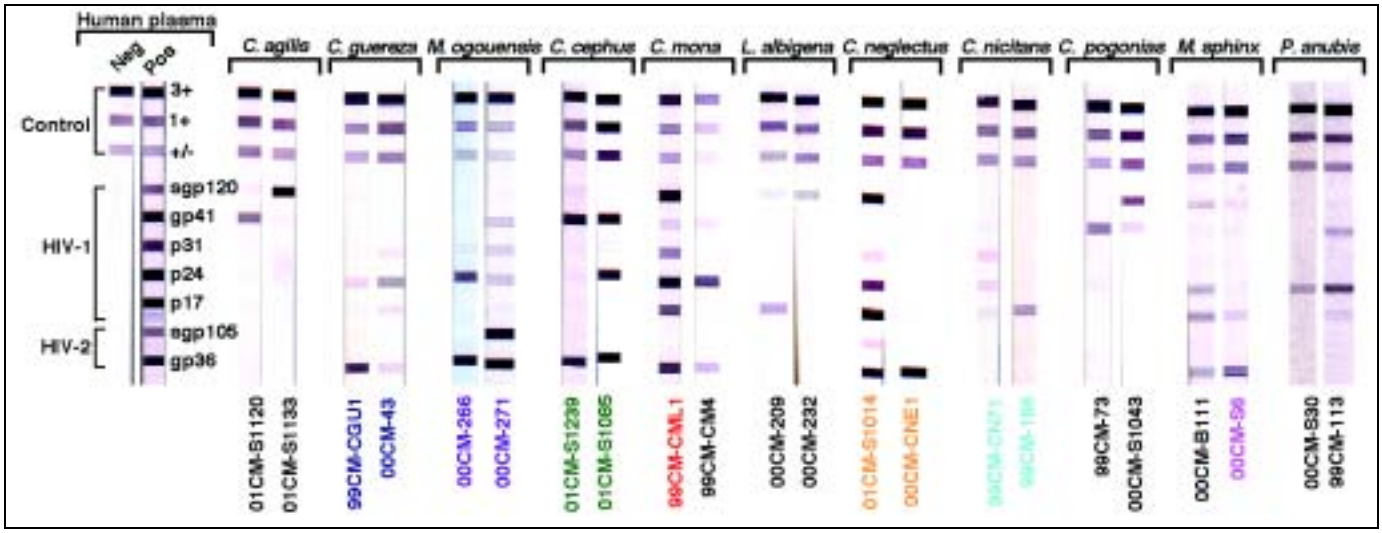

Figure 1. Detection of HIV-1/HIV-2 cross-reactive antibodies in sera from 11 primate species by using a line immunoassay (INNO-LIA HIV Confirmation, Innogenetics, Ghent, Belgium). Varying patterns of reactivity to HIV peptides and proteins (HIV-1 gp120, gp41, p31, p24, and p17; HIV-2 gp130, and gp36) are shown. Samples from which *Simian immunodeficiency virus* (SIV) sequences were subsequently amplified by polymerase chain reaction are color-coded as in Figure 2. Plasma samples from HIV-1/HIV-2-negative and -positive persons are shown as controls on the left. The 3+, 1+ and +/- bands at the top of all test strips control for sample addition (presence of plasma immunoglobulin) and test performance (binding of secondary antibody).

Table 2. HIV-1/HIV-2 cross-reactive antibodies[a] detected in primate species, Cameroon

			Pet animals		Primate bushmeat		Total	
Genus	Species	Common name	pos/tested	ind/tested	pos/tested	ind/tested	pos/tested	ind/tested
Cercocebus	*agilis*	Agile mangabey	1/19	1/19	5/33	7/33	6/52	8/52
	torquatus	Red-capped mangabey	0/1	0/1	0/1	0/1	0/2	0/2
Lophocebus	*albigena*	Grey-cheeked mangabey	0/6	0/6	2/15	3/15	2/21	3/21
Cercopithecus	*cephus*	Mustached guenon	1/29	3/29	48/273	9/273	49/302	12/302
	mona	Mona monkey	1/7	0/7	1/2	0/2	2/9	0/9
	neglectus	De Brazza's monkey	1/8	0/8	9/26	1/26	10/34	1/34
	nictitans	Greater spot-nosed monkey	6/44	0/44	22/122	3/122	28/166	3/166
	pogonias	Crested mona	0/6	0/6	9/67	4/67	9/73	4/73
	preussi	Preuss's monkey	0/1	-	-	-	0/1	-
Chlorocebus	*tantalus*	Tantalus monkey	3/18	0/18	-	-	3/18	0/18
Miopithecus	*ogouensis*	Gabon talapoin	2/11	1/11	2/8	0/8	4/19	1/19
Erythrocebus	*patas*	Patas monkey	1/19	0/19	-	-	1/19	0/19
Colobus	*guereza*	Mantled guereza	0/2	0/2	7/24	1/24	7/26	1/26
Mandrillus	*leucophaeus*	Drill	0/2	0/2	-	-	0/2	0/2
	sphinx	Mandrill	7/20	0/20	1/2	1/2	8/22	1/22
Papio	*anubis*	Olive baboon	2/22	0/22	-	-	2/22	0/22
Total			25/215	5/215	106/573	29/573	131/788	34/788
(%)			11.6%	2.3%	18.4%	5.1%	16.6%	4.3%

[a]Plasma samples were tested for antibodies cross-reactive with HIV-1 and HIV-2 antigens by using a recombinant-based line immunoassay (INNO-LIA HIV Confirmation, Innogenetics, Ghent, Belgium). Positive (pos) and indeterminant (ind) INNO-LIA scoring criteria as described in Methods.

The INNO-LIA profiles from members of the same as well as different primate species varied extensively (Figure 1). Some sera reacted only with HIV core and/or Pol proteins, while others reacted with Gag and/or Pol and/or Env proteins from either HIV-1 or HIV-2 or both. Other than classifying sera as INNO-LIA reactive or nonreactive, no banding pattern or algorithm could be derived that would have been predictive of infection of any given primate species.

Confirmation of SIV Infection by PCR and Discovery of Novel SIV Lineages

A total of 342 samples, including INNO-LIA positive (n=91), indeterminant (n=23), or negative (n=228) specimens, were subjected to PCR analysis (16,32), which yielded amplification products for 28 blood samples from seven primate species: *Cercopithecus mona*, *C. neglectus*, *C. nictitans*, *C. cephus*, *Colobus guereza*, *Miopithecus ogouensis*, and *Mandrillus sphinx* (Table 3). All these amplification products were of appropriate size. Moreover, subsequent sequence and phylogenetic analysis confirmed SIV infection (Figure 2). Most of the newly derived sequences did not fall into any of the known SIV groups. Viral sequences from *C. mona* (SIVmon), *C. neglectus* (SIVdeb), *C. nictitans* (SIVgsn), *C. cephus* (SIVmus), and *Miopithecus ogouensis* (SIVtal) formed species-specific monophyletic clusters that were roughly equidistant from each other as well as from all previously defined SIV lineages in this region of the *pol* gene. Viruses from the remaining two species (*Colobus guereza* and *Mandrillus sphinx*) grouped with previously reported SIVcol and SIVmnd-2 strains, respectively.

The single sequence of SIVmon was given lineage status because of its high degree of genetic diversity from the other SIV strains. We maintained the lineage designation of SIVtal previously assigned to a virus thought to be derived from a zoo animal of the species *M. talapoin* (28) because that sequence and the two newly derived talapoin viruses from *M. ogouensis* cluster together in a phylogenetic tree derived from additional *pol* nucleotide sequences (not shown). Thus, our new SIVtal sequences confirm the existence of this lineage in the wild .

SIV sequences were confirmed in 26 of 91 INNO-LIA-positive samples, as well as in 1 of 23 indeterminate and 1 of 223 negative samples (Table 3). Because many blood samples were obtained under poorly controlled circumstances, especially from the bushmeat markets, we tested the possibility of DNA degradation. Whole blood and PBMC DNA preparations were subjected to single-round PCR with primers designed to amplify introns 4 and 5 of the nuclear G6PD gene (1,100 bp). Of the 65 LIA-positive samples that did not yield a virus-specific PCR product, 11 also failed to yield a G6PD amplification product. Similarly, 4 of 17 INNO-LIA-indeterminate and SIV PCR-negative samples, as well as 25 of 102 INNO-LIA-negative samples, were also negative by G6PD amplification.

Table 3. Polymerase chain reaction (PCR) amplification of *Simian immunodeficiency virus* (SIV) sequences

Genus	Species	INNO-LIA pos[a] PCR pos/tested	INNO-LIA ind PCR pos/tested	INNO-LIA neg PCR pos/tested
Cercocebus	*agilis*	0/6	0/8	0/13
	torquatus	–	–	0/1
Lophocebus	*albigena*	0/2	0/2	0/7
Cercopithecus	*cephus*	2/25	0/7	0/56
	mona	1/2	–	0/2
	neglectus	8/9	–	0/4
	nictitans	3/21	1/1	0/61
	pogonias	0/9	0/3	0/34
Chlorocebus	*tantalus*	0/1	–	0/2
Miopithecus	*ogouensis*	2/3	–	0/10
Erythrocebus	*patas*	–	–	0/7
Colobus	*guereza*	6/6	0/1	1/16
Mandrillus	*sphinx*	4/5	0/1	0/4
Papio	*anubis*	0/2	–	0/11
Total		26/91	1/23	1/228

[a]DNA was extracted from a subset of seropositive (pos), indeterminant (ind) and negative (neg) blood samples and subjected to nested PCR amplification by using HIV/SIV consensus *pol* primer pairs. In each column, the number of PCR-positive samples per total number of samples tested is indicated. The authenticity of all amplification products was confirmed by sequence analysis.

These results indicate that, in addition to using only a single set of nested *pol* primer pairs, low PCR amplification rates from LIA-positive and -indeterminant samples were also due to DNA degradation, the presence of PCR inhibitors, or both.

Discussion

Zoonotic transfers of SIV to humans have been documented on no fewer than eight occasions (5,9), but no previous study has examined to what extent African primates that are frequently hunted or kept as pets are infected with SIV. Although our serologic screening approach has limitations (i.e., an unknown extent of antigenic cross-reactivity between HIV proteins and SIV antibodies), we were able to detect cross-reactive antibodies suggesting SIV infection in 16.6% of all tested animals, including members of four species not previously known to harbor SIV (*C. agilis*, *Lophocebus albigena*, *C. pogonias*, and *Papio anubis*). PCR confirmation and molecular identification of SIV infection were obtained in seven species, and phylogenetic analyses showed the presence of highly divergent viruses that grouped according to their species of origin. Four of these SIV lineages from mona *(C. mona*), De Brazza's (*C. neglectus*), mustached (*C. cephus*), and greater spot-nosed (*C. nictitans*) monkeys have not previously been recognized. Finally, we confirmed the SIVtal infection of wild talapoin monkeys (*Miopithecus ogouensis*). These data establish for the first time that a considerable proportion of wild-living primates in Cameroon are infected with SIV, posing a potential source of infection to those who come in contact with them. Our findings bring to 30 the number of African nonhuman primate species known or strongly suspected to harbor primate lentiviruses (5).

Our data likely still underestimate the prevalence and diversity of naturally occurring SIV infections in Cameroon. First, not all native primate species were tested, and many were undersampled because they were either rare in the regions of Cameroon where we sampled for this study or too small to be regularly hunted. For example, the absence of reactive sera from drills and red-capped mangabeys, two species known to harbor SIV (15,23), must be due to the low number of blood samples (5/788) analyzed. In addition, the INNO-LIA test sensitivity is clearly not 100%, as one negative sample contained SIV sequences as determined by PCR amplification. Finally, our PCR approach, which utilized only a single set of nested primers, likely amplified only a subset of viral sequences. Thus, the true prevalence of SIV infection in the various primate species will require the development of SIV lineage-specific assays with known sensitivities and specificities.

Human infection with SIVcpz and SIVsm is thought to

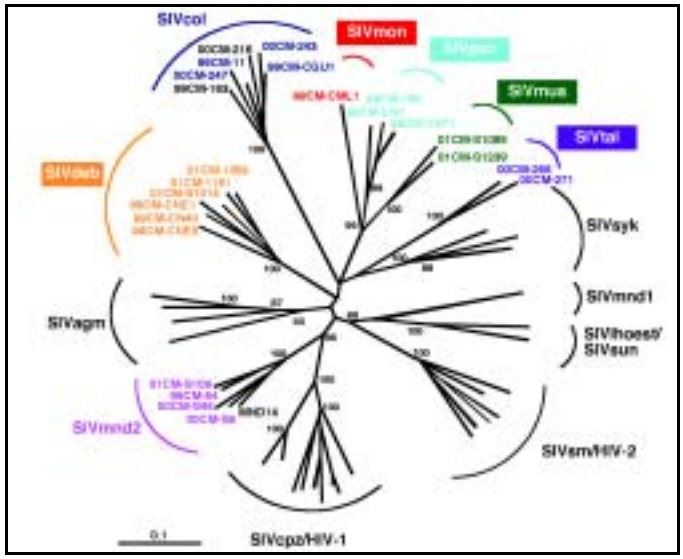

Figure 2. Identification of diverse *Simian immunodeficiency virus* (SIV) lineages in primate bushmeat. A 650-bp *pol* fragment was amplified from monkeys representing seven primate species, sequenced, and subjected to phylogenetic tree analysis by the neighbor-joining method. The positions of 21 SIV sequences from the present study (in color) are shown in relation to HIV/SIV reference sequences from the Los Alamos HIV/SIV Sequence Database (in black). The consensus length of the final alignment used for tree construction was 555 bp. The new species-specific SIV lineages are generally identified by a lower-case three-letter code corresponding to the initial letters of the common species name (e.g., SIVgsn for greater spot-nosed monkeys [*Cercopithecus nictitans*], SIVmus for mustached guenons [*C. cephus*] and SIVmon for mona monkeys [*C. mona*]). Lineages are defined as clusters of viral sequences from the same primate species that group together with significant (>80%) bootstrap values. We maintained the lineage designation of SIVtal previously assigned to a virus thought to be derived from a zoo animal of the species *Miopithecus talapoin* (28) since that sequence, and the two newly derived talapoin viruses from *M. ogouensis,* cluster together in a phylogenetic tree derived from additional *pol* nucleotide sequences (not shown). Branch lengths are drawn to scale (the bar indicates 10% divergence). The numbers at the nodes indicate the percent bootstrap values supporting the cluster to the right (only values >80% are shown).

have resulted from cutaneous or mucous membrane exposure to infected blood during the hunting and butchering of chimpanzees and sooty mangabeys for food (5). Bites from pet animals and possibly contact with fecal and urine samples may have also been involved (5). Our study shows that many primate species in addition to chimpanzees and sooty mangabeys are hunted and that 20% (or more) of these animals likely harbor SIV. Thus, if contact with infected blood or other secretions is indeed the primary route of transmission, hunters and food handlers may be at risk of infection with many more SIVs than just those from chimpanzees and sooty mangabeys.

Bushmeat hunting, to provide animal proteins for the family and as a source of income, has been a longstanding common component of household economies in the Congo Basin and, more generally, throughout subSaharan Africa (33-35). However, the bushmeat trade has increased in the last decades. Commercial logging, which represents an important economic activity in Cameroon as well as many other west-central African countries, has led to road constructions into remote forest areas, human migration, and social and economic networks supporting this industry (36). Hunters are now penetrating previously inaccessible forest areas, making use of newly developed infrastructure to capture and transport bushmeat from remote areas to major city markets (37). Moreover, villages around logging concessions have grown from a few hundred to several thousand inhabitants in just a few years (37). These socioeconomic changes, combined with our estimates of SIV prevalence and genetic complexity in wild primates, suggest that the magnitude of human exposure to SIV has increased, as have the social and environmental conditions that would be expected to support the emergence of new zoonotic infections.

Whether any of the newly identified SIVs have the ability to infect humans remains unknown since molecular evidence is lacking for SIV cross-species transmissions from primates other than chimpanzees and sooty mangabeys. However, such infections may have been unrecognized by HIV-1/HIV-2 screening assays. A case in point is the recent identification of a Cameroonian man who had an indeterminant HIV serology but reacted strongly (and exclusively) with an SIVmnd V3 loop peptide (32). Although viral sequences were not confirmed in this man, the finding suggests that at least some naturally occurring SIVs have the potential to cross the species into the human population. In fact, several recently reported SIV isolates, including SIVlhoest, SIVsun, SIVrcm, and SIVmnd2, replicate well in primary human lymphocytes in vitro (23,26,27,32,38) as do SIVcpz (25) and SIVsm (24). Thus, to determine whether additional zoonotic transmissions of SIVs have already occurred, virus type- and/or lineage-specific immunoassays and PCRs will have to be developed. Such work should receive high priority given the extent of human exposure to different SIV lineages as a result of the expanding bushmeat trade and the impact of two major human zoonoses (HIV-1 and HIV-2). Recombination between newly introduced SIVs and circulating HIVs poses still another human risk for novel zoonoses.

In summary, the current HIV-1 pandemic provides compelling evidence for the rapidity, stealth, and clinical impact that can be associated with even a single primate lentiviral zoonotic transmission event. We document for the first time that humans are exposed to a plethora of primate lentiviruses through hunting and handling of bushmeat in Cameroon, a country at the center of HIV-1 groups M, N, and O endemicity that is home to a diverse set of SIV-infected nonhuman primates. To what extent wild monkey populations in other parts of Africa are also infected with diverse SIVs is unknown. A complete and accurate assessment of all SIV-infected nonhuman primate species is needed, as well as a determination of the virus lineage(s) present in each species. Studies are also needed to determine whether zoonotic transmissions of SIVs from primates other than chimpanzees and mangabeys have already occurred and what clinical outcomes were associated with these infections. Results from these studies will yield critical insights into the circumstances and factors that govern SIV cross-species transmission and thus allow determination of human zoonotic risk for acquiring these viruses.

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Epidemiology of Urban Canine Rabies, Santa Cruz, Bolivia, 1972–1997

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We analyzed laboratory data from 1972 to 1997 from Santa Cruz, Bolivia, to determine risk factors for laboratory canine samples' testing positive for *Rabies virus* (RABV). Of 9,803 samples, 50.7% tested positive for RABV; the number of cases and the percentage positive has dropped significantly since 1978. A 5- to 6-year cycle in rabies incidence was clearly apparent, though no seasonality was noted. Male dogs had significantly increased odds of testing positive for RABV (odds ratio [OR]=1.14), as did 1- to 2-year-old dogs (OR=1.73); younger and older dogs were at lower risk. Samples submitted from the poorer suburbs of the city were more likely to test positive for RABV (OR=1.71). Knowledge of the distribution of endemic canine rabies in an urban area will help focus control measures in a resource-poor environment.

Europe and North America have successfully controlled rabies in domestic animals, leaving wildlife as the main reservoir of concern (1,2). Nevertheless, rabies remains a serious public health hazard in many developing countries, where dog bites continue to be the main mode of transmission of the disease to humans. Throughout the world, an estimated 35,000 to 100,000 people a year die of rabies (1,3). The disease also elicits fear in communities, and subsequent control measures are drains on public health budgets (4,5). Rabies is a particular problem in the larger cities of less-developed countries, with sprawling, impoverished suburbs and high densities of dogs (3,6,7). Controlling rabies in urban dog populations is seen as a more cost-effective, long-term approach to prevent human rabies than reliance on postexposure human treatment (8). To achieve control, knowledge of the epidemiology of rabies in dog populations has long been recognized as crucial (9).

In South America several larger urban areas have successfully eliminated rabies through legislation, education, and mass vaccination of dogs (10). Cities in poorer countries such as Bolivia, however, lag behind in control efforts, in large part because resources are scarce, and programs are poorly focused. In these situations, control efforts and resources must be more directed. Knowledge of risk factors for canine rabies in urban settings is needed to assess the danger to public health.

We analyzed 26 years of laboratory data on rabies diagnosis in dogs in the city of Santa Cruz, Bolivia, where rabies is endemic. We interpreted the results in light of possible biases to determine risk factors and temporal trends for rabies in the general dog population.

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Materials and Methods

Study Area

Santa Cruz is a city of 1 million inhabitants, located in the department of Santa Cruz in the lowlands of eastern Bolivia. The city has been rapidly expanding at an average rate of 6.7% per year since 1976 (11). The city center is circled by eight concentric ring-roads; municipal services and general socioeconomic status drop as distance increases from the city center. When we extrapolate from a study of the dog population conducted in 1996 (Laboratorio de Investigación y Diagnóstico Veterinario [LIDIVET], unpub. data), the canine population in 1999 in Santa Cruz was an estimated 276,034 dogs (1 dog per 4 inhabitants). Dogs are routinely vaccinated by private veterinarians and the municipality and Ministry of Health staff during 1- to 2-day annual public vaccination campaigns. Nevertheless, vaccination coverage data are unreliable. Additional municipal control measures for rabies include a dog pound, which collects and euthanizes up to 200 stray or aggressive dogs each month.

Canine rabies is a major problem in Santa Cruz, accounting for >90% of all animal rabies. In 1997, more than 2,178 people who had been bitten by dogs attended the municipal clinic for rabies prophylaxis; 1,464 required specific antirabies treatment. In 1997, three persons died of rabies, for an incidence of $0.30/10^5$ population, compared with the reported incidence of $0.025/10^5$ for Latin America in the same year (3). (Derived from 114 cases cited in that reference and using estimated Latin American population of 500 million.)

Data Collection

LIDIVET receives samples of brain tissue for rabies diagnosis from suspected cases in animals and humans. For animal samples, species, age group, sex, and bite history have been noted since 1972. Since 1994, the location where the animal was found has also been recorded by ring-road.

Canine samples come from three sources: 1) dogs that have bitten people and have been killed or have died during the 10-day observation period; 2) dogs brought in by the public or private veterinarians because they showed suspicious symptoms; and 3) stray dogs routinely collected and euthanized by the pound. Impression smears of brain tissue from the cerebellum, Ammon's horn, and medulla are examined after staining by fluorescein-labeled anti-rabies globulin (Centocor, Malvern, PA).

Data Analysis

All the records of canine samples examined at LIDIVET for rabies from within the municipal boundaries of Santa Cruz from 1972 through 1997 were analyzed. Secular trends were investigated with linear regression with EXCEL (Microsoft, Redmond, WA), using time in months as the independent variable (x) and number of cases per month as the dependent variable (y). A t-test was used to test for the significance of the slope (b). A centered moving average was applied to monthly numbers of positive cases to assess cyclicity in the data. To assess seasonality of rabies incidence, analysis of variance and the F-test were used to test for significant differences in the mean number of positives and mean percentage positive for each of the 12 calendar months. Associations between numbers of positive samples and age group, sex, and ring-road were examined by the chi-square test for unequal odds and linear trend across groups (Epi-Info 6.04, Centers for Disease Control and Prevention, Atlanta, GA).

Results

From 1972 through 1997, 9,308 samples of canine brain tissue were analyzed in Santa Cruz; the annual number of tests varied from 66 to 764. Of all samples, 4,694 (50.4%) were positive for *Rabies virus* (RABV). The annual number of positive cases varied from 45 to 383, and the annual percentage of samples positive for RABV ranged from 22.81% to 87.05%. The annual percentage of positive samples dropped during the study period, as did the annual number of cases confirmed as positive; this trend was especially apparent in the last 5 years (Figure 1).

The equation of the regression line was fitted only from 1978 because the low number of tests from 1972 through 1978 was uncharacteristic of the rest of the data. The number of monthly cases dropped significantly after 1978 (b = 0.04: t-test for slope= -4.53, 239 degrees of freedom [df], p<0.001) (Figure 2).

The cyclicity of rabies incidence suggested by Figure 1 is clearly shown by the 24-month centered moving average on cases per month in Figure 2. Since 1972, the mean number positive and mean percentage positive by calendar month (1972–1997) showed no significant variation (F-test= 0.47, p=0.92).

Gender

Twice as many samples and reported rabies cases were associated with male dogs (Table). The association between male dogs and sample positivity was significant, with an odds ratio [OR] of 1.14 (chi square=8.27, p=0.004).

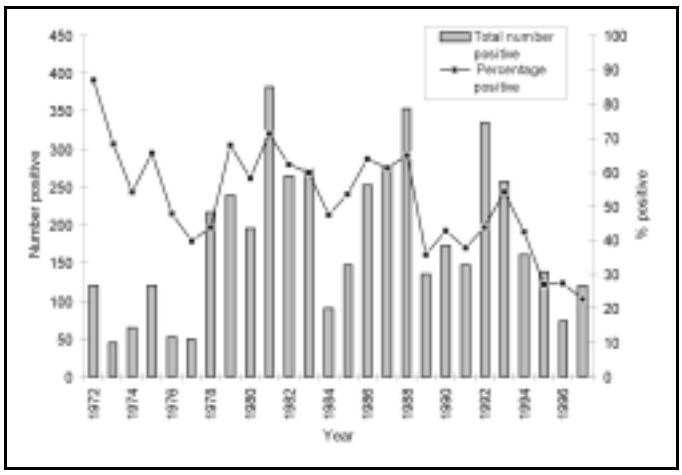

Figure 1. Percentage of rabies-positive samples and total number of positive samples* from dogs, Santa Cruz, Bolivia, 1972–1997.
* By direct fluorescent antibody test.

Age

Of the 8,352 samples for which age was recorded, dogs <1 year old accounted for 4,893 (58.6%) samples and for 2,475 (60.3%) of 4,104 positive samples (Table). The odds for positive samples varied significantly between age groups (chi-square unequal odds = 189, p<0.001). Dogs 1 to 2 years of age were significantly more likely to test positive than dogs ≤3 months old (OR=1.73, chi square = 43, p<0.001). Testing positive for RABV was less likely in dogs >2 years of age, with dogs >3 years old at lower risk than those ≤3 months old (OR=0.69, chi square = 23, p<0.001).

Location

The percentage of positive samples increased significantly as distance increased from the town center (chi-square trend = 25, 1 df p<0.001). This trend was especially evident after the sixth ring.

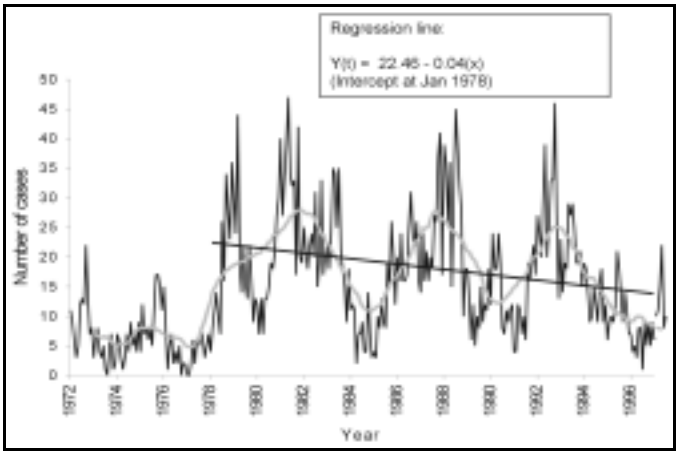

Figure 2. Positive rabies samples by month with a moving 24-month centered mean, Santa Cruz, Bolivia, 1972–1997, and regression line, 1978–1997.

Table. Canine laboratory samples examined for rabies and odds ratios for positive result, Santa Cruz, Bolivia, 1972–1997

Clinical and demographic characteristics	% of canine population[a]	No. of samples	No. (%) positive	OR (95% CI[b])
Gender				
Female	50	2,969	1,419 (47.9)	1
Male	50	5,655	2,887 (51.1)	1.14 (1.04 to 1.25)
Unknown		684	368 (56.7)	
Age group				
<3 mo	20.6	1,624	713 (43.9)	1
3 mo to l yr	16.7	3,273	1,762 (53.8)	1.49 (1.32 to 1.68)
<l yr to 2 yrs	18.8	743	802 (57.5)	1.73 (1.49 to 2.00)
>2 yrs to 3 yrs	13.2	1,395	364 (49.0)	1.23 (1.03 to 1.47)
>3 yrs	30.7	1,317	463 (35.5)	0.69 (0.59 to 0.81)
Unknown		956	590 (61.7)	
Ring road[c]				
Within 4th	50.4	631	132 (20.9)	1
4th and beyond	49.6	488	152 (31.4)	1.71 (1.29 to 2.26)
Unknown		8,189	4,410 (53.9)	
Total	100	9,308	4,694 (50.4)	

[a] Percentages of total population of owned dogs (228,170) as estimated in 1996 (LIDIVET, unpub. data).
[b] Confidence interval.
[c] Data only available since 1994.

Despite similar estimated owned dog populations, fewer samples were received from outside the fourth ring than within. Samples from dogs outside the fourth ring were significantly more likely to test positive (Table; chi square = 14.67. 1 df, $p<0.001$).

Discussion

The overall percentage of confirmed rabies in dogs submitted for diagnosis in Santa Cruz (50.4%) was similar to the 44% found in another urban study in Ghana (12). Other studies, not specifically urban, have shown percentages of samples positive varying from 54% to 67% (13-15). Canine rabies incidence appeared to decrease during the study period, especially since 1992. The significant drop in both number and percentage of positive samples suggested that this was not a reporting artifact. This decrease in incidence may be a result of vaccination, although vaccine coverage data are unreliable, and public sector vaccination has not been focused in recent years.

The data strongly suggested a 5- to 6-year cyclicity of rabies incidence; this cycle was most clearly apparent with a centered moving average of number of cases diagnosed per month. The cyclicity was independent of any changes in control measures and might explain the recent downturn in rabies cases. Some previous studies have reported a cyclical nature of rabies incidence (14,16), but this feature was not noted in an urban study in Delhi (17). Cyclicity is usually explained by increasing numbers of young, susceptible, unrestrained dogs in a population with low vaccine coverage. These factors lead to a drop in herd immunity, allowing rapid spread of the disease (6,16). However, even if underreporting is taken into account, the canine deaths from rabies in an epidemic year are unlikely to have substantially affected the number of susceptibles. A reduction in susceptibles may also be due to dogs' becoming immune after recovering from clinical or inapparent infections. Recovery of dogs from rabies is well documented (18-20); one study showed 20% of experimentally infected dogs recovered (21). Also, serologic evidence shows that almost 20% of unvaccinated dogs in Thailand have been exposed to rabies (22).

Gender does seem to be a risk factor for sample positivity: male dogs had a significantly higher percentage of samples diagnosed positive (OR 1.14). This increased risk may be explained by males' fighting over females. Studies in Mexico and India (6,17) show higher numbers of male dogs being affected, though the authors concluded that gender was not a risk factor, perhaps because of low numbers studied.

Rabies was not evenly distributed in Santa Cruz. The percentage of positive samples increased significantly with distance from the city center and as socioeconomic status dropped. In addition, a higher number of positives were reported from beyond the fourth ring-road, despite a similar-sized dog population. Some of the increase in percentage positive (but not in number positive) as distance increased from the center may be due to reporting bias. Fewer samples were submitted from outside the fourth ring, and these samples probably included a higher proportion of dogs that showed specific signs of rabies. An association of increased risk for canine rabies and areas of low socioeconomic status has also been shown in Mexico (6). Lower vaccination coverage and increased densities of unrestrained dogs have previously been reported to be associated with poorer urban areas (23), a characteristic also shown in a recent survey of the canine population in Santa Cruz (LIDIVET, unpub. data).

Age was a clear risk factor for sample positivity in our study. The median age of a dog that tested positive for rabies was up to 1 year, as found in Mexico (6). The age group most at risk of testing positive for rabies, however, was 1- to 2-year-old dogs (OR 1.73). Dogs 3 months to 1 year of age were at intermediate risk (OR 1.49); however, this risk for rabies in dogs up to 1 year old may be underestimated. Perhaps because of the die-off of dogs of that age from all causes and the relative ease of carrying a puppy to the laboratory, the proportion of submissions (68% of all samples) from dogs <1 year was high, even relative to the population (37% of all dogs). This disproportion led to the finding of more positive samples but also to less specific reporting, with proportionally more nonrabid dogs with vague symptoms; such dogs would not have been submitted had they been older. This discrepancy may

have decreased the percentage positive and thus underestimated the comparative risk for rabies in dogs <1 year old. It is nonetheless plausible that the risk for contracting rabies in dogs <1 year is lower than for 1- to 2-year-old dogs. Although dogs <1 year are less likely to have been vaccinated, sexually immature dogs are also less likely to roam, interact, and fight with other dogs. Puppies <3 months old may also benefit from passive immunity from their mothers. The large population of puppies, however, and their increased contact with children and adults make them a particular public health risk. The decreasing odds of sample positivity after 2 years of age may be due to increased likelihood of vaccination and less fighting among older dogs. Older dogs are also more likely to be owned.

We have shown that laboratory data can provide important information on risk groups and temporal trends for rabies in an urban environment. Especially if combined with additional work on the epidemiology of dog bites and seroepidemiologic studies, such data can help to effectively focus rabies-control efforts.

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Clonal Groupings in Serogroup X *Neisseria meningitidis*

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The genetic diversity of 134 serogroup X *Neisseria meningitis* isolates from Africa, Europe, and North America was analyzed by multilocus sequence typing and pulsed-field gel electrophoresis. Although most European and American isolates were highly diverse, one clonal grouping was identified in sporadic disease and carrier strains isolated over the last 2 decades in the United Kingdom, the Netherlands, Germany, and the United States. In contrast to the diversity in the European and American isolates, most carrier and disease isolates recovered during the last 30 years in countries in the African meningitis belt belonged to a second clonal grouping. During the last decade, these bacteria have caused meningitis outbreaks in Niger and Ghana. These results support the development of a comprehensive conjugate vaccine that would include serogroup X polysaccharide.

Bacterial meningitis due to *Neisseria meningitidis* (meningococcus) causes epidemics in Africa usually associated with serogroup A meningococci. Sporadic cases, outbreaks, and hyperendemic disease in Europe and the United States are usually caused by serogroups B and C (1). Occasionally, however, endemic disease and outbreaks are caused by bacteria belonging to other serogroups, including W135, Y, and X. Serogroup X *N. meningitidis* was described in the 1960s (2,3), and serogroup X meningitis has been observed in North America (4), Europe (5,6), Australia (7), and Africa (8,9). Serogroup X outbreaks have been reported in Niger (10,11) and Ghana (12). In some cases, serogroup X disease has been associated with a deficiency of particular complement components (13,14) or with AIDS (15).

Asymptomatic nasopharyngeal carriage of *N. meningitidis* is common, and in only a small percentage of colonized persons do the bacteria invade the bloodstream and cerebrospinal fluid to cause disease. Meningococcal populations are highly diverse, and lineages of meningococci with increased capacity to cause invasive disease are thought to arise periodically and spread, sometimes globally (16). Relatively few of these hyperinvasive lineages or clonal groupings are responsible for most meningococcal disease worldwide (17). These clonal groupings diversify during spread (18,19), primarily as a result of frequent horizontal genetic exchange (19-21). However, many variants are isolated only rarely or from a single country and are not transmitted further because of bottlenecks associated with geographic spread and competition (19,22). The population structure of *N. meningitidis* is effectively panmictic as a result of frequent horizontal genetic exchange (23), but that of some groupings, such as epidemic serogroup A meningococci, is largely clonal (24). The population structure of serogroup X meningococci has not yet been investigated in detail.

After an epidemic of serogroup A disease in 1997-1998 in northern Ghana (9), we conducted a longitudinal carriage study to investigate the dynamics of meningococcal carriage during an interepidemic period (12). We observed a sharp increase in nasopharyngeal carriage of serogroup X meningococci by healthy persons, accompanied by several cases of serogroup X meningitis. To investigate the phylogenetic relationships of these bacteria, we compared the isolates from Ghana with other serogroup X meningococci isolated during recent decades in Africa, Europe, and North America.

Materials and Methods

Bacterial Strains

We analyzed 134 *N. meningitidis* isolates of serogroup X by pulsed-field gel electrophoresis (PFGE) (130 isolates) or multilocus sequence typing (MLST) (41 isolates). Of these bacteria, 102 were isolated in Africa from 1970 to 2000: from meningitis patients (9 isolates) and healthy carriers (70 isolates) in Ghana, 1998-2000; from healthy carriers in Mali in 1970 (9 isolates) and 1990-91 (4 isolates); and from patients in Chad (1995, 1 isolate), Niger (1997-1998, 4 isolates), and Burkina Faso (1996-1998, 5 isolates). Six isolates were not tested serologically; the other 96 were NT:P1.5.

In addition, 32 serogroup X strains isolated from 1988 to 2000 in the United Kingdom (22 isolates), Germany (3 isolates), United States (4 isolates), France (1 isolate), Norway (1 isolate), and the Netherlands (1 isolate) were included in the analysis. The 26 strains subjected to further testing had diverse serotypes (2b, 4, 4/21, 14, 16, 21, 22) and serosubtypes (nonsubtypable [NST], P1.5, P1.5,10, P1.7, P1.12, P1.14, P1.15, P1.16) in various combinations.

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Molecular Typing of Bacteria

PFGE was done by digesting chromosomal DNA prepared in agarose blocks with *NheI* and *SpeI* as described (22), and MLST by sequencing gene fragments of *abc*Z, *adk*, *aro*E, *fumC*, *gdh*, *pdh*C, and *pgm*, also as described (16; http://www/mlst.net). The detailed MLST results and sources of isolates have been deposited in a public database (http://www.mlst.net). Additional MLST data for 31 isolates in 30 sequence types were obtained with permission from http://www.mlst.net.

Data Analysis

A neighbor-joining tree was constructed by using the numbers of MLST allele differences with Bionumerics 2.0 (25).

Results

PFGE with two discriminatory rare-cutting enzymes (*NheI* and *SpeI*) was used to identify groups of closely related strains in 134 isolates of serogroup X *N. meningitidis* from countries in Africa, Europe, and North America. All but 3 of 102 isolates from Africa had similar PFGE patterns (Figure 1, clonal grouping X-I). In contrast, 19 of 32 isolates from Europe and North America had distinct PFGE patterns (Figure 2) that differed from those of the African isolates. However, among the latter 32 strains, similar PFGE patterns were observed for 13 isolates from the United Kingdom, Germany, the Netherlands, and the United States (Figure 2, clonal grouping X-II).

Forty-one isolates, each representing a distinct PFGE pattern, were analyzed by MLST. For bacteria from which multiple isolates with a similar PFGE pattern had been detected, we tested at least one representative from each year and country of isolation. Together with other data in the MLST WEB site (http://www.mlst.net), 39 distinct sequence types (STs) have been found in 50 serogroup X meningococci. The general structure of a neighbor-joining tree of allelic differences resembles a bush, with little phylogenetic structure (Figure 3). However, isolates with similar PFGE patterns were assigned to closely related STs. All 29 clonal grouping X-I isolates analyzed by MLST were in STs ST181, ST182, or ST751 (Figure 3), which differ by one to three of the seven gene fragments (Table). Similarly, all five clonal grouping X-II isolates were in STs 24 and 750, which differ by one of the seven gene fragments (Table). The three unusual African isolates (strain designations D87, D91, and D93) were in ST188, which is very distinct from STs of clonal grouping X-I (Figure 3). These results show that numerous serogroup X isolates from Africa and nearly half the serogroup X isolates from Europe and North America belong to two clonal groupings, while other serogroup X isolates from Europe or North America are quite diverse.

Serologic Results

African isolates of clonal grouping X-I were NT:P1.5. The 11 North American and European isolates of clonal grouping X-II for which serologic data were available were 21:P1.16. Diverse serotype and serosubtype patterns were found for the other isolates from North America and Europe

The PFGE patterns distinguished two finer groups (Ia and Ib) in clonal grouping X-I, which differ consistently in four *NheI* and three *SpeI* fragments (Figure 1). All 14 group Ia strains tested were either ST181 or ST182, which differ at one of the seven gene fragments (Table). All 15 group Ib strains tested were ST751, which differs from ST181 and ST182 at two to three loci (Table). Group Ia included 10 isolates from Mali (1970-1990), 4 isolates from Niger (1997-1998), and the sole isolate from Chad (1995), as well as one of 79 isolates

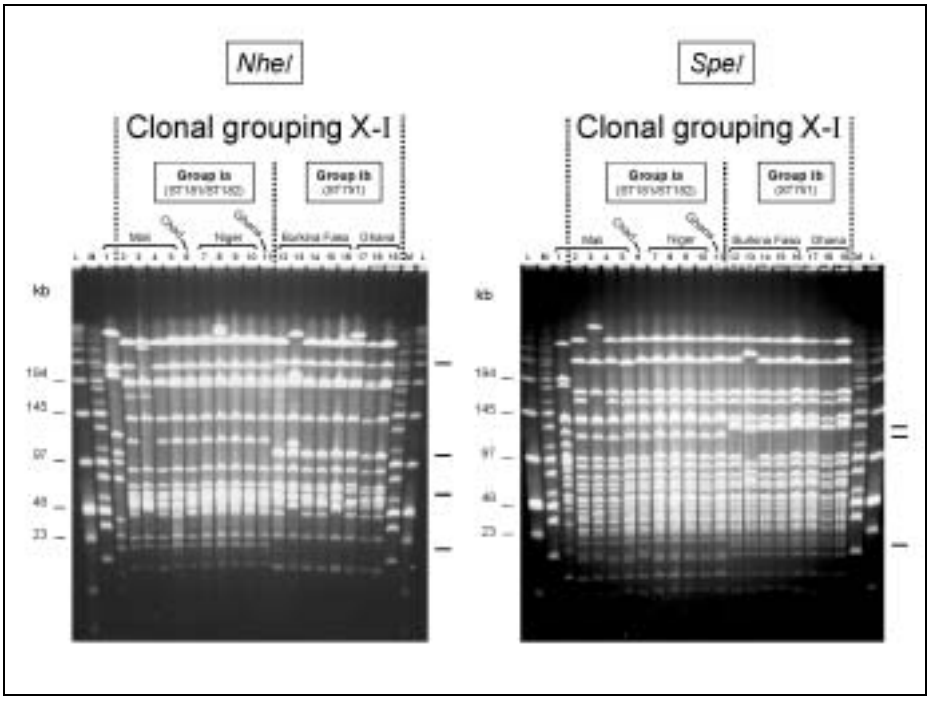

Figure 1. Two groups of pulsed-field gel electrophoresis patterns among *NheI*- and *SpeI*-digested chromosomal DNA from selected serogroup X *Neisseria meningitidis* strains isolated in Africa. Lane: strain: 1: D93 (ST188); 2: 1970; 3: 3187; 4: 3529; 5: D5; 6: LNP13407; 7: LNP14964; 8: LNP15040; 9: 97013; 10: 97014; 11: Z9413; 12: LNP14297; 13: LNP15061; 14: BF2/97; 15: BF5/97; 16: BF1/98; 17: Z7091; 18: Z8336; 19: Z9291. Molecular weight markers were loaded in the flanking lanes as indicated (L: low-range marker; M: midrange marker); their molecular weights are indicated at the left. Characteristic band differences are indicated on the right.

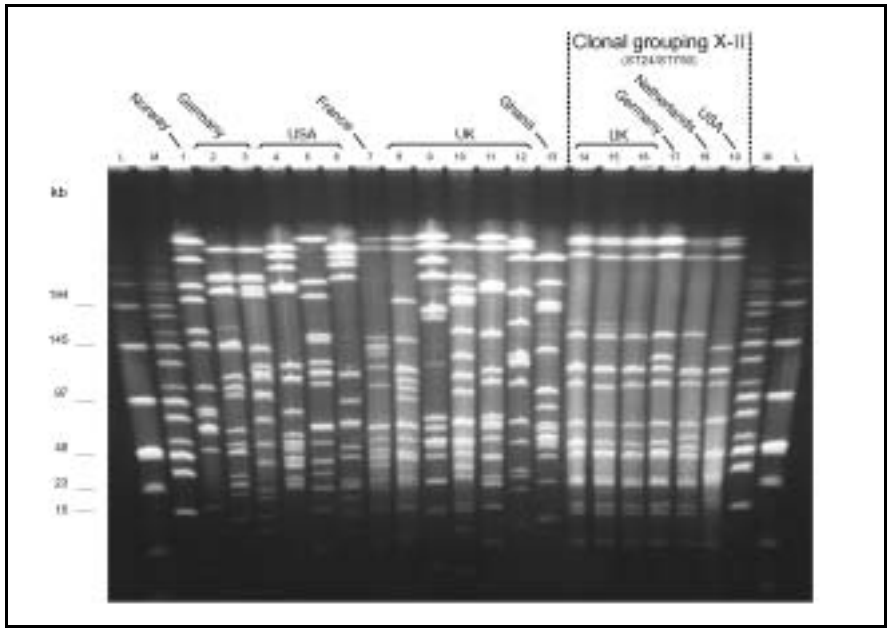

Figure 2. Pulsed-field gel electrophoresis patterns of *Nhe*I-digested chromosomal DNA of selected serogroup X *Neisseria meningitidis* isolates from Europe and the United States, plus a prototype isolate from Ghana (lane 13). Lane: strain: 1: E26; 2: X4571; 3: X4890; 4: M2526; 5: M4222; 6: M3772; 7: LNP17351; 8: J88-603; 9: K89-1395; 10: L92-1413; 11: M98-253172; 12: M00-240465; 13: Z9291; 14: M98-252848; 15: M98-252718; 16: M99-240899; 17: X5967; 18: 860060; 19: M4370.

from Ghana (2000). All five isolates from Burkina Faso (1996-1998) and 78 of 79 isolates from Ghana (1998-2000) were in group Ib.

Discussion

The general population structure of *N. meningitidis* is panmictic as a result of the frequent import of alleles from unrelated neisseriae (20,23). Furthermore, several MLST studies have demonstrated that meningococci from healthy carriers are highly diverse (16,26). Phylogenetic trees of different housekeeping genes from *N. meningitidis* are no more congruent with each other than with random trees (27). Our results for sequence typing of housekeeping genes of serogroup X meningococci also fit this pattern. Phylogenetic analysis of allele differences resulted in a bushlike tree that does not seem to contain any deep phylogenetic information. However, two clonal groupings were found in this otherwise panmictic group of bacteria. The same isolates were assigned to both clonal groupings by two independent methods, MLST and PFGE, indicating that these assignments reflect real genetic relationships and do not depend on the methods used.

Similar concordant genetic relationships were discerned in epidemic serogroup A *N. meningitidis* by multilocus enzyme electrophoresis (MLEE), random amplification of polymorphic DNA (RAPD), and MLST; population genetic analyses confirmed that the population structure of these bacteria is clonal (28). Concordant groupings were also discerned by MLEE and MLST among the so-called hypervirulent serogroup B and C isolates of the ET-5 complex, ET-37 complex, lineage III, and cluster A4 (16). Although their apparent clonality may reflect an epidemic population structure (23), this possibility has been excluded for epidemic serogroup A meningococci (28). Therefore, multiple clonal groupings exist in *N. meningitidis*, even though the population structure of most of the species is panmictic.

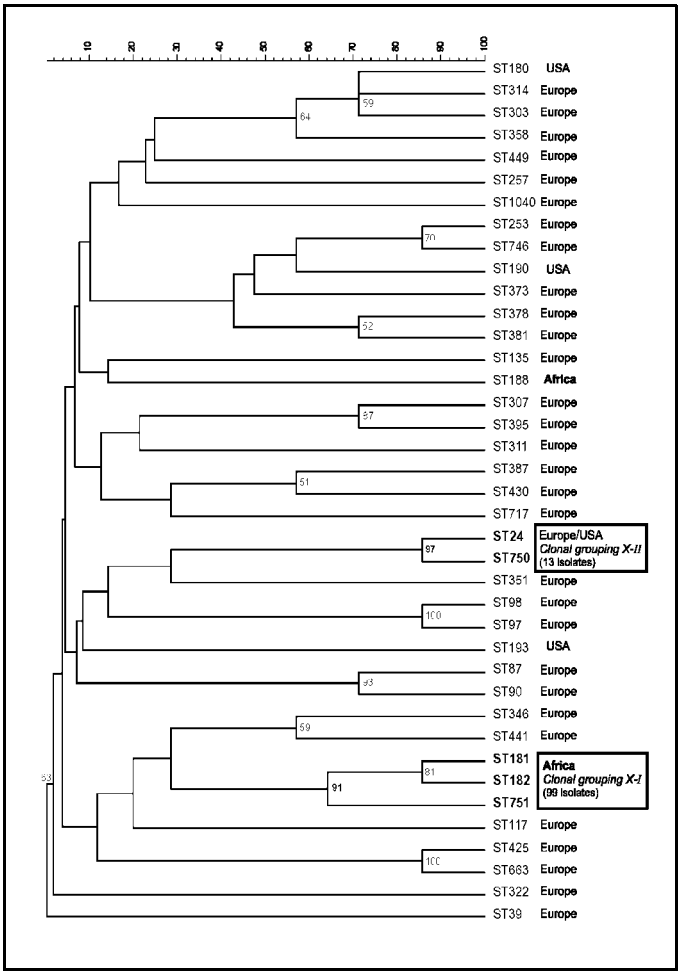

Figure 3. Neighbor-joining phenogram of allelic identities in 39 multilocus sequence typing (MLST) sequence types from serogroup X *Neisseria meningitidis*. Numbers at nodes are the percentages of 1,000 bootstrap replicates in which these nodes appeared. Only nodes with percentages >50% were included. The two boxes indicate clonal groupings that were detected by MLST or pulsed-field gel electrophoresis.

Table. Multilocus sequence typing results of two serogroup X *Neisseria meningitidis* clonal groupings

	Allele numbers								
ST	AbcZ	Adk	aroE	FumC	gdh	pdhC	Pgm	Country (no. of isolates)	Yr
24	2	5	2	7	15	20	5	Netherlands (1), United States (1)	1986, 1993
750	2	5	2	9	15	20	5	United Kingdom (2), Germany (1)	1998-1999
181	10	3	15	7	5	41	31	Mali (6), Chad (1), Niger (2), Ghana (1)	1970-2000
182	10	3	15	26	5	41	31	Mali (4)	1970
751	10	3	15	7	8	41	6	Burkina Faso (3), Ghana (12)	1996-2000

The population structure of subgroup III serogroup A meningococci seems to represent continual, sequential replacement of fit genotypes by related variants during periods of several years to decades (19). In subgroup III, nine genoclouds, each consisting of a frequent genotype plus its rarer, less fit variants, have been identified during 3 decades of pandemic spread. Our PFGE data for clonal grouping I of serogroup X suggest that clonal grouping X-I also has a genocloud structure. Two sets of PFGE variants (group Ia and Ib), which might each represent a genocloud, were detected in different countries (Mali, Chad, and Niger; and Burkina Faso and Ghana, respectively). Additional analyses of polymorphic genes are necessary to clarify the uniformity of these groups and to test the similarity of their population structure compared with that of subgroup III.

Both serogroup X clonal groupings described here were isolated over decades, on multiple occasions, and from diverse locations. Clonal grouping X-I (1970-2000) was isolated from different countries in West Africa, and clonal grouping X-II (1986-1999) was isolated from Europe and North America. For clonal grouping X-I in Ghana, the disease rate in healthy carriers was estimated to be 3/10,000 (12). Clonal grouping X-1 is thus of considerably lower virulence than serogroup B ET-5 complex bacteria (disease/carrier rate of 2,100/10,000 [29]) or serogroup A subgroup III bacteria during a postepidemic period in a vaccinated population (100/10,000 [9]).

The relationship between bacterial fitness and clonality has not yet been investigated extensively in natural isolates. Variation in virulence between bacterial genotypes leads to more uniformity in disease isolates than in carriage organism in *Streptococcus pneumoniae* (30) and *Staphylococcus aureus* (31). However, our data suggest that the clonal structure of certain meningococcal genotypes need not reflect virulence but rather is associated with genotypes that are particularly fit at colonizing the nasopharynx and spreading from person to person.

Although clonal grouping X-I bacteria are less virulent than serogroup A and B meningococci, they are still pathogenic. Most strains described here were isolated from asymptomatic carriers or patients with rare endemic cases. However, group Ia caused a meningitis outbreak with >60 cases in 1997 in Niger (11). Group Ib caused a smaller outbreak in 2000 in Ghana (12). These results suggest that X-I meningococci may even be capable of causing epidemics. Meningococci are naturally transformable, and horizontal DNA transfer is frequent in these bacteria (20-22). Meningococcal carriage is usually low in interepidemic periods in Africa (1,12,32,33), offering less opportunity for horizontal genetic exchange, which could account for the low genetic variability in serogroup X meningococci in Africa.

For more than a decade, many countries in the African meningitis belt have vaccinated extensively with A/C polysaccharide vaccines (34). Recently, mass vaccination with conjugated serogroup C vaccines has been implemented in the United Kingdom, and strong initial protection has been reported (35)**.** However, if effective, these vaccines may well select for the spread of bacteria for which they are not protective (36), including unusual causes of disease such as serogroups Y, W135, and X. Capsule switching due to DNA transformation has been documented (37,38), and effective vaccination against serogroups A and C may select for capsule switch variants of fit genotypes expressing a capsular polysaccharide not included in the vaccination program. The recent outbreaks after the 2000 Hajj pilgrimage, caused by W135 ET-37 complex meningococci (39,40), may reflect exactly such selection. These findings support the development of comprehensive conjugate vaccines that include capsular polysaccharides from formerly rare causes of disease such as serogroup X.

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genetic characterization of *Neisseria meningitidis* and *Mycobacterium tuberculosis.*

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Genetic Variants of *Ehrlichia phagocytophila*,[1] Rhode Island and Connecticut

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Primers were used to amplify a 561-bp region of the 16S rRNA gene of *Ehrlichia phagocytophila* from *Ixodes scapularis* ticks and small mammals collected in Rhode Island and Connecticut. DNA sequences for all 50 *E. phagocytophila*-positive samples collected from 1996 through 1998 in southwestern Connecticut were identical to the sequence reported for *E. phagocytophila* DNA from confirmed human cases. In contrast, the sequences from 92 of 123 *E. phagocytophila*-positive Rhode Island samples collected from 1996 through 1999 included several variants differing by 1-2 nucleotides from that in the agent infecting humans. While 11.9% of 67 *E. phagocytophila*-positive ticks collected during 1997 in Rhode Island harbored ehrlichiae with sequences identical to that of the human agent, 79.1% had a variant sequence not previously described. The low incidence of human ehrlichiosis in Rhode Island may in part result from these variant ehrlichiae's interference with the maintenance and transmission of the true agent of human disease.

Members of the genus *Ehrlichia* are obligate, intracellular bacteria in the order *Rickettsiales*. Although ehrlichial infections of veterinary importance were first described in 1935, the first case of human ehrlichiosis in the United States was reported in 1987 (1). The human pathogen was subsequently identified as *Ehrlichia chaffeensis* (2), and the number of reported human cases now exceeds 740 (3). In 1994, a second ehrlichial infection in humans was reported, called human granulocytic ehrlichiosis (HGE) because of its proclivity to infect neutrophils (4). Most HGE cases have been diagnosed in the northeastern and upper midwestern United States, although a few cases have been reported in Europe and northern California (5-12).

The close genetic and antigenic relationship of the HGE agent to two previously characterized species (*E. phagocytophila*, noted for infections of ruminants in Europe, and *E. equi*, the agent of equine granulocytic ehrlichiosis) has led to the suggestion that these three be classified as a single species, with *E. phagocytophila* as the precedent name. The 16S rRNA gene has been amplified and sequenced from confirmed human cases in both North America and Europe, and all sequences have been identical to the original published sequence for the HGE agent (4,8), with the exception of two cases recently reported from northern California that were the same as the *E. equi* 16S rRNA gene sequence (12). A variant that differed by 2 bp from the sequence of the HGE agent was reported in white-tailed deer in Maryland and Wisconsin and in *Ixodes scapularis* ticks collected in Rhode Island (13,14). Likewise, the 16S rRNA sequences determined from documented infections of horses and ruminants by various *E. phagocytophila* strains have differed by several bp from the sequences of the HGE agent. None of the variant forms have been shown to cause human disease. Another ehrlichia found in nature, which is closely related to *E. phagocytophila* but apparently does not cause human disease, is the white-tailed deer agent (15). Ehrlichiae closely related to *E. phagocytophila* recently have been identified in Colorado, where human ehrlichiosis is not endemic (16). These data suggest that only a subset of the *E. phagocytophila* strains that exist in nature can cause human disease. Although the HGE agent is now considered a member of the species *E. phagocytophila*, hereafter we will designate the isolates responsible for human disease as *E. phagocytophila*-human agent (EP-ha) to differentiate them from the 16S rRNA sequence variants we describe in this report.

Rhode Island and Connecticut, adjacent northeastern states, have similar frequency and distribution of vector *I. scapularis* ticks and primary reservoir hosts (white-footed mice and chipmunks) (17-19). However, the number of reported human infections with *E. phagocytophila* is dramatically higher in Connecticut than in Rhode Island. Through 1997, the average annual incidences (per one million population) of HGE in Connecticut and Rhode Island were 15.90 and 0.67, respectively (3). We investigated the frequency and distribution of *E. phagocytophila*, including EP-ha and *E. phagocytophila*-related variants, in potential reservoir and vector populations in Rhode Island and Connecticut.

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[1]Since this study was conducted, new nomenclature (*Anaplasma phagocytophila*) has been proposed; see Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia*, and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol 2001;51:2145-65.

Materials and Methods

Tick and Mammal Collections

Questing nymphal and adult black-legged ticks (*I. scapularis*) collected from four sites in South Kingstown, Rhode Island, and one site in Bridgeport, Connecticut, were analyzed for the presence of granulocytic ehrlichiae (Figure). Collections of adult- or nymphal-stage ticks or both were available from ongoing tick surveillance conducted in each region from 1996 to 1999. Questing nymphal ticks collected from Bluff Point in southeastern Connecticut in 1997 were also available. The Rhode Island sites are all located in the state's zone of highest *I. scapularis* density (17). Tick density was also high at the Bridgeport site. Ticks were collected by following standardized sampling procedures (17). All ticks were stored for <2 years in 70% ethanol until tested. Small rodents, including white-footed mice (*Peromyscus leucopus*) and chipmunks (*Tamias striatus*) live-trapped at the same locations were bled following procedures approved by the institutional animal care and use committees of each institution. Briefly, animals were trapped from July to September along transect lines or in trapping grids. An additional collection was made during May 1998 at the Bridgeport site. Blood was stored in EDTA at -80°C until tested for ehrlichiae by polymerase chain reaction (PCR) techniques and DNA sequencing.

Sample Preparation

DNA was extracted directly from blood samples by using a QIAamp Blood Extraction Kit (Qiagen, Chatsworth, CA), according to manufacturer's instructions. Briefly, detergent lysis was done in the presence of proteinase K for 10 min at 70°C. The lysed material was applied to a spin column containing a silica gel-based membrane and then washed twice. Purified DNA was eluted from the columns in 200 µL Tris-HCl (10 mM, pH 8.0) and stored at 4°C until used as template for PCR amplification. DNA was extracted from *I. scapularis* ticks by a modification of the manufacturer's protocol for the QIAamp Tissue Kit (Qiagen) as described (13).

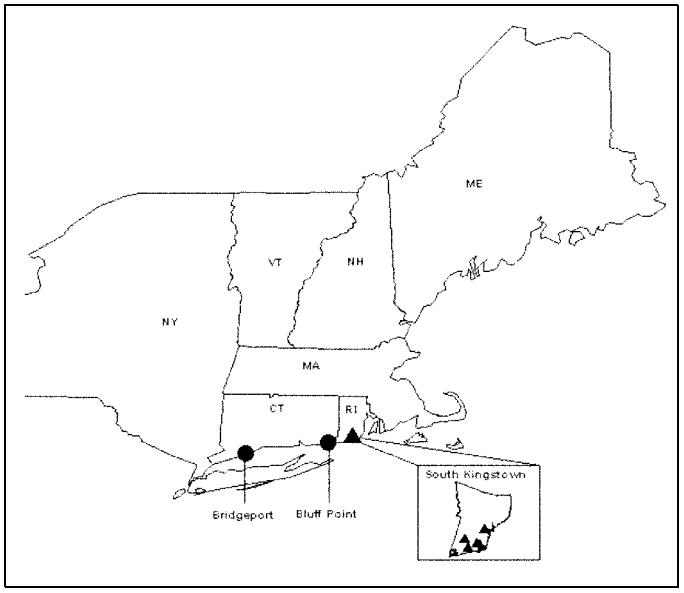

Figure. Map of northeastern United States, showing location of tick and rodent sampling sites.

PCR Analysis

A nested PCR that amplified a 546-bp portion from the 5´ region of the 16S rRNA gene was used to identify granulocytic ehrlichiae in tick and wildlife samples (13). Briefly, primary amplifications consisted of 40 cycles in a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer, Applied Biosystems Division, Foster City, CA), with each cycle consisting of a 30-sec denaturation at 94°C, 30-sec annealing at 55°C, and a 1-min extension at 72°C. The 40 cycles were preceded by a 2-minute denaturation at 95°C and followed by a 5-minute extension at 72°C. Primary amplifications used primers ge3a and ge10r and reagents from the GeneAmp PCR Kit with AmpliTaq DNA Polymerase (Perkin-Elmer). Each reaction contained 5 µL of purified DNA as template in a total volume of 50 µL, as well as 200 µM each deoxynucleoside triphosphate (dNTP) (dATP, dCTP, dGTP, and dTTP), 1.25 units Taq polymerase, and 0.5 µM each of primer. Reaction products were subsequently maintained at 4°C until analyzed by agarose gel electrophoresis or used as template for nested reactions.

Nested amplifications used primers ge9f and ge2 and 1 µL of the primary PCR product as template in a total volume of 50 µl. Each nested amplification contained 200 µM each dNTP (dATP, dCTP, dGTP, and dTTP), 1.25 units Taq polymerase, and 0.2 µM each of primer. Nested cycling conditions were as described for the primary amplification, except 30 cycles were used. Reactions were subsequently maintained at 4°C until analyzed by agarose gel electrophoresis or purified for DNA sequencing (13).

DNA Sequencing and Data Analysis

All samples producing positive PCR products were subjected to DNA sequencing reactions with fluorescent-labeled dideoxynucleotide technology (Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer, Applied Biosystems Division). Sequencing reaction products were separated, and data were collected with an ABI 377 automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division). The full sequence was determined for both strands of each DNA template to ensure maximum accuracy of the data. Sequences were edited and assembled with the Staden software programs (20) and analyzed with the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI) (21).

Results

Fifty (13.3%) of 375 *I. scapularis* ticks from Bridgeport, Conn., were PCR positive for ehrlichiae (Table 1). The percentage positive in each of the 4 years ranged from 6.1% in nymphs in 1998 to 23.3% in adults in 1996. Less year-to-year variation was noted in adult ticks, in which infection prevalence ranged from 11.7% (1997) to 23.3% (1996). PCR analysis of EDTA blood samples from white-footed mice collected

Table 1. Spatial and temporal variation in the occurrence and distribution of *Ehrlichia phagocytophila*-human agent (EP-ha) and *E. phagocytophila* variants, Connecticut and Rhode Island, 1996–1999

Collection site	Year	No.	Adult	Nymph	No. of PCR-positive ticks (%)		No. of PCR products sequenced	%Proportion infected with—		
								EP-ha	Variant 1	Variant 2
Bridgeport, CT	1996	30	+		7	(23.3)	7	100		
	1997	60	+		7	(11.7)	7	100		
	1998	82		+	5	(6.1)	5	100		
	1998	101	+		19	(18.8)	19	100		
	1999	102	+		12	(11.8)	12	100[a]	8.3[a]	
Bluff Point, CT	1997	79		+	9	(11.4)	9	55.6	44.4	
South Kingstown, RI	1996	31	+		5	(16.1)	5	60	40	
	1997	112		+	52	(46.4)	30	10	3.3	86.7
	1997	120	+		46	(38.3)	37	13.5	13.5	73
	1998	91		+	5	(5.5)	5	20	80	
	1999	103		+	5	(4.9)	5	80	20	
	1999	81	+		10	(12.3)	10	80	20	

[a]Includes one tick that was positive for both the human granulocytic ehrlichiosis agent and variant 1.
PCR, polymerase chain reaction.

in Connecticut during summer and fall 1997 and spring 1998 showed that 17 (36.2%) of 47 in 1997 and 3 (60%) of 5 in 1998 were positive (22). The amplification products were sequenced for each *Ehrlichia* PCR-positive mouse and tick. All products from samples collected in the Bridgeport, Conn., area from 1996 through 1998 had sequences identical to the 16S rRNA gene (EP-ha) previously amplified and sequenced from documented human infections in the Northeast and Upper Midwest United States and in Europe (4). The 16S rRNA sequence determined from adult ticks collected from Bridgeport in 1999 showed that all 12 positive samples also contained the human agent (EP-ha), although one of the ticks produced a mixed sequence, suggesting the presence of more than one agent. The PCR products from this tick were cloned, and individual clones were purified and sequenced. These data confirmed the presence of a mixed population of ehrlichiae containing some 16S rRNA sequences that matched EP-ha and some that differed from EP-ha by two nucleotides. The latter sequence was identical to a variant (called variant 1) previously described in ticks in Rhode Island and deer in Maryland and Wisconsin (13,14) (Table 2). In contrast to ticks and rodents from the Bridgeport area, nymphal ticks collected in 1997 from Bluff Point in southeastern Connecticut contained a nearly equal distribution of EP-ha (5 [55.6%] of 9 positives) and variant 1 (4 [44.4%] of 9 positives) ehrlichiae.

Rhode Island samples from *I. scapularis* ticks, white-footed mice, and chipmunks contained *E. phagocytophila* variants as well as EP-ha. A total of 123 (22.9%) of 538 ticks were positive for *E. phagocytophila* by PCR, including 61 (26.3%) of 232 adults and 62 (20.3%) of 306 nymphs. DNA sequencing was performed on 92 of these PCR products, and overall, only 24 (26.1%) showed sequences identical to those of EP-ha. Fifteen (16.3%) ticks showed sequences corresponding to variant 1. The rest of the ticks (53 [57.6%]) had a novel sequence differing from EP-ha by two nucleotides and from variant 1 by four nucleotides (hereafter called variant 2) (Table 2).

PCR testing of blood samples from 19 Rhode Island chipmunks in 1996 detected 11 (57.9%) positives. DNA sequencing of these PCR products showed that nine were identical to the sequence of EP-ha; the remaining two represented novel variant sequences, each differing from EP-ha by a single nucleotide (variants 3 and 4; Table 2). Although both the white-tailed deer agent and the *E. equi*/CA human sequence variant (Table 2) are amplified by the PCR assay used in this study, neither agent has been detected in potential rodent reservoir populations in Connecticut or Rhode Island. Host and vector associations of EP-ha and the four *E. phagocytophila* variants found in Rhode Island are shown in Table 3.

The prevalence of *E. phagocytophila* in *I. scapularis* ticks (adults and nymphs combined, years 1996-1999) was higher in Rhode Island (22.8%) than in Bridgeport (13.3%) ($p<0.001$; Fisher's exact test). This finding was also true for adult ticks: 26.3% were infected in Rhode Island compared with 15.4% in

Table 2. Variation within nucleotide region 74 to 446 in 16s rRNA gene sequences obtained for *Ehrlichia phagocytophila*-human agent (EP-ha),[a] the Rhode Island variants, and *E. equi*

	Position no.					
	76	84	157	176	284	299
EP-ha	A	G	A	G	C	A
RI variant 1	**G**[b]	**A**	A	G	C	A
RI variant 2	A	G	A	**A**	**T**	A
RI variant 3	A	G	**G**	G	C	A
RI variant 4	A	G	A	G	C	**G**
E. equi/CA human	A	**A**	A	G	C	A

[a] The number designations for the EP-ha 16S rDNA sequence correspond to those reported by Chen et al. (4). GenBank accession no. U02521.
[b] Variant base pairs are shown in bold.

Table 3. Host and vector associations of *Ehrlichia phagocytophila* and the four 16S rDNA sequence variants detected in Rhode Island,1996–1999

Host or tick species	EP-ha	Variant 1[a]	Variant 2	Variant 3	Variant 4
Ixodes scapularis ticks	+	+	+	–	–
White-footed mouse	+	–	+	–	–
Eastern chipmunk	+	–	–	+	+
White-tailed deer	–	+	–	–	–
Human[b]	+	–	–	–	–

[a]Variant 1 detected only in ticks in Rhode Island and Connecticut; positive deer samples were collected in Maryland and Wisconsin (13,14).
[b]Based on samples from 35 confirmed human infections from various states, including Rhode Island and Connecticut.

Bridgeport (p=0.002). Using either the total number of ticks tested or adult ticks only, the prevalence of *E. phagocytophila* in Rhode Island compared with Bridgeport was 1.7. However, if the 1997 Rhode Island data, which were skewed by an unusually large number of variants, are removed from the calculations, the percentage of *E. phagocytophila*-positive ticks (adults and nymphs) was significantly higher in Bridgeport (13.3%) than in Rhode Island (8.2%) (p=0.03). The same analysis, when restricted to the adult tick population, showed no significant difference between Connecticut (15.4%) and Rhode Island (13.4%) EP-positive ticks (p=0.6). If the 1997 Rhode Island data are excluded, the prevalence of *E. phagocytophila* in that state compared with Bridgeport was 0.6 for the total number of ticks tested and 0.8 for adult ticks only.

Infection prevalence data were available for adult and nymphal ticks from the same site for 3 years: Rhode Island in 1997 and 1999 and Bridgeport in 1998 (Table 1). For two of these, Rhode Island in 1999 and Bridgeport in 1998, the prevalence of *E. phagocytophila* was significantly higher (Rhode Island 1999; p=0.01) or borderline higher (Connecticut 1998; p=0.065) in adults than in nymphs. *E. phagocytophila* infection rates in nymphal and adult ticks from Rhode Island in 1997 did not differ significantly (p=0.21).

Temporal trends in tick infection rates showed that the prevalence of *E. phagocytophila* in Rhode Island nymphs was highest in 1997 and then declined in 1998 and 1999 (p<0.001; chi-square test for trend). However, the high number of variants found in both adult and nymphal ticks from Rhode Island in 1997 influenced this analysis, as *E. phagocytophila* prevalence in Rhode Island in 1997 was significantly higher than in all other years combined (p<0.001). In Bridgeport, no significant temporal trends were noted in *E. phagocytophila* infection rates in adult ticks (p=0.3), nor were significant prevalence or temporal trends noted in the rodents tested from any of the sites.

Analysis of the proportion of *E. phagocytophila*-positives that were variants showed that prevalence of the variants in Rhode Island (73.9% variants) was significantly higher than in Bridgeport (0.02% variants) (p<0.001). When the proportion of *E. phagocytophila*-positives that were variants was compared with the total number of positives for the two Connecticut sites, the ticks from Bluff Point (44.4% variants) showed significantly higher rates than ticks from Bridgeport (0.02% variants) (p<0.001).

Discussion

Strains of *E. phagocytophila* found in nature are capable of causing disease in sheep, cattle, horses, dogs, cats, and humans. These strains, including the species previously known as the HGE agent and *E. equi*, are grouped as a single species on the basis of their close relationship at the genetic and antigenic levels. However, biological and ecological differences clearly exist between strains of *E. phagocytophila*, including varying host pathogenicity, vectors, DNA sequence, and geographic distribution. Small ribosomal subunit (16S) DNA sequences are highly conserved in bacteria and are often used to identify and differentiate bacterial species. The 16S rRNA gene sequences amplified from every confirmed human case, except for two isolated cases in northern California, have been identical to the *E. phagocytophila*-human agent (EP-ha) sequence determined by Chen et al. (4). PCR-positive white-footed mice (n=20) and *I. scapularis* ticks (n=38) collected in Bridgeport from 1996 through 1998 also harbored only *E. phagocytophila* identical in sequence to EP-ha for a 546-bp region of the 16S rRNA gene (4). Sequence analysis of PCR products from two Connecticut deer blood samples showed DNA identical to the EP-ha *p44* gene sequence (23). However, an *Ehrlichia* organism with a 16S rRNA gene sequence differing from EP-ha by a single nucleotide has been identified in white-tailed deer from Maryland and Wisconsin and in *I. scapularis* from Rhode Island (13,14).

In contrast to our results from Bridgeport, where we consistently found EP-ha, mice and ticks from Rhode Island had a significantly lower percentage of isolates identical to EP-ha, but several *E. phagocytophila* variants with novel 16S rRNA gene sequences. These data indicate that variant forms of *E. phagocytophila*, not yet associated with human or veterinary disease, frequently occur in Rhode Island. The same or additional *E. phagocytophila* variants may also occur in other regions of the United States, but this concept remains to be investigated.

Most PCR assays amplify products from the variant agents that are the same size as the EP-ha PCR product, so that variants are indistinguishable when the products are analyzed only by agarose gel electrophoresis. Therefore, results from other human-infection prevalence surveillance studies in ticks and rodents that have not included PCR product sequencing may be misleading. For example, if we had not sequenced our PCR products for 1997, we would have concluded that 46.4% of nymphal and 38.3% of adult *I. scapularis* ticks collected in southern Rhode Island were positive for EP-ha. Actually, only 11.9% of the positives that were sequenced and an estimated 5.0% of the total ticks tested were EP-ha positive, with the rest of the 1997 Rhode Island positives consisting of genetic variants not yet associated with human disease.

The 16S rRNA sequences obtained from tick and rodent samples collected from Bridgeport from 1996 through 1998

were identical to EP-ha. However, in 1999, one tick collected in that site was positive for both EP-ha and a variant (variant 1) previously found in Rhode Island. In a retrospective analysis of ticks collected at another eastern Connecticut site (Bluff Point) close to the Rhode Island border, variant 1 was found in 1997. Our inability to detect variant 1 despite extensive testing of samples collected in Bridgeport from 1996 to 1998 and its subsequent appearance in 1999 suggests that its geographic range may be expanding westward. Additional studies with larger sample sizes of ticks and rodents from Bridgeport and other locations in Connecticut are needed to assess the prevalence of EP-ha and the variants, as our Bridgeport results may not be representative of the entire state. In fact, the Bluff Point data suggest that other areas of Connecticut may have EP-ha/variant populations quite different from those in Bridgeport and more similar to the distribution noted in Rhode Island.

The identification of the coinfected tick in Connecticut represents the first detection of more than one strain of *E. phagocytophila* in a single tick vector in the United States, although the coinfection of *I. xodes ricinus* ticks by *E. phagocytophila* strains has been reported in Europe (24). These data indicate that two strains of the agent are capable of coexisting in a single tick, at least transiently, and that they can survive the molting process, since the coinfection was found in an unfed, host-seeking adult tick.

The results from Rhode Island samples collected in 1997 are unusual in several regards. First, the rate of *E. phagocytophila*-positive ticks (42.2% nymphs and adults) was very high relative to all other tick populations sampled from 1996 through 1999, and many of the positives were variant 2 (79.1% of PCR-positives sequenced). Second, the 1997 Rhode Island ticks represent the only population in which *E. phagocytophila* prevalence was higher in nymphs than adults (46.4% nymphs and 38.3% adults). Finally, variant 2 sequences were also seen in samples collected in 1997 from white-footed mice and chipmunks but were not detected before and have not been detected after 1997. The fact that both nymphal and adult questing ticks were positive for variant 2 suggests that the variant was present in reservoir species during both larval and nymphal feedings and may have been present in reservoirs from late summer 1996 through summer 1997. Why this variant appeared only in Rhode Island during 1997, was the most prevalent strain infecting ticks that year, and then completely disappeared are matters of speculation. Variant 2 may be a more common infection in a reservoir species that we did not examine, which may be less commonly targeted by host-seeking ticks. Expression of variant 2 in the tick population could have resulted if, during 1996-1997, host populations preferred by immature *I. scapularis* (i.e., white-footed mice and chipmunks) were lower than normal, resulting in a higher proportion of ticks feeding on such atypical hosts harboring variant 2. After molting, nymphs infected as larvae the previous year could have transmitted variant 2 to the more preferred hosts of immature ticks, resulting in the variant 2-positive mice found in 1997. Subsequent reestablishment of normal host populations may then have diluted the prevalence of variant 2, as immature tick feeding reverted to the preferred hosts.

Although the function and biological importance of the genetic differences in *E. phagocytophila* strains are unknown, we hypothesize that the variants may be interfering with maintenance and transmission of the human disease-causing agent (EP-ha). Even if an increased human ehrlichiosis case surveillance effort in Connecticut is taken into account, the number of confirmed and suspected cases differs dramatically between the two neighboring states: several hundred cases were reported in Connecticut compared with fewer than 25 cases in Rhode Island during the same time period. From 1995 to 1997, 178 cases were confirmed or suspected (25), and case reports in Connecticut increased substantially in 1998 (228 provisional, 104 confirmed, Connecticut Dept. of Public Health). These adjacent states share many of the ecologic factors that support natural maintenance of both *Borrelia burgdorferi* and granulocytic ehrlichiae, such as populations of the tick vector *I. scapularis* and reservoir rodents, including white-footed mice (*P. leucopus*) (19,22). The incidence of Lyme disease in Connecticut and Rhode Island has been the highest in the nation for several years, with Connecticut having a reported incidence only approximately 1.3-1.5 times higher than Rhode Island's (26). In contrast, through 1997 there was a 24-fold difference in the incidence of reported HGE cases in the two states (Connecticut 15.9; Rhode Island 0.67). Therefore, the *E. phagocytophila* variants may have a competitive advantage over the EP-ha, possibly in infecting certain reservoir or vector populations. A lower incidence of EP-ha and less human disease would therefore be expected in areas where the variants predominate, since a lower proportion of ticks would harbor EP-ha.

Rickettsia rickettsii, the etiologic agent of Rocky Mountain spotted fever, was first identified in the early 1900s on the basis of its association with human disease (27). Subsequent studies of veterinary infections and tick populations identified numerous *Rickettsia* species closely related to *R. rickettsii*, all clearly members of the spotted fever group but not associated with human disease. These species include *R. montana*, *R. rhiphicephali*, *R. parkeri*, *R. bellii*, and the "east side agent" *R. peacockii* (28,29). Nonpathogenic rickettsiae are thought to interfere with the development of more virulent *R. rickettsii* in *Dermacentor* ticks and may be found more often in ticks than are the more virulent species (30,31). Our data suggest that a similar situation may exist among the granulocytic ehrlichiae, with both pathogenic and nonpathogenic genetic variants coexisting in nature. Isolation of the new variants will allow us to address this competitive-advantage hypothesis experimentally in both ticks and mice through the use of mixed infections in the laboratory.

Identification and use of novel gene targets more variable than the 16S rRNA gene will eventually permit better assessment of variability between strains of *E. phagocytophila* (32-34). Future studies should include *E. phagocytophila* from additional geographic areas where a substantial number of human cases of granulocytic ehrlichiosis are reported (e.g., New

York, Wisconsin, Minnesota) compared with areas (e.g., New Jersey, Pennsylvania, Delaware, Maryland, California) with similar vector densities but with little or no human disease.

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Trends in Fluoroquinolone (Ciprofloxacin) Resistance in *Enterobacteriaceae* from Bacteremias, England and Wales, 1990–1999

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The Public Health Laboratory Service receives antibiotic susceptibility data for bacteria from bloodstream infections from most hospitals in England and Wales. These data were used to ascertain resistance trends to ciprofloxacin from 1990 through 1999 for the most prevalent gram-negative agents: *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Proteus mirabilis*. Significant increases in resistance were observed for all four species groups. For *E. coli,* ciprofloxacin resistance rose from 0.8% in 1990 to 3.7% in 1999 and became widely scattered among reporting hospitals. The prevalence of resistance in *Klebsiella* spp. rose from 3.5% in 1990, to 9.5% in 1996 and 7.1% in 1999, while that in *Enterobacter* spp. rose from 2.1% in 1990 to 10.5% in 1996 and 10.9% in 1999. For both *Klebsiella* and *Enterobacter* spp., most resistance was localized in a few centers. Resistance was infrequent and scattered in *P. mirabilis*, but reached a prevalence of 3.3% in 1999.

Fluoroquinolone antimicrobial drugs were a major therapeutic advance of the 1980s because they have 100-fold greater activity than their parent compound, nalidixic acid (1). Unlike nalidixic acid, which is used only for urinary infections and occasionally shigellosis, the fluoroquinolones have a broad range of therapeutic indications and are given as prophylaxis, e.g., for neutropenic patients. In veterinary medicine fluoroquinolones are used as treatment and metaphylaxis but not as growth promoters. Early researchers thought that fluoroquinolone resistance was unlikely to evolve, largely because resistant *Escherichia coli* mutants are exceptionally difficult to select in vitro (2) and because plasmid-mediated quinolone resistance remained unknown even after 30 years of nalidixic acid usage. Nevertheless, mutational fluoroquinolone resistance emerged readily in staphylococci and pseudomonads, which are inherently less susceptible than *E. coli*. More recently, fluoroquinolone resistance has emerged in *E. coli* and other *Enterobacteriaceae*, contingent on multiple mutations that diminish the affinity of its topoisomerase II and IV targets in various ways, reduce permeability, and upregulate efflux (3). Plasmid-mediated quinolone resistance has been reported, but it is exceptional (4).

We report here resistance trends to ciprofloxacin, the most widely used fluoroquinolone in the United Kingdom, in the prevalent *Enterobacteriaceae* species from bacteremias in England and Wales during the 1990s.

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Data Sources

Data Collection

The surveillance, described previously, depends on the voluntary reporting of bloodstream isolates by hospital laboratories in England and Wales (5). The number of laboratories reporting data has grown steadily: by 1998, 208 (91%) of the 229 establishments in England and Wales listed by the Association of Medical Microbiologists were participating. Participation by laboratories in Scotland and Northern Ireland is limited, and their data were excluded from our analysis. Most laboratories used variants of Stokes' disc method (6) for susceptibility tests in the period reviewed, but a minority used breakpoint tests. Results reported as intermediate were counted as resistant. Quality control was provided by the laboratories' participation in the National External Quality Assurance Scheme and by comparison to results for the smaller numbers of *E. coli* isolates from bloodstream infections tested at the Central Public Health Laboratory (7).

Prescribing Data for Fluoroquinolones

Prescribing data for fluoroquinolones, as defined daily doses (8), were estimated for retail pharmacies by using IMS HEALTH's British Pharmaceutical Index (BPI) and for hospitals by using Medicare Audit's Hospital Pharmacy Audit (HPA). The BPI records pharmaceutical sales to retail pharmacies and dispensing doctors in the United Kingdom, Channel Islands, and the Isle of Man. Approximately 97% of wholesaler sales to retail and physician outlets and >80% of direct sales by manufacturers are recorded; other sales are estimated from a sample of approximately 600 pharmacies. The number

of pharmacies represented in the BPI remained constant during the study period.

The HPA provides information on pharmaceutical consumption by National Health Service hospitals, which account for >95% of hospital care in the United Kingdom. Most hospitals participate: approximately 93% of beds are currently covered. Since 1995, HPA data have been collected monthly from the stock control systems of participating hospitals. Most data are supplied electronically, which minimizes reporting errors. Data include usage of pharmaceuticals among in- and outpatient departments and for private patients in NHS hospitals but not for private patients in designated private hospitals. Before 1995, HPA data were collected from wholesalers, manufacturers, and a panel of hospitals: approximately 90% of indirect sales to hospitals were received from wholesalers and approximately 40% of direct sales from manufacturers. The panel of hospitals covered approximately 80% of beds in 1990 and 84.5% in 1995.

Statistical Analyses

Poisson regression was performed by using the log (total number of isolates with resistance information) as an offset to determine if the proportion of ciprofloxacin-resistant isolates was changing with any type of pattern over time. S-Plus (Mathsoft Inc., Seattle, WA) was used for calculation.

Results

Species Prevalence and Reporting Patterns

During the 1990s, the Public Health Laboratory Service received nearly 392,551 reports of bacteremia in England and Wales, including 132,311 that indicated *E. coli*, klebsiellae, *Enterobacter* spp., and *P. mirabilis* as the pathogens isolated. These four species groups thus accounted for 32% to 36% of all bacteremia results in each year and for 71% to 72% of those concerning gram-negative bacteria (Table 1). *E. coli* was the most frequently reported pathogen, causing 22% to 25% of all bacteremias in each year, whereas *Klebsiella*, *Proteus*, and *Enterobacter* spp. were among the 10 most frequent isolates. The number of bacteremia reports rose each year (Table 1), reflecting improved reporting rather than an increased incidence of disease. A fall in the proportion of reports with susceptibility data in 1997 reflected early problems after a switch to electronic reporting and was not exclusive to ciprofloxacin.

Resistance Trends for Ciprofloxacin

Among the reports for *E. coli*, klebsiellae, *Enterobacter* spp., and *P. mirabilis*, 75,168 (56.8%) had susceptibility data for ciprofloxacin, confirming widespread testing. Ciprofloxacin resistance was extremely rare when surveillance began but subsequently increased for all four organisms (Figure 1). The proportion of *E. coli* isolates reported as resistant rose slowly but steadily, from 0.8% in 1990 to 3.7% in 1999. For *Klebsiella* spp., the resistance rate rose from 3.5% of reports in 1990 to 9.5% in 1996, before declining to 7.1% by 1999. *Enterobacter* spp. showed a similar pattern to klebsiellae: the prevalence of resistance rose from 2.1% in 1990 to 10.5% in 1996, then dipped to 7.9% in 1998 before rising to 10.9% in 1999. Only a few *P. mirabilis* isolates were reported resistant in any year before 1999. Poisson regression showed strong evidence of a trend to increasing resistance for all four organisms and suggested that these increases had a nonlinear component for *E. coli*, enterobacters, and klebsiellae. If the trends nevertheless were approximated to be linear, the average annual increases in the proportion of resistant isolates were as follows: *E. coli*, 21.54% (95% confidence intervals [CI] 18.86-24.30); *Klebsiella* spp., 6.97% (CI 4.41-9.59); *Enterobacter* spp. 13.97% (CI 10.46-17.58); and *P. mirabilis*, 21.31% (CI 11.38-32.13).

Distribution of Resistance

To assess the distribution of resistance, we counted, for each organism in each year: 1) the number of laboratories reporting resistant isolates, 2) any laboratories contributing >10% of all reports of resistance, and 3) the proportion of reports of resistance from the top three contributors (Table 2). The last two criteria were applied only when >30 resistant isolates of a species were reported in a year, so that a hospital would not appear as a "major contributor" on the basis of three or fewer resistant isolates.

The number of laboratories reporting resistant *E. coli* rose from 25 in 1990 to 89 in 1999, and no single laboratory ever contributed >10% of all reports of resistance in a year for this species. Laboratories reporting five or more resistant *E. coli* in years before 1998 mostly served major teaching hospitals, but many district general hospitals reported five or more resistant *E. coli* isolates in 1998 and 1999. Resistance was more localized and more prevalent in *Klebsiella* and *Enterobacter* spp. than in *E. coli*. The number of laboratories reporting resistant klebsiellae fluctuated from 36 to 57 after 1992, without obvious trend. During a peak in resistance prevalence, from 1995 to 1997, one or two laboratories each contributed >10% of all reports of resistant klebsiellae, and the top three contributors accounted for 32% to 39% of reports of resistance. For *Enterobacter* spp., laboratories reporting resistance increased from 10 in 1990 to 36 in 1992, then fluctuated with little trend until 1997, before rising to 40 in 1998 and 58 in 1999. In the peak of resistance in 1995 and 1996, two laboratories each accounted for >10% of all reports of resistant enterobacters, and 30% to 32% of reports of resistance came from the top three contributors. Resistance was uncommon in *P. mirabilis*, and clusters were not evident.

In a further analysis, we identified eight laboratories that frequently reported large numbers of resistant *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. during the entire surveillance period. These were in major metropolitan areas and served teaching hospitals. These laboratories accounted for 7.7%, 11.2%, and 10.3% of reports with ciprofloxacin data for *E. coli*, *Klebsiell*a, and *Enterobacter* spp. respectively, but for 18.2%, 30.9%, and 22.4%, respectively, of reports of

Table 1. Ciprofloxacin-resistant *Enterobacteriaceae* reported from bacteremias, England and Wales, 1990–1999

	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999
Escherichia coli										
Total no. reports	7,610	7,377	7,849	7,872	8,274	8,465	9,155	10,143	11,248	11,573
No. with cipro. results	4,171	4,456	5,036	5,071	5,136	5,143	4,559	3,706	6,282	6,708
No. reported ciproR	33	32	47	65	88	108	119	144	244	246
Klebsiella spp.										
Total no. reports	1,544	1,634	1,710	1,725	1,791	1,957	2,143	2,383	2,816	2,802
No. with cipro. results	821	1,082	1,124	1,141	1,173	1,256	1,137	900	1,551	1,578
No. reported ciproR	29	48	55	77	77	115	108	80	125	112
***Enterobacter* spp.**										
Total no. reports	895	912	1,013	948	1,118	1,089	1,229	1,480	1,638	1,629
No. with cipro. results	582	636	743	759	815	723	617	534	908	949
No. reported ciproR	12	26	36	29	54	65	65	55	72	103
Proteus mirabilis										
Total no. reports	868	898	911	925	984	942	1,244	1,131	1,241	1,145
No. with cipro. results	454	578	560	573	635	673	578	447	715	658
No. reported ciproR	2	3	1	7	14	7	4	5	14	22
No. of other organisms	19,866	20,458	21,335	22,968	23,559	24,545	27,908	31,258	34,517	34,216
Total bacteremia reports	30,783	31,279	32,838	34,438	35,726	36,948	41,679	46,395	51,100	51,365

Cipro, ciprofloxacin; R, resistant.

resistance in these organisms, confirming a major excess of resistance.

The prevalence of ciprofloxacin resistance was examined in relation to patients' ages for *E. coli*, since those aged ≤14 years should not receive fluoroquinolones. Taking the period 1995 through 1999 as a whole, 12 (3.9%) of 305 *E. coli* with data from patients 1 to 14 years old were reported as ciprofloxacin resistant, compared with 778 (3.2%) of 24,302 *E. coli* isolates from patients aged ≥15 years. These data indicated a relative risk of 1.22 (95% CI 0.7-2.1) for the younger patients. Similar calculations were not performed for other species because of the small numbers of source patients ages 1-14 years.

Use of Fluoroquinolones

Fluoroquinolone use increased in the earlier years of surveillance, nearly doubling from 1990 to 1993. However, usage has been relatively stable from 1997 onwards, with community use declining slightly (Figure 2). Although most use is still in the community, hospital use has grown steadily in absolute terms and as a proportion, constituting 31.5% of total use in 1999 compared with 18.9% in 1992. Ciprofloxacin was the dominant fluoroquinolone throughout the period (not shown).

Conclusion

When this surveillance began in 1990, the ciprofloxacin resistance rates in *E. coli* and *P. mirabilis* were <1%, and rates for enterobacters and klebsiellae were 2.1% and 3.5%, respectively. The prevalence of resistance in *E. coli* subsequently rose slowly and progressively to reach 3.7% in 1999; this resistance was widely scattered in hospitals. Resistance also increased significantly ($p<0.01$, chi-square test for trend) in enterobacters and klebsiellas. The prevalence rates for these two genera were strongly influenced by clusters of resistant isolates reported by a few laboratories. Thus, the prevalence of ciprofloxacin resistance in klebsiellae peaked at 9.5% in 1996, when three laboratories accounted for 35% of reports of resistance. A subsequent decline was associated with the absence of clusters but not with a decline in the number of hospitals that reported resistance. For enterobacters, the proportion of resistant isolates rose from 1990 to 1996, but the number of laboratories reporting resistance was relatively constant from 1992 to 1997. Peak rates of resistance in 1995 and 1996 were in a period when the top three contributors accounted for 30% to 32% of reports. Resistance in *P. mirabilis* was infrequent and scattered but rose significantly ($p<0.01$) in prevalence.

Although our analysis of resistance prevalence depended on the compilation of susceptibility results obtained at different sites by different methodologic variants, there is no suggestion that definitions of resistance to ciprofloxacin have become more conservative in the United Kingdom. Moreover, a rising prevalence of ciprofloxacin resistance is evident in the smaller numbers of *E. coli* isolates tested by a standardized method at the Central Public Health Laboratory, supporting the trends found here (7,9).

Several factors may explain the greater prevalence and clustering of resistance in enterobacters and klebsiellae. Most

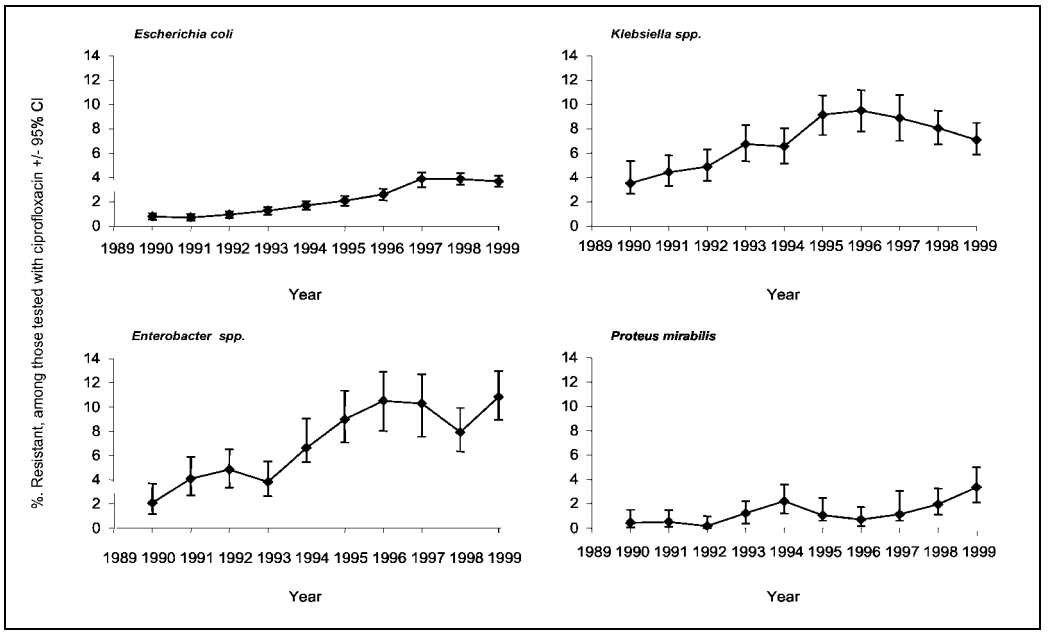

Figure 1. Resistance trends in *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., and *Proteus mirabilis*, England and Wales, 1990–1999.*

*Bars indicate 95% confidence intervals.

importantly, *Enterobacter* and *Klebsiella* spp. are primarily hospital pathogens, whereas *E. coli* bacteremias are more often community acquired. Thus, *E. coli* accounted for 22.8% of all bacteremias in this surveillance, which included both hospital- and community-acquired infections, but only 12.5% of hospital-acquired bacteremias, as recorded by the Nosocomial Infection National Surveillance Scheme (10). Although most fluoroquinolone use is in the community (Figure 2), the most intensive use and therefore the greatest selection pressure relative to numbers and concentration of patients is in hospitals. Moreover *Klebsiella* and *Enterobacter* infections are more often clonal than those involving *E. coli;* single strains, perhaps resistant, spread to numerous patients (11). Clonal outbreaks seem the likely explanation when small numbers of hospitals contributed substantially to resistance totals—as was often the case for *Enterobacter* and *Klebsiella* spp. (Table 2)—but cannot be proved without retained isolates. Bacteremias caused by quinolone-resistant *E. coli* may or may not be clonal, even when multiple cases occur in a unit (12,13). The laboratories reporting clusters of resistant *Enterobacter* and

Table 2. Distribution of reports of ciprofloxacin resistance for *Enterobacteriaceae* from bacteremia in hospitals, England and Wales, 1990–1999

	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999
Escherichia coli										
No. labs reporting ciproR isolates	25	29	39	40	57	52	58	68	94	89
Labs contributing >10% of ciproR total (n)[a]	1	0	0	0	0	0	0	0	0	0
% of all ciproR reports from top three contributors[a]	33	19	17	20	13	19	26	17	11	12
***Klebsiella* spp.**										
No. labs reporting ciproR isolates	23	48	38	42	42	47	42	36	57	50
Labs contributing >10% of ciproR total (n)[a]	-	0	1	0	0	1	2	2	1	0
% of all ciproR reports from top three contributors[a]	-	17	29	15	21	32	35	39	23	21
***Enterobacter* spp.**										
No. labs reporting ciproR isolates	10	19	36	27	37	30	35	33	39	58
Labs contributing >10% of ciproR total (n)[a]	-	-	0	-	0	2	2	0	0	0
% of all ciproR reports from top three contributors[a]	-	-	28	-	22	32	30	22	22	16
Proteus mirabilis										
No. labs reporting ciproR isolates	2	2	1	6	12	6	3	6	12	20
Labs contributing >10% of ciproR total (n) [a]	-	-	-	-	-	-	-	-	-	-
% of all ciproR reports from top three contributors[a]	-	-	-	-	-	-	-	-	-	-

[a]Not calculated if ≤30 resistant isolates.
Cipro, ciprofloxacin; R, resistant; labs, laboratories.

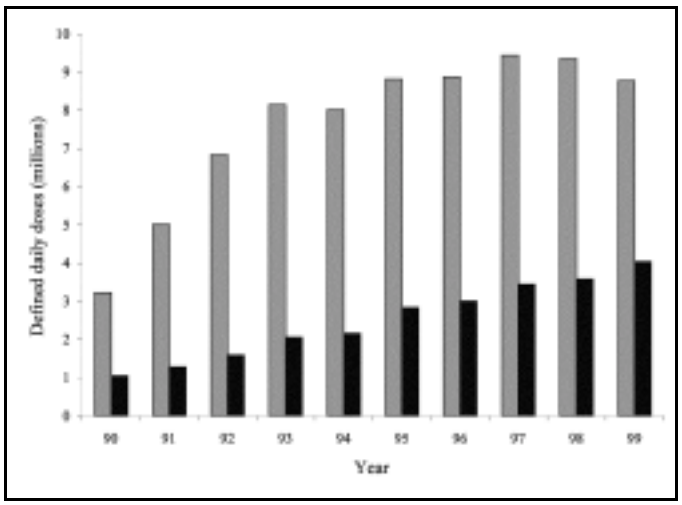

Figure 2. Fluoroquinolones dispensed by retail (grey) and hospital (black) pharmacies, United Kingdom, 1990–1999.

Klebsiella spp. mostly served major teaching hospitals, where fluoroquinolone prophylaxis by hematology departments has been associated with a reduced incidence of bacteremias in neutropenic patients (14) but with more bacteremias being caused by fluoroquinolone-resistant strains (15,16).

We did not attempt to comprehensively relate resistance and prescribing, but three general points can be made. First, the recent decline in community prescribing of fluoroquinolones (Figure 2) has not affected the upward resistance trend in *E. coli*, although most *E. coli* bacteremia is believed to involve non-nosocomial strains. Second, the rising hospital use of fluoroquinolones has not been mirrored by an acceleration in upward trend of resistance in *Klebsiella* and *Enterobacter* spp. Third, the prevalence of resistant *E. coli* from bacteremias in patients 1-14 years old was similar to or higher than that in older patients, although the younger patients should not receive fluoroquinolones. These observations imply complex relationships between use and resistance, demanding prospective investigation.

Except for *P. mirabilis*, the resistance prevalence rates found here resemble those for bacteremias in the United States, a country with much heavier fluoroquinolone use than the United Kingdom. The Surveillance Network database (http://www.mrlworld.com) shows resistance trends (with intermediate counted as resistant) in bloodstream isolates from 250 U.S. hospitals as follows: *E. coli*, 1.8% in 1996 and 4.3% in 1999; *Klebsiella* spp., 7.1% in 1996 and 6.7% in 1999; *Enterobacter* spp., 6.6% in 1996 and 6.5% in 1999; and *P. mirabilis*, 5.7% in 1996 and 12.7% in 1999. Much higher rates are reported from Barcelona, Spain, where 17% of *E. coli* isolates from community infections were ciprofloxacin resistant (17), and India, where up to 50% of hospital *E. coli* are reported resistant (18). High rates in *E. coli* may reflect contamination via the food chain: the Spanish study found quinolone-resistant *E. coli* in 90% of chicken feces and noted similar fecal carriage rates of resistant *E. coli* in children and adults. Acquisition of resistant *E. coli* via the food chain may also explain why, in our study, resistant *E. coli* were reported from age groups who should not receive fluoroquinolone therapy and its contingent selection pressure.

Ciprofloxacin remains a potent antibiotic; but the slow accumulation of resistant *Enterobacteriaceae* is disturbing, not least because resistance is a class effect, affecting all fluoroquinolones. Ultimately, this resistance may be partly overcome by inhibiting the efflux pumps that contribute to the resistance (19), but this strategy is still several years from fruition. In the interim, the best approach lies in the prudent use of fluoroquinolones in humans and animals, coupled with an emphasis on preventing patient-to-patient spread of resistant strains.

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Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Healthy Children: Implications for the Use of Heptavalent Pneumococcal Conjugate Vaccine

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We assessed the prevalence of *Streptococcus pneumoniae* serotypes in the nasopharynx of healthy children, antimicrobial susceptibility patterns, risk factors for carriage, and the coverage of heptavalent pneumococcal conjugate vaccine. In 2,799 healthy infants and children, the *S. pneumoniae* carrier rate was 8.6% (serotypes 3, 19F, 23F, 19A, 6B, and 14 were most common). Most pneumococci (69.4%) were resistant to one or more antimicrobial classes. The rate of penicillin resistance was low (9.1%); macrolide resistance was high (52.1%). Overall, 63.2% of the isolates belonged to strains covered by the heptavalent pneumococcal vaccine. This percentage was higher in children <2 years old (73.1%) and in those ages 2-5 years (68.9%). Sinusitis in the previous 3 months was the only risk factor for carrier status; acute otitis media was the only risk factor for the carriage of penicillin-resistant *S. pneumoniae*. Most isolated strains are covered by the heptavalent conjugate vaccine, especially in the first years of life, suggesting that its use could reduce the incidence of pneumococcal disease.

The nasopharynx of children has resident microbial flora that do not usually harm the child but, in some cases, constitute a reservoir of pathogens implicated in respiratory tract infections and invasive diseases (1,2). The bacteria carried in the nasopharynx of healthy children reflect the infection-causing strains currently circulating in the community (3), and so studies of the prevalence of different pathogens and their resistance patterns can provide useful indications for more rational therapeutic and preventive strategies.

The asymptomatic nasopharyngeal carriage of *Streptococcus pneumoniae* is widely prevalent in young children and has been related to the development of disease and the spread of the pathogen (4,5); furthermore, nasopharyngeal colonization by antibiotic-resistant *S. pneumoniae* has steadily increased over the last few years (6,7). Antibiotic-resistant strains are more often carried by infants and young children than adults and belong to a limited number of serotypes that are also some of the most common causes of invasive pediatric diseases (8-10).

A heptavalent conjugate vaccine, which includes the most common serotypes involved in invasive diseases, effectively induces protection against pneumococcal nasopharyngeal carriage (11,12). However, while the vaccine is statistically effective in preventing carriage of vaccine-related strains, a number of reports show an increase in the percentage of nonvaccine strains in immunized patients (13,14).

We assessed the prevalence of different *S. pneumoniae* serotypes in the nasopharynx of healthy children attending day-care centers and primary schools, determined their antimicrobial susceptibility to a wide range of therapeutic compounds, identified the risk factors for carrier status, and defined the possible coverage provided by the heptavalent pneumococcal conjugate vaccine during the first years of life.

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Patients and Methods

Study Population

From April 15 through June 15, 2000, a single nasopharyngeal specimen per child was obtained from children attending day-care centers and the first years of primary schools in 13 Italian cities (3 northern, 4 central, 6 southern). Only one pediatrician, belonging to the main pediatric department in each city, was responsible for the study. Two day-care centers (one for infants and children $\leq$3 years of age and one for children ages 4-5 years) and one primary school (children ages 6-7 years) were also included. All of the children attending each participating center were considered eligible unless they had an underlying chronic illness (immunologic diseases; neoplastic disorders; renal, cardiac, hepatic, or hematologic diseases; bronchodysplasia; Down syndrome; chronic otitis media with effusion) or even a mild acute upper or lower respiratory tract infection at the time of enrollment.

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The study protocol was approved by the ethics committees of the pediatric departments in charge of health control of the day-care centers and schools in each city, and written informed consent was obtained from a parent or guardian of each child. The questionnaires used to obtain demographic and clinical characteristics of the enrolled children were completed by trained reviewers in the presence of parents. The questions included: 1) the duration of breast-feeding; 2) living conditions (urban vs. rural); 3) information about previous day-care attendance; 4) the number and age of family members; 5) birth rank; 6) smoking habits of the family members living together; 7) the number and type of respiratory infections (including rhinitis, tonsillitis, laryngitis, acute otitis media, sinusitis, acute bronchitis, and pneumonia) during the previous 3 months; and 8) the number and type of antimicrobial drugs administered during the previous 3 months. The information was gathered without knowing the child's carrier status.

Specimen Collection

Nasopharyngeal specimens were obtained by the same trained investigator in each center on the basis of a previously described and validated protocol (15): a Mini-Culturette (Becton Dickinson, Cockeysville, MD) extra-thin flexible wire swab with its tip bent at an angle of approximately 30° was inserted through the mouth and placed 1 to 1.5 inches into the nasopharynx without touching the uvula or the tongue and kept in place for at least 5 seconds. The children were not allowed to eat or drink for 3 hours before specimen collection.

Microbiologic Procedures

The same microbiologic procedures were used by all of the participating centers on the basis of previously validated guidelines (15,16). The nasopharyngeal cultures were injected into Stuart transport medium tubes (Venturi Transystem, Brescia, Italy), which were sent to the microbiology laboratory of the individual hospitals within 1-3 hours and immediately processed. *S. pneumoniae* was isolated and identified using standard laboratory procedures (16). The strains were stored in serum-glycerol freezing medium at -80°C, and the frozen samples were sent to the University of Genoa's Institute of Microbiology to confirm their identity and to test their antimicrobial susceptibility by Gram strain morphology, catalase reaction, optochin susceptibility, and bile solubility. The strains were serotyped by their quellung reaction with sera produced by the Statens Seruminstitute (Copenhagen, Denmark) according to established procedures (16).

The strains susceptibility to penicillin, amoxicillin, amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, meropenem, erythromycin, clarithromycin, azithromycin, tetracycline, trimethoprim-sulfamethoxazole, rifampicin, and chloramphenicol was tested using the agar dilution method described by the National Committee for Clinical Laboratory Standards (16). MICs of the antimicrobial drugs were determined using the Sensititre microbroth incorporation technique with an inoculum of approximately 10^5 CFU/mL in a medium of Mueller-Hinton broth supplemented with saponin-lysed horse blood and Factor V. Further details concerning the microbiologic method we used are given in the article describing the results of the Alexander Project (16). All of the laboratory work was carried out in a blinded manner; susceptibility was tested by a technician unaware of the serotypes, and the samples were typed by a technician unaware of the susceptibility results.

Data Management and Analysis

The association between the characteristics of the children and the carriage of *S. pneumoniae* was first analyzed by a series of univariate analyses. Then, to control simultaneously for the possible confounding effects of the different variables, the risk of being an *S. pneumoniae* carrier was estimated by multiple logistic regression analysis with stepwise variable selection. The univariate and multivariate analyses of antibiotics and antibiotic resistance assessed individual drugs as well as all antibiotics together. In both analyses, the association was expressed in odds ratios (OR) and 95% confidence intervals (CI). Logistic regression analyses were made to evaluate the correlates of the carriage of different *S. pneumoniae* strains. On the basis of previous studies (12,17-19), strains 14, 6, 19, 18, 23, 9, 1, 7, 4, 5, 3, and 24 were considered invasive, and strains 4, 6, 9, 14, 18, 19, and 23 covered by or cross-reactive with the heptavalent vaccine. All of the reported p values are two-sided and refer to a significance level of 0.05.

Results

Study Participation

The study involved 2,799 children, whose demographic and clinical characteristics are shown in Table 1. Most of them lived in an urban area, attended a large day-care center full-time, belonged to small families, and had at least one respiratory tract infection in the previous 3 months.

Recovery of *S. pneumoniae* from Nasopharyngeal Cultures

The pneumococcal carrier rate and the recovery of invasive strains or strains covered by the heptavalent vaccine are shown in Table 2. The total *S. pneumoniae* carrier rate was 242 (8.6%) out of 2,799, with no significant difference between the age groups; the most common strains were 3 (11.6%), 19F (11.2%), 23F (11.2%), 19A (10.7%), 6B (9.9%), and 14 (6.6%). Furthermore, 77.3% of the examined strains belonged to invasive serogroups (with no significant difference between the age groups) and 63.2% to serogroups covered by or cross-reactive with the heptavalent pneumococcal vaccine. The potential coverage related to the use of the heptavalent vaccine was significantly higher in children ages <2 (73.1%) or 2-5 years (68.9%) than in those aged >5 years (51.2%: <2 vs. >5 years: p=0.040; 2-5 vs. >5 yrs: p=0.0008). The proportion of invasive strains covered by the heptavalent vaccine was 153 (81.8%) of 187.

Table 1. Characteristics of the 2,799 children tested for nasopharyngeal carriage of *Streptococcus pneumoniae*, 13 Italian cities, April 15–June15, 2000

Characteristics	No. of children (% of 2,799)
Male	1,459 (52.1)
Race (white)	2,760 (98.6)
Age	
<2 yrs	420 (15.0)
2-5 yrs	1,389 (49.6)
>5 yrs	990 (35.4)
Breast-feeding ≥3 mo.	1,477 (52.8)
Urban residence	2,537 (90.6)
Full-time child-care attendance[a]	2,571 (91.9)
No. of subjects in each child-care center	
<20	927 (33.1)
20-29	1,600 (57.2)
>29	272 (9.7)
No. of siblings	
0	752 (26.9)
1	1,450 (51.8)
>2	597 (21.3)
First-born	1,366 (48.8)
Passive smoking	1,320 (47.2)
URTIs in the last 3 mo.[b]	
Rhinitis	1,759 (62.8)
Tonsillitis	636 (22.7)
Laryngitis	501 (17.9)
Acute otitis media	558 (19.9)
Acute sinusitis	174 (6.2)
LRTIs in the last 3 mo.[b]	
Acute bronchitis	579 (20.7)
Pneumonia	167 (5.9)
Antibiotic therapy in the last 3 mo.[c]	
Aminopenicillins	244 (8.7)
Amoxicillin-clavulanate	214 (7.6)
Macrolides	247 (8.8)
Cephalosporins	507 (18.1)
At least one antibiotic	1,032 (36.9)

[a]5-6 days/week, 6-8 hrs/day.
[b]One or more episodes; URTIs = upper respiratory tract infections; LRTIs = lower respiratory tract infections.
[c] One or more courses.

Table 2. Recovery of *Streptococcus pneumoniae* in the nasopharynx by age, 13 Italian cities, April 15–June 15, 2000

	Age group (% of total/age group)			
Carriers	<2 yrs	2-5 yrs	>5 yrs	Total
Total/age group	420	1,389	990	2,799
Total carriers	26 (6.2)	132 (9.5)	84 (8.5)	242 (8.6)
Carriers of invasive strains	20 (74.1)	106 (81.5)	61 (71.2)	187 (77.3)
Carriers of strains covered by the heptavalent vaccine	19 (73.1)[a]	91 (68.9)[b]	43 (51.2)[a,b]	153 (63.2)

[a]Carriers <2 yrs vs. carriers >5 yrs: p=0.040.
[b]Carriers 2-5 yrs vs. carriers >5 yrs: p=0.0008.

Susceptibility Patterns

The antibiotic resistance pattern of the *S. pneumoniae* strains is shown in Table 3. Only 74 strains (30.6%) were susceptible to all of the antibiotics tested, 69 (28.5%) were resistant to one antimicrobial agent, 70 (28.9%) were resistant to two, and 29 (12.0%) to more than two.

Only 22 *S. pneumoniae* isolates (9.1%) were penicillin-resistant: 18 intermediately resistant (MIC 0.1-1.0 μg/mL) and four fully resistant (MIC ≥2 μg/mL); the serogroups most resistant were 9V (40.9%) and 23F (22.7%). Seventeen (77.3%) of these penicillin-resistant strains were preventable by the heptavalent pneumococcal conjugate vaccine; none of the five strains not covered by the vaccine is usually considered invasive. The incidence of penicillin resistance was significantly higher in younger children (25.9% in children aged <2 years vs. 7.6% in those aged 2-5 years: p=0.024; 25.9% in children aged <2 years vs. 5.9% in those aged >5 years: p=0.007).

Table 3. Antibiotic resistance pattern of *Streptococcus pneumoniae* strains, 13 Italian cities, April 15–June 15, 2000

Antibiotic	Resistant strains (%)
Penicillin	22 (9.1)
Amoxicillin	0
Amoxicillin-clavulanate	0
Cefotaxime	9 (3.7)
Ceftriaxone	8 (3.3)
Meropenem	12 (4.9)
Azithromycin	126 (52.1)
Clarithromycin	126 (52.1)
Erythromycin	126 (52.1)
Tetracyclin	74 (30.6)
Thrimethoprim-sulphamethoxazole	58 (23.9)
Rifampicin	0
Chloramphenicol	26 (10.7)

Table 4. Univariate analysis of the variables potentially associated with the nasopharyngeal carriage of *Streptococcus pneumoniae*

	Total carriers		Carriers of invasive strains		Carriers of strains covered by the heptavalent vaccine	
Risk factor	ORs[a]	95% CI[b]	ORs	95% CI	ORs	95% CI
Sex	1.0	0.8-1.4	0.9	0.7-1.4	0.9	0.7-1.3
Age, yrs						
2-5	0.7	0.1-1.7	1.3	0.8-2.3	1.5	0.9-2.7
>5	1.5	0.9-2.5	0.9	0.5-1.6	1.1	0.6-1.9
Breast-feeding ≥3 mo.	0.9	0.6-1.4	1.0	0.6-1.7	1.1	0.7-1.7
Urban residence	0.8	0.1-2.2	1.2	0.6-2.6	1.5	0.7-2.9
Full-time day-care attenance	0.4	0.1-1.5	0.8	0.6-1.2	1.0	0.7-1.4
Child-care center ≥20 subjects	0.9	0.6-1.3	1.2	0.8-1.9	1.2	0.8-1.9
At least one sibling	0.9	0.7-1.3	0.7	0.4-1.2	0.7	0.4-1.3
First-born	1.0	0.8-1.4	1.1	0.8-1.6	1.1	0.8-1.6
Passive smoking	1.0	0.7-1.3	1.0	0.7-1.4	1.0	0.7-1.4
At least one URTIcin the previous 3 mo.						
Rhinitis	0.9	0.7-1.3	1.0	0.7-1.5	1.0	0.7-1.5
Tonsillitis	0.8	0.6-1.3	0.9	0.6-1.5	1.0	0.6-1.5
Laryngitis	0.6	0.3-1.1	0.8	0.5-1.5	0.8	0.4-1.5
Acute otitis media	1.3	0.9-1.9	1.5	0.9-2.3	1.4	0.9-2.2
Acute sinusitis	2.3	1.1-4.6[d]	3.1	1.4-6.4[d]	3.3	1.6-6.9 [d]
LRTIs[e] in the previous 3 mo.						
Acute bronchitis	0.7	0.4-1.1	0.6	0.3-1.1	0.6	0.4-1.1
Pneumonia	0.8	0.2-2.6	0.4	0.1-3.1	0.9	0.2-3.7
Antibiotic therapy in the previous 3 mo.						
Aminopenicillins	0.9	0.6-1.5	1.0	0.5-1.8	1.0	0.5-1.8
Amoxicillin-clavulanate	0.8	0.4-1.3	0.7	0.3-1.5	0.8	0.4-1.5
Macrolides	0.6	0.3-1.1	0.8	0.4-1.6	0.7	0.4-1.5
Cephalosporins	0.9	0.6-1.3	0.8	0.5-1.3	0.9	0.6-1.5
At least one antibiotic	0.8	0.7-1.2	1.0	0.7-1.4	1.0	0.7-1.4

[a]OR = odds ratio.
[b]95%CI= confidence interval.
[c] URTIs= upper respiratory tract infections.
[d] $p<0.05$.
[e] LRTIs=lower respiratory tract infections.

Resistance to erythromycin, clarithromycin, and azithromycin (MIC_{50} 0.25, MIC_{90} ≥64) was very common (52.1%, 126 isolates); the most resistant serogroups were 6B (16.7%), 19F (15.9%), 14 (14.3%), and 19A (12.7%). Of these macrolide-resistant strains, 94 (74.6%) were preventable by the heptavalent pneumococcal conjugate vaccine; none of the 32 uncovered strains is usually considered invasive. Although no significant association was found, the macrolide-resistant strains were more often isolated in children aged <2 years (60.4%) than in those ages 2-5 years (56.3%) or >5 years (44.6%). Fourteen isolates (5.8%) were both penicillin and macrolide resistant.

Risk Factors for Nasopharyngeal Carriage of *S. pneumoniae*

Table 4 shows the results of the univariate analysis of the potential risk factors for the nasopharyngeal carriage of *S. pneumoniae*. One or more episodes of sinusitis in the previous 3 months was the only risk factor for total carrier status and the carriage of both invasive strains and the strains covered by the heptavalent vaccine. None of the other variables was significantly associated with pneumococcal nasopharyngeal carriage, regardless of the strain. Multivariate analysis also indicated at least one episode of sinusitis in the previous 3 months as the only risk factor for the nasopharyngeal carriage of *S. pneumoniae* (total carriers: OR 2.48; 95% CI 1.11-5.0; carriers of

invasive strains: OR 3.04; 95% CI 1.23-6.53; carriers of strains covered by the heptavalent vaccine: OR 3.30; 95% CI 1.41-6.83).

In terms of antibiotic resistance, univariate analysis identified one or more episodes of acute otitis media in the previous 3 months as the only risk factor (OR, 2.8; 95% CI 1.2-6.8) and an age >5 years as a protective factor (OR, 0.3; 95% CI, 0.1-1.0) for the carriage of penicillin-resistant *S. pneumoniae*. Multivariate analysis confirmed the role of a previous history of acute otitis media (OR 2.7; 95% CI 1.0-6.6). Neither univariate or multivariate analysis identified an association between the carriage of macrolide-resistant strains and any risk or protective factor.

Discussion

S. pneumoniae was carried by 8.6% of the 2,799 healthy children. Possible reasons for this low prevalence, similar to that previously reported by us (15), include the fact that the survey only included healthy children, the subjects were enrolled for a very short time, winter (a period of frequent respiratory illness) was not the season of enrollment, the large sample prevented any focus on specific situations, and the fact that human genetic traits may play a role (15,17,20). Furthermore, although a sampling or laboratory error is unlikely because all of the investigators were carefully pretrained and the microbiologic procedures were monitored throughout the study, some continuing colonization titers may have been below the sensitivity threshold of the culture method (13).

The serotypes most frequently colonizing our healthy population (3, 19F, 23F, 19A, 6B, and 14) were those commonly involved in invasive pneumococcal diseases (12,17-19,21), highlighting the importance of nasopharyngeal colonization in the development of serious community infections.

The low rate of penicillin resistance (9.1%) and the high rate of macrolide resistance (52.1%) detected in our study population are in contrast to the data reported in other countries (22-25) but consistent with previous Italian reports regarding adults and children with lower respiratory tract infections and invasive diseases (19,26,27). Comparison of the present data with those coming from our previous survey of a similar population of healthy subjects revealed an increased prevalence of antibiotic-resistant pneumococci, especially in children ages <2 years (15). As in other studies, we found that resistance to penicillin was associated with serotypes 9V and 23F, whereas resistance to macrolides was related to a wide range of serotypes (particularly 6B, 19F, 14, and 19A) (19,26,27).

Univariate and multivariate analyses indicate that infections of the nasal sinuses and the middle ear may favor *S. pneumoniae* carriage and may play a role in the spread of the organisms. However, considering that the data on the characteristics of our study population were obtained from parental recollection, the role of the different risk factors in pneumococcal colonization needs to be further confirmed. The protective effect of an age of >5 years on the carriage of penicillin-resistant strains is in agreement with other published data (17). As we have previously observed (15), but unlike other authors' findings (10,28), nasopharyngeal carriage of *S. pneumoniae* and antibiotic use per se or the type of drug used in the previous 3 months were not related. In our previous survey (15), we found that having one or more older siblings and a history of full-time day-care attendance were risk factors for the nasopharyngeal carriage of *S. pneumoniae*, whereas living in a rural area was a protective factor. The differences observed in this study may be because of changes in the epidemiologic characteristics of pneumococcal carriage in Italy and confirm the importance of constant local surveillance.

Our data on the efficacy of the heptavalent pneumococcal conjugate vaccine indicate that it could have a considerable impact on the incidence of nasopharyngeal carriage and a major effect on invasive and antibiotic-resistant pneumococcal diseases, especially in the first years of life.

In conclusion, our study shows that, although nasopharyngeal carriage is low in healthy children, the most common circulating serotypes are invasive and antibiotic resistant. No risk factor other than sinusitis and acute otitis media seems to be related to pneumococcal colonization and to the carriage of penicillin-resistant *S. pneumoniae*, respectively. The fact that most isolated strains are covered by the heptavalent conjugate vaccine, especially in the first years of life, suggests that its broader use could reduce the incidence of pneumococcal disease.

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Hospital-Based Diagnosis of Hemorrhagic Fever, Encephalitis, and Hepatitis in Cambodian Children

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Surveillance was conducted for three clinical syndromes (hemorrhagic fever, encephalitis, and hepatitis) in Cambodian children admitted to the National Pediatric Hospital in Phnom Penh from July 1996 through September 1998. Acute- and convalescent-phase sera, and cerebrospinal fluid, when applicable, underwent diagnostic evaluation for infections with *Dengue virus* (DENV), *Japanese encephalitis virus* (JEV), and Hepatitis A, B, C, and E viruses. Of 621 children admitted with hemorrhagic fever, 499 (80%) were confirmed to have either primary or secondary DENV infection. DENV rates were as high as 10.6/100 hospital admissions in September 1998. Of 50 children with clinical encephalitis, 9 (18%) had serologic evidence of JEV infection. Forty-four children had clinical hepatitis, most (55%) due to *Hepatitis A virus* (HAV). One patient had *Hepatitis B virus*, and no patients had hepatitis C or E. This study identified a large number of children with vaccine-preventable diseases (JEV and HAV).

Infectious diseases continue to pose a major threat to populations in developing countries in tropical regions. Dengue is the most important arbovirus infection in Southeast Asia (1). It is spread by the bite of the vector mosquito, *Aedes aegypti*, and causes asymptomatic infection, mild to severe influenza-like symptoms (dengue fever), and plasma leakage and hemorrhage, which are sometimes fatal (dengue hemorrhagic fever). In 1995, Rathavuth et al. conducted 2 days of surveillance for hemorrhagic fever in children admitted to the National Pediatric Hospital (NPH) in Phnom Penh (2). Their findings of a high frequency of secondary *Dengue virus* (DENV) infection, a low mean age of admission, and the presence of all four dengue serotypes suggested that dengue was highly endemic in Cambodia.

Bacterial, viral, fungal, and parasitic agents are all causes of encephalitis or encephalopathy in children in Southeast Asia. Tsai reported that the main causes of encephalitis in rural Asia included tuberculosis, typhoid fever, cerebral malaria, and viruses such as DENV, herpes simplex, measles, *Enterovirus*, and HIV (3). Few reports have been published about the incidence or possible causes of encephalitis in Cambodian children. In a report by Sunara et al., surveillance at two pediatric hospitals in Phnom Penh from 1990 through 1994 showed >300 cases of acute encephalitis in children (4). While the cause for many of these cases was suspected to be *Japanese encephalitis virus* (JEV), laboratory confirmation was lacking.

Only one report has discussed the prevalence of markers for Hepatitis viruses A, B, and C (HAV, HBV, HCV), in Cambodia. In 1991, Thuring et al. conducted a serologic study for markers of viral hepatitides and HIV in Takeo, a southern province (5). In that study, adults and children, both healthy and those ill with liver or kidney disease, were examined. HAV was the most frequent cause of acute hepatitis in these children, occurring in 11 (32%) of 34 pediatric patients. Ongoing infection with HBV accounted for 18%, and one child tested positive for HCV-specific antibody. That study did not screen for *Hepatitis E virus* (HEV) infection.

Our study was undertaken to characterize the extent of disease in Cambodian children, specifically for the following three syndromes: hemorrhagic fever (HF), encephalitis, and hepatitis.

Materials and Methods

Surveillance was conducted at NPH in Phnom Penh. This hospital, one of two pediatric referral hospitals in Cambodia, serves a population of approximately 2.7 million children ages ≤14. Enrolled in this study were children admitted with any of the following clinical signs: HF (fever, headache, or rash, and on physical examination, a positive tourniquet test, ascites, pleural effusion bleeding, or shock); encephalitis (headache, fever, or neck stiffness, and alteration of consciousness or focal neurologic signs); and hepatitis (lethargy, anorexia, nausea or vomiting, abdominal pain, hepatomegaly, scleral icterus, or jaundice). Case definitions were kept broad to capture as many cases as possible.

On the basis of published criteria from the World Health Organization, cases of DHF were classified into one of four grades of severity. Grade 1 includes fever with nonspecific symptoms; the only hemorrhagic manifestation is a positive tourniquet test, easy bruising, or both. Grade 2 includes Grade 1 manifestations plus spontaneous bleeding (usually skin hem-

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orrhages). Grade 3 includes circulatory failure (rapid, weak pulse, hypotension) and cold, clammy skin. Grade 4 is manifested by profound shock with undetectable blood pressure or pulse. The last two grades are considered to be dengue shock syndrome (DSS).

Sera were collected on the day of admission, at the time of discharge, and, in some cases, on follow-up exam. However, due to the nature of the population, a follow-up visit was not always possible, and therefore diagnosis relied on only an admission and discharge sample. Sera were stored at -70°C until transported to the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand, on dry ice. Clinical criteria for admission diagnosis directed the subsequent diagnostic workup. All DHF and encephalitis cases were tested for both JEV and DENV. Sera were tested for immunoglobulin (Ig) G and IgM antibody against DENV and JEV by use of an antibody-capture enzyme-linked immunoassay (EIA) and previously published criteria of acute and primary or secondary dengue (6). Virus isolation was attempted with acute-phase serum specimens, as previously described (7).

Sera were screened for IgM antibody to HAV, IgM antibody to hepatitis B core antigen (HbcAg), hepatitis B surface antigen (HbsAg), and total Ig to HCV by using commercially available kits (HAVAB-EIA, Corzyme-M, AUZYME Monoclonal, and HCV EIA Third Generation; Abbott Laboratories, Abbott Park, IL). Assays were performed as recommended by the manufacturer.

All samples were tested for total Ig and IgM to HEV by an indirect second-generation EIA developed at the Department of Virus Diseases, Walter Reed Army Institute of Research. The assay quantifies total Ig and IgM reactive with recombinant HEV capsid protein expressed using a baculovirus system expressed in U/mL (8). To control interassay variation, all specimens were tested in duplicate wells, with all specimens from a single patient tested together on the same plate. A patient was considered to have HEV infection if there was virologic (HEV RNA positive) or serologic (IgM >100 U/mL, total Ig >500 U/mL) evidence of acute infection.

Statistics

All statistical procedures were performed by using SPSS for Windows, Version 10.0 (SPSS Inc., Chicago, IL). Fisher's exact test (two-tailed) was used to determine significant difference in the number of boys with encephalitis and diagnosed as having JEV compared with girls with the same syndrome and final diagnosis.

Results

Figure 1 shows the rates of HF, encephalitis, and hepatitis per 100 hospital admissions, by month and year. From July 1996 through September 1998, 621 children were admitted with a diagnosis of HF (Table 1). Of those, 495 were confirmed to have a secondary DENV infection by serologic tests; 14 had primary dengue. Figure 2 illustrates the number of confirmed DENV patients compared with the total number of patients with HF.

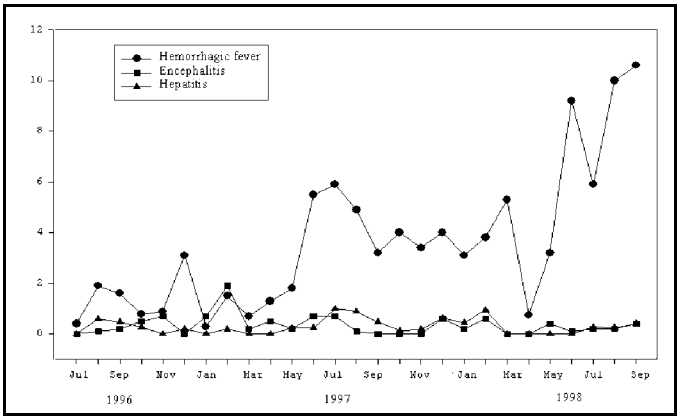

Figure 1. Admission rates by disease syndrome and month, National Pediatric Hospital, 1996–1998.*
*Rates are given as number of cases per 100 hospital admissions.

The severity of DHF can be classified into four grades based on two pathophysiologic findings: hemorrhage and shock. In this study, 41 of the 509 total confirmed dengue patients had DHF Grade 1; 145 patients had DHF Grade 2; 180 patients had DHF Grade 3; and 29 patients had DHF Grade 4.

Of the 75 samples tested, 22 were polymerase chain reaction (PCR)-positive for virus. DENV-2 was isolated from 14 samples, DENV-3 from seven samples, and DENV-4 from one sample. All three serotypes were found in children living in Phnom Penh. DENV-2 and -3 were found in Kampong Speu

Table 1. Characteristics of pediatric patients with clinical hemorrhagic fever, encephalitis, or hepatitis, National Pediatric Hospital, Phnom Penh, Cambodia, July 1996–September 1998[a]

Syndrome	Total no. of cases	Males	Females	Mean age (range)	Outcome
Total hemorrhagic fever	621	288	332	7 yrs (5 mo-15 yrs)	11[b]
Secondary dengue	495	222	272	7 yrs (8 mo-15 yrs)	4[b]
Primary dengue	14	8	6	4 yrs (5 mo-9 yrs)	—
Total encephalitis	50	15	35	4 yrs (3 mo-14 yrs)	17[b]
					12[c]
JE	9	6	3	6 yrs (3-10 yrs)	2[b]
					2[c]
Total hepatitis	44	21	23	6 yrs (2 mo-14 yrs)	—
Hepatitis A	24	12	12	5 yrs (2 mo-9 yrs)	—
Hepatitis B	1	1	0	10 yrs	—

[a]Total number of hospital admissions during this period was 16,492 children.
[b]Deaths.
[c]Disabled.
JE, Japanese encephalitis; —, all recovered.

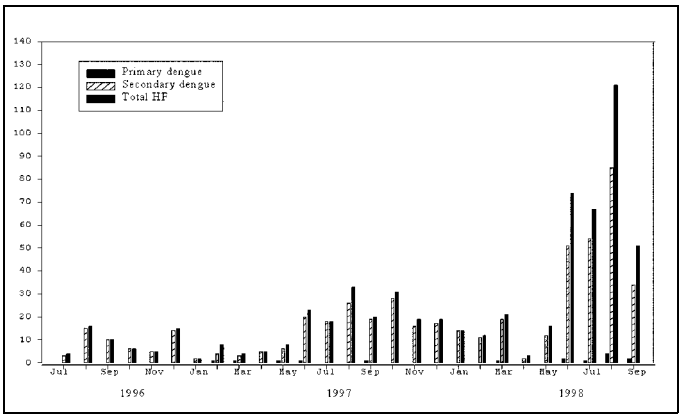

Figure 2. Cases of hemorrhagic fever (HF), secondary and primary dengue, National Pediatric Hospital, 1996–1998

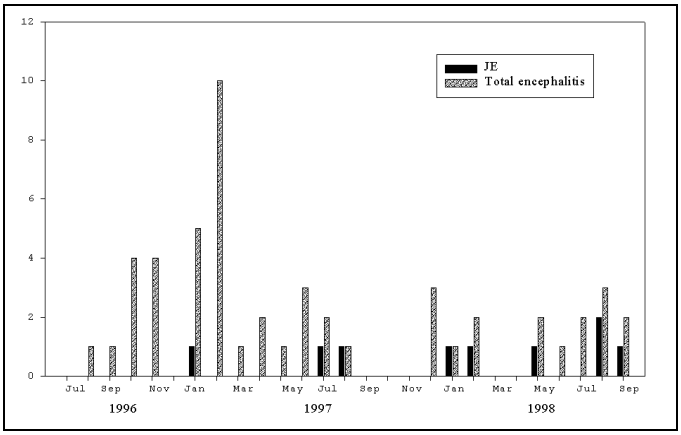

Figure 3. Total number of cases of encephalitis versus Japanese encephalitis, National Pediatric Hospital, 1996–1998.

and Kampong Cham. The other provinces appeared to have only one circulating dengue serotype, based on the small number of positive samples.

During this same period, 50 children were admitted to NPH with a diagnosis of encephalitis; 9 (18%) were due to JEV (Table 1) and 2 to acute secondary dengue infection. There was no evidence of concurrent infections. Figure 3 illustrates the number of cases of JEV, by month and year, compared with the total encephalitis cases. While over twice as many girls as boys had this syndrome, significantly more boys were diagnosed with JEV infection (p=0.015). The final outcome for children seen at NPH with encephalitis was poor: death or disability occurred in 29 (58%) of the children.

Forty-four children were admitted with a diagnosis of hepatitis. Twenty-four cases were confirmed to be due to acute HAV infection on the basis of elevated HAV IgM antibodies (Table 1). One patient had serologic evidence of acute hepatitis B, and no serologic evidence for HCV or HEV infections. Figure 4 illustrates the number of HAV patients compared with the total number of patients admitted to NPH with a clinical diagnosis of hepatitis. Of the 24 children hospitalized with hepatitis A, 17 (71%) had onset in July, August, and September. Most children admitted to NPH came from Phnom Penh (339 patients) or its adjacent province, Kandal (146 patients). Table 2 shows the distribution of inpatients by province and syndrome on admission.

Discussion

This surveillance was undertaken to characterize the extent of disease in Cambodian children with respect to three specific syndromes: hemorrhagic fever, encephalitis, and hepatitis. To our knowledge, this is the first such study conducted in Cambodia. As in other Southeast Asian countries, DHF accounted for a large percentage of hospitalizations and deaths of Cambodian children. Dengue was confirmed in 82% of children admitted to NPH with symptoms that suggested dengue fever. Serologic results for the remaining 112 HF cases suggested DENV infection in most instances, but lack of a convalescent-phase sample prevented definitive diagnosis. DHF has been reported as a leading cause of hospitalization and death of children throughout Asia in 1998 (9). Our surveillance shows that in August and September 1998, the hospitalization rate for children with HF exceeded 10 per 100 hospital admissions.

Similarly, Japanese encephalitis has been reported to occur in nearly every country in Asia (10). While the disease is presumed to be endemic in Cambodia, no laboratory-confirmed data on disease frequency have been published until now. During this 2-year study, 18% of children admitted to NPH with encephalitis had JEV infection. Similar to reports elsewhere (11), more cases of JEV infection occurred in boys than in girls (six versus three, respectively). Transmission of disease is usually seasonal, from late summer to early fall. In our study, 67% of cases were reported from May to October; the remainder occurred in January or February.

HAV infection is highly endemic in developing countries that lack adequate clean water and have poor sanitary conditions (12). According to the 1998 Cambodian General Population Census, only 29% of the population has access to safe water (range 23.7%-60.3%) (13). In poorer countries, most children develop antibodies to HAV by age 10. A seroprevalence study conducted on 200 healthy Cambodian children from 1990 through 1991 showed that 97% were positive for anti-HAV IgG by 15 years of age (5). While hepatitis A is

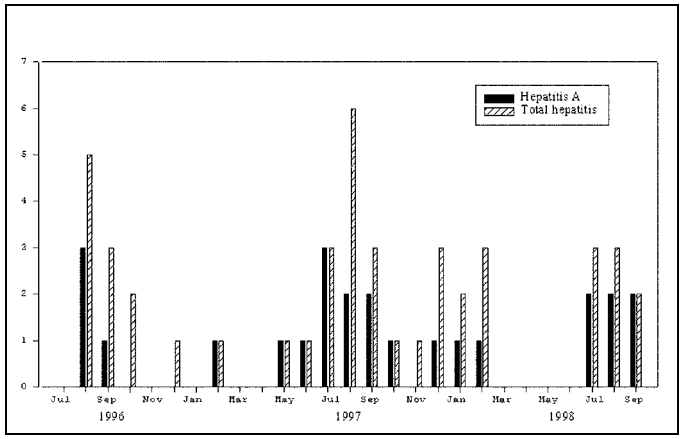

Figure 4. Total number of cases of hepatitis versus hepatitis A, National Pediatric Hospital, 1996–1998.

Table 2. Distribution of inpatients by most widely represented provinces, National Pediatric Hospital, Cambodia

	No. (%) of patients by province						
Syndrome upon admission	Phnom Penh	Kandal	Kampong Speu	Kampong Cham	Takeo	Prey Veng	Total
Hemorrhagic fever	309 (50)	116 (19)	9 (3)	40 (6)	20 (3)		494 (81)
Encephalitis	15 (30)	15 (30)	8 (16)		5 (11)		43 (87)
Hepatitis	15 (34)	15 (34)	3 (7)	3 (7)		5 (11)	41 (93)
Total	339	146	20	43	25	5	

largely considered a subclinical infection in children in Thuring's study, it accounted for 32% of acute hepatitis in hospitalized children in our study; hepatitis A accounted for 55% of the pediatric patients hospitalized with suspected hepatitis.

One (2%) of 44 children showed evidence of ongoing HBV infection. This contrasts with Thuring's earlier study, in which 18% of children with acute hepatitis were actively infected with HBV (HbsAg positive). However, similar to Thuring's study, we saw no evidence of HCV in this population.

No indication of acute hepatitis E was found in our study, nor did any of the children admitted to NPH with hepatitis have evidence of prior exposure to HEV. Similar to hepatitis A, hepatitis E is common in countries that lack adequate clean water and in which general sanitation is poor. In Southeast Asia, epidemics of HEV have been reported in Myanmar, Vietnam, and Indonesia (14). In most disease-endemic areas, up to 5% of the children are positive for anti-HEV antibodies. While clinical attack rates of hepatitis E are reported to be highest in young adults (15-40 years), a recent report from India noted anti-HEV antibodies in >60% of children <5 years old (15). Cambodia is surrounded by countries (except Thailand) that are endemic for HEV; therefore, the total lack of anti-HEV IgG in this Cambodian population was unexpected.

Currently, Cambodian children are given BCG, polio, diphtheria-tetanus-pertussis, and measles vaccines, although coverage varies throughout the country. Instituting childhood HAV immunizations could benefit countries that have shown a decrease in age-related HAV seroprevalence concomitant with improved socioeconomic development (16,17). While the seroprevalence for HAV in Thuring's 1991 study approached 100% in children by age 15, no HAV seroprevalence data have been gathered since then. Further studies are warranted to determine if additional vaccines, such as those for HAV and JEV, should be added to the nation's immunization program.

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Excess Mortality Associated with Antimicrobial Drug-Resistant *Salmonella* Typhimurium

Morten Helms,* Pernille Vastrup,* Peter Gerner-Smidt,* and Kåre Mølbak*

In a matched cohort study, we determined the death rates associated with drug resistance in *Salmonella* Typhimurium. We linked data from the Danish Surveillance Registry for Enteric Pathogens with the Civil Registration System and the Danish National Discharge Registry. By survival analysis, the 2-year death rates were compared with a matched sample of the general Danish population, after the data were adjusted for differences in comorbidity. In 2,047 patients with *S.* Typhimurium, 59 deaths were identified. Patients with pansusceptible strains of *S.* Typhimurium were 2.3 times more likely to die 2 years after infection than persons in the general Danish population. Patients infected with strains resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline were 4.8 times (95% CI 2.2 to 10.2) more likely to die, whereas quinolone resistance was associated with a mortality rate 10.3 times higher than the general population.

Foodborne *Salmonella* infections have become a major problem in most industrialized countries. Of particular concern is the increasing number of infections with antimicrobial drug-resistant *Salmonella*, including the recent emergence of drug-resistant *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) definitive phage type 104 (DT104). This strain is usually resistant to at least five drugs: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT) and has become a predominant *Salmonella* type in many countries, including the United States, United Kingdom, Germany, and France (1-4). In spite of its rapid international dissemination (5) and the fact that antimicrobial drug-resistant *Salmonella* was associated with human infections before the recent spread of DT104, the available data are inconclusive regarding a possible increased virulence of DT104. Whether antimicrobial drug resistance in DT104 contributes to enhanced illness or death is unclear (5-7). Few studies have addressed the health impact of drug resistance in types of zoonotic *Salmonella* other than DT104 (8-10), and these studies suggest that drug resistance may be associated with increased illness and death rates.

Excess mortality associated with drug resistance in zoonotic *Salmonella* is difficult to quantify. Death is a relatively rare event and may not occur until months after the initial diagnosis. Furthermore, a number of factors, including chronic and malignant diseases, may contribute to death from salmonellosis. The objective of this study was to determine death associated with antimicrobial drug resistance in *S.* Typhimurium. The study was based on a large, unbiased sample of Danish patients registered in a national database. We linked these data with those in the Danish civil registry, which has complete information about survival status. Furthermore, by completing the data with information from hospital discharge registries, we were able to adjust for preexisting condition.

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Materials and Methods

Surveillance

In Denmark the diagnosis of human *Salmonella* infections is made at Statens Serum Institut (SSI) or at 10 clinical microbiology laboratories. The SSI receives notifications of positive findings as well as isolates from the microbiology laboratories. If a specific *Salmonella* serotype is found more than once from the same person during a period of up to 6 months, only the first positive sample is registered. As a part of this laboratory-based surveillance system, monitoring for antimicrobial resistance in *S.* Typhimurium was initiated in 1995. In 1995 and 1996, a sample of strains was tested, but from 1997 on, all *S.* Typhimurium strains received at SSI were tested for antimicrobial susceptibility. This study included all isolates of *S.* Typhimurium examined from January 1, 1995, through October 31, 1999.

Isolates were tested by tablet diffusion on Danish Blood Agar (SSI Diagnostica, Hillerød, Denmark) with the use of Rosco Neosensitabs (Rosco, Roskilde, Denmark). The panel included 13 drugs from the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (11). Because reduced susceptibility to ciprofloxacin is difficult to detect by the tablet diffusion test, the E-test (Biodisk, Solna, Sweden) was used as well whenever the tablet diffusion test identified nalidixic acid resistance. In this paper, quinolone resistance refers to strains resistant to the first-generation quinolone nalidixic acid (12).

Registry Linkage Study

All live-born children and citizens of Denmark are assigned a personal identification number, uniquely identifying every person the Danish Civil Registration System (13). Demographic data, including vital status, marriage status, emigration/immigration, and address of residence, are kept in this Civil Registration System.

The matched cohort study used the data from the Civil Registration System to compare the death rates of patients with culture-confirmed *S.* Typhimurium infections to the death rates of persons in the general Danish population. For each patient, we randomly selected 10 people matched by age, sex, and county of residence. People who were born during the same month and year as the patient and were alive on the date of sample receipt were eligible for the reference group. From the Danish Civil Registration System, we obtained information on vital status, date of change of vital status, (i.e., date of death or emigration) and area of residence (county level) for the patients and the persons included in the reference group.

Data on admissions to hospital and discharge diagnosis were obtained by using the data from the Danish National Patient Registry (14) and the Cancer Registry for all persons included in this study, thereby allowing us to control for preexisting illness (comorbidity). Danish National Patient Registry contains data on all patients discharged from non-psychiatric departments since January 1, 1977. Diagnoses and procedures are coded according to the International Classification of Diseases 8 or International Classification of Diseases 10 (from 1993). Diagnoses obtained during 10 years before infection were used to calculate the comorbidity index.

Statistical Methods

The comorbidity index used the principles described by Charlson et al. (15). This index is a sum of severity scores (weights) corresponding to the number and severity of comorbidity conditions. In the first step, we analyzed the data from the background population to calculate the relative rate associated with each of the diagnostic groups summarized in Table 1. These relative rates served as the weights in the further survival analyses. The index was calculated by adding log-transformed weights, thus taking into account multiple hospital discharges. Diagnostic groups associated with a relative mortality rate <1.2 were not included in the models. By including this index in the survival analyses, any difference between the death rates of *Salmonella* patients and the general population quantifies excess mortality beyond what is attributable to underlying illness.

To compare mortality rates of *S.* Typhimurium patients with those of the general population, the data were stratified so that each stratum contained 1 patient and 10 persons from the reference group. To control for age, sex, and county of residence, we used conditional proportional hazard regression. Death up to 2 years after infection was determined, after adjusting the data for comorbidity as described. To assess death rates associated with antimicrobial drug resistance, interaction by drug resistance on *Salmonella* cases was determined. We used the Wald test to test for homogeneity of the rate ratios. The analyses were conducted by the use of the PHREG procedure of the SAS system (Version 6.12, SAS Inst. Inc., Cary, NC). Death rate ratios (RR) are expressed as the relative death rates of patients compared with the matched sample of the general Danish population, and the term “referents” refers to this unexposed matched sample.

Table 1. The distribution of comorbidity diagnosis of 2,047 patients with *S.* Typhimurium infection and a sample of the general Danish population of 20,456 persons

Diagnostic group	No. (%) of *S.* Typhimurium patients	No. (%) in the general population	Severity score (weight) in comorbidity index
Lymphoma or leukemia	19 (0.9)	23 (0.1)	3.40
Metastatic cancers	8 (0.4)	19 (0.1)	2.02
Liver disease	10 (0.5)	35 (0.2)	1.97
Tuberculosis	0	13 (0.1)	1.78
Movement disorders and epilepsy	4 (0.2)	40 (0.2)	1.56
Diabetes	44 (2.2)	186 (0.9)	1.50
Renal disease	31 (1.5)	114 (0.6)	1.37
Inflammatory bowel disease	39 (2.4)	66 (1.7)	1.34
Other neurologic diseases[a]	12 (0.6)	76 (0.4)	1.32
Hemoglobin abnormalities	14 (0.7)	62 (0.3)	1.23
Congestive heart failure	22 (1.1)	103 (0.5)	1.22

[a]Neurologic or neuromuscular diseases other than Alzheimer's disease, multiple sclerosis, Parkinson's disease, Huntington's disease, and epilepsy.

Results

Of 4,075 cases of *S.* Typhimurium infection reported in Denmark from January 1995 to October 1999, the antimicrobial-drug susceptibility was determined in isolates from 2,059 cases, and a successful link to the Civil Registry System was obtained for 2,047 (99.4%). In the period up to 2 years after entry in the study, 59 deaths were identified in *S.* Typhimurium patients and 221 deaths among 20,456 referents. The median age of the 59 persons were 74.1 years (range 18.1 to 90.1). In the first 30 days after entry in the study, the cumulative mortality proportion (Kaplan-Meier estimate) was 0.73% for *S.* Typhimurium patients and 0.04% for the referents (RR 15.4, 95% confidence interval [CI] 6.1 to 39.2). In the period 30 to 720 days after entry, cumulative mortality was 2.75% in *S.* Typhimurium patients and 1.51% in referents (RR 1.8, 95% CI 1.3 to 2.6). On this basis, we used the period 0 to 720 days in the remaining analyses.

Overall, patients with *S.* Typhimurium were 3.0 times (95% CI 2.2 to 4.0) more likely to die than referents in the 2 years following infection. After the data were adjusted for

comorbidity, the relative rate was 2.3 (95% CI 1.7 to 3.2). This relative death rate was independent of age (p=0.84).

A total of 631 (30.8%) patients were hospitalized in connection with the *S.* Typhimurium infection. In the reference group, 577 (2.8%) were hospitalized within 60 days of entry. Five of those had gastroenteritis as their primary diagnosis.

Two hundred seventeen (10.6%) of *S.* Typhimurium patients and 954 (4.7%) persons from the referent group had at least one of the diagnoses listed in Table 1, which summarizes the various diagnostic groups and their weights in relation to the comorbidity index. A total of five HIV infections were found, three among patients and two in the reference group. All five were still living at the end of the study.

In the 2,047 strains, 953 (46.6%) were pansusceptible, 1,094 (53.4%) resistant to at least one drug in the panel, and 639 (30.8%) were resistant to at least two drugs. Resistance to sulfonamides was found in 47.3% of the patient isolates, tetracycline in 25.1%, streptomycin in 22.4%, ampicillin in 19.2%, chloramphenicol in 17.0%, kanamycin in 9.6%, quinolone in 4.1%, trimethoprim in 3.0%, gentamicin in 2.2%, and ceftriaxon in 1.4%. No ciprofloxacin-resistant strains were found. The MIC of ciprofloxacin in the quinolone-resistant isolates ranged from 0.06 to 0.38 mg/L (median 0.09 mg/L).

R-type ACSSuT was found in 283 (13.8%) isolates, and patients infected with this type were 6.9 times more likely to die than the general population, compared with a RR of 2.6 in patients with strains of other R-types (p =0.02). Also, chloramphenicol (7.4 vs. 2.4, p=0.003), quinolones (9.9 vs. 2.8, p=0.05), and ampicillin (5.1 vs. 2.7, p=0.09) were associated with higher death rates in resistant than sensitive strains.

Table 2 shows the relative death rate associated with antimicrobial resistance after the data was adjusted for coexisting diseases. Infections with pansusceptible strains were 2.3 times (95% CI 1.5 to 3.5) more likely to die than the general population, whereas infection with R-type ACSSuT was associated with 4.8 times (95% CI 2.2 to 10.5) higher mortality. Patients infected with quinolone-resistant strains (R-type Nx) were 10.3 times (95% CI 2.8 to 37.8) more likely to die, and R-type ACSSuTNx was associated with 13.1 times (95% CI 3.3 to 51.9) higher mortality. Three other antimicrobial drugs (trimethoprim, gentamicin, and ceftriaxone) were examined, but because of a low number of resistant strains, valid statistical inference could not be carried out. All the strains resistant to these drugs exhibited R-type ACSSuT. Most (82%) of the chloramphenicol-resistant strains and 72% of the ampicillin-resistant strains were also R-type ACSSuT.

A total of 270 of the isolates with R-type ACSSuT were phage-typed, and 217 (80.4%) were DT104, 18 (6.7%) DT12, 11 (4.1%) DT120, and the rest were other or unknown phage types. Strains with other R-types were distributed over a number of different phage types. A total of 1,667 were examined, and the three most common were DT12 (46.8%), DT66 (6.0%), and U288 (4.9%). Thirty-nine (2.3%) were DT104. In the patients with R-type ACSSuT, no difference in the death rate between persons infected with DT104 (relative death rate 4.4, 95% CI 1.7 to 11.6) and other phage types (relative death rate 6.4, 95% CI 1.3 to 32.4) was found; both estimates were adjusted for comorbidity.

No difference in age and sex distribution between patients infected with R-type ACSSuT and other antibiograms were found. The median age in both groups was 33 years (range 1 to 87 and 0 to 95, respectively, p=0.89).

Finally, we analyzed a model with three levels of resistance: non-ACSSuT, R-type ACSSuT (Nx-sensitive), and R-type ACSSuTNx. The figure shows the survival curve of the referents and patients according to these three groups. In the

Table 2. Two-year relative death rate of patients infected with *Salmonella* Typhimurium, by antimicrobial susceptibility pattern. Registry linkage study including 2,047 patients and a random matched sample of 20,456 people from the Danish general population

	Resistant		Susceptible[a]		
	Deaths/cases	RR[b] (95% CI[c])	Deaths/cases	RR (95% CI)	p-value[d]
Resistant to ≥1 drug	31/1,094	2.4 (1.6–3.7)	28/953	2.3 (1.5-3.5)	0.86
Ampicillin	13/393	3.5 (1.7-7.2)	46/1,654	2.1 (1.5-3.0)	0.21
Chloramphenicol	16/347	5.1 (2.6-10.2)	43/1,700	1.9 (1.4-2.8)	0.01
Streptomycin	13/458	2.1 (1.1-4.1)	46/1,589	2.4 (1.7-3.4)	0.76
Sulfonamides	31/969	2.5 (1.6-3.9)	28/1,078	2.1 (1.4-3.3)	0.60
Tetracycline	15/513	2.2 (1.2-4.1)	44/1,534	2.4 (1.7-3.4)	0.85
Kanamycin	3/108		23/1,018	3.9 (2.2-6.7)	
Quinolone	5/83	10.3(2.8-37.8)	54/1,964	2.1 (1.6-3.0)	0.02
R-type ACSSuT	12/283	4.8 (2.2-10.5)	47/1,764	2.1 (1.5-2.9)	0.06
R-type ACSSuTNx	5/40	13.1 (3.3-51.9)	7/243[e]	2.9 (1.1-7.9)	0.09

[a]Susceptible refers to strains susceptible to the given antimicrobial drugs or combination of antimicrobial drugs; first cell refers to pansusceptible strains.
[b]Death rate relative to the general population, as estimated by conditional proportional hazards regression analysis, controlling for underlying illness; RR=rate ratio.
[c]CI=confidence interval.
[d]p-value for test of homogeneity, i.e., RR for resistant being the same as for susceptible strains.
[e]Strains with R-type ACSSuT, but quinolone sensitve.

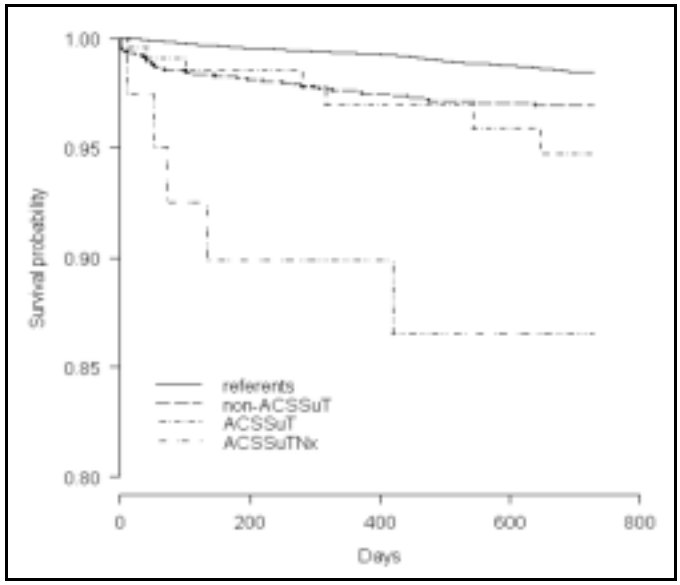

Figure. Survival comparison of patients infected with *Salmonella* Typhimurium (by resistance level) to referents. The patients and referents were matched by age, gender, and county of residence.

group of 40 cases with R-type ACSSuTNx, we identified five deaths within the 2-year period after infection, one of those within the first month of infection, three within 6 months, and one within 18 months. The relative risk associated with an infection with R-type ACSSuTNx was 12.4 without adjusting the data for comorbidity. After adjustment, the RR associated with this resistance pattern was 13.1. The median age in this group was 43 years (range 1 to 89), 10 years higher than the R-type ACSSuT quinolone-sensitive group.

Discussion

Since the 1990s, the frequency of antimicrobial drug resistance in zoonotic *Salmonella* and the number of drugs to which the strains are resistant have increased, primarily as a consequence of antimicrobial use in food production (1,9,16-18). The recent development of fluoroquinolone resistance is of particular concern (16-21). At present, a fluoroquinolone is the drug of first choice for extraintestinal and serious intestinal *Salmonella* infections in adults, and resistance to this drug may potentially reduce the efficacy of early empirical treatment. The health impact of antimicrobial drug resistance in zoonotic *Salmonella* needs to be determined (21,22). We used data from registries created for other purposes to avoid bias and were able to explore long-term death rates and adjust the data for comorbidity.

The comorbidity index was based on discharge diagnoses from patients admitted to hospitals in Denmark and to a lesser degree on data from outpatient clinics but did not include data from general practitioners. Any patient with a coexisting disease severe enough to alter the outcome of a *Salmonella* infection is likely to have had contact with a hospital or an outpatient clinic within the 10-year period before infection. The backbone for the construction of the comorbidity index was the National Discharge Registry. A validation of this registry showed that there was agreement between the registry and hospital records of 75% to 90%, using 3-digit level International Classification of Diseases diagnoses (14).

In general, patients with *S.* Typhimurium infections were 2.3 times more likely to die than the matched sample of the Danish population during a 2-year follow-up. This figure is likely to reflect both long-term consequences of *S.* Typhimurium as well as underlying diseases and conditions not fully described by our comorbidity score based on hospital discharge diagnosis. The excess mortality was independent of age, a finding which warrants further studies. The cumulative mortality in the first 30 days, 0.7%, is comparable with the case-fatality rate of 0.8% for all nontyphoidal *Salmonella* serotypes found in data from FoodNet 1996-97 (23).

We found that *S.* Typhimurium with R-type ACSSuT was associated with higher death rates than other strains. Similar tendencies were found for chloramphenicol and ampicillin, both being markers for R-type ACSSuT. Patients infected with R-type ACSSuT were seven times more likely to die than the general population, but when the data were adjusted for underlying illness, this figure was reduced to fivefold higher mortality. This reduction was expected; a part of the excess mortality associated with R-type ACSSuT was attributable to underlying illness. However, the excess mortality still tended to be elevated after adjustment. Patients with quinolone-resistant strains had a marked and substantial excess mortality, which could not be explained by imbalances in comorbidity. All the quinolone-resistant strains in this study were designated as fluoroquinolone-susceptible by NCCLS cut-offs for ciprofloxacin. Several patients in the study were part of an outbreak of *S.* Typhimurium DT104 R-type ACSSuTNx traced back to swine herds in the Danish island of Zealand (17).

Most deaths occurred in relation to infections with *S.* Typhimurium DT104, and we were not able to demonstrate any statistically significant variation among different phage types. In our initial model, we took age into account, expecting a relatively higher mortality among the elderly. But again, we could not demonstrate such an effect. In other words, no additive effect was found between age and drug resistance compared with age and being infected by sensitive strains of *S.* Typhimurium.

A study from England suggests that the isolation rates of drug-resistant DT104 from blood cultures are not higher than those of other *S.* Typhimurium phage types and that the frequency is comparable with the incidence of blood culture isolates of *Salmonella* Enteritidis (7). The study suggests that *S.* Typhimurium of R-type ACSSuT does not cause invasive disease more often than *Salmonella* Enteritidis. However, the overall mortality in relation to *S.* Typhimurium infection is higher. Two studies based on outbreaks of resistant *Salmonella* in the United States and the United Kingdom have found case fatality rates of 4.2% and 3.0%, respectively (6,8). Even though they were based on outbreak investigations, the cumulative death rate is comparable to our results (2.9% after 6 months of infection).

Antimicrobial drug resistance in zoonotic *Salmonella* may be associated with adverse consequences in several ways, including treatment failures. However, treatment failures have, until now, been infrequently reported (17,21). We had no data on treatment with antimicrobial drugs. Therefore, exploring the extent to which the excess mortality of patients infected with quinolone-resistant strains was caused by reduced efficacy of drugs was impossible. We estimate that approximately 20% of the patients were prescribed empiric treatment in connection with the collection of specimens and that some of the deaths may have been associated with reduced efficacy of flouroquinolones, as described in Mølbak et al. (17).

Resistant bacteria have a selective advantage in ecosystems where antimicrobial drugs are used. Studies have shown that treatment with antimicrobial drugs (for any reason) is a major risk factor for infections with antimicrobial drug-resistant bacteria, and that this association may result in increased incidence and illness severity (9,24,25). Infection with drug-resistant *S.* Typhimurium in patients treated for other infections may contribute to the excess mortality we found.

Infections with resistant *Salmonella* may be associated with increased severity for reasons that are poorly understood. An increased virulence of drug-resistant *Salmonella* has not been well characterized. Two earlier studies found increased rates of hospitalizations (10) and death (8), but these studies had limitations. Lee et al. (10) were only able to control for comorbidity in a limited way, and none of the earlier studies were restricted to a single serotype and able explore the impact of specific resistance patterns as we did.

The use of antimicrobial drugs in food production is one of the major factors in the emergence and dissemination of antimicrobial drug-resistance in foodborne bacterial pathogens. We were able to determine death rates in a large sample of patients with *S.* Typhimurium and to control for confounding factors in the analyses. We associated resistance in *S.* Typhimurium with excess mortality, and the demonstration of a hazard to human health underscores the need for restrictions in the use of antimicrobial drugs in the production of food from animals. A particular risk was associated with quinolone resistance, indicating that the use of fluoroquinolones for food production animals should be discontinued.

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Dr. Helms is a research fellow at the Department of Epidemiology Research, Statens Serum Institut, studying health outcomes in relation to foodborne bacterial infections, in particular the hazards associated with drug-resistant bacteria in our food supply.

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Sentinel Surveillance: A Reliable Way To Track Antibiotic Resistance in Communities?

Stephanie J. Schrag,* Elizabeth R. Zell,* Anne Schuchat,* and Cynthia G. Whitney*

We used population-based data to evaluate how often groups of randomly selected clinical laboratories accurately estimated the prevalence of resistant pneumococci and captured trends in resistance over time. Surveillance for invasive pneumococcal disease was conducted in eight states from 1996 to 1998. Within each surveillance area, we evaluated the proportion of all groups of three, four, and five laboratories that estimated the prevalence of penicillin-nonsusceptible pneumococci (%PNSP) and the change in %PNSP over time. We assessed whether sentinel groups detected emerging fluoroquinolone resistance. Groups of five performed best. Sentinel groups accurately predicted %PNSP in five states; states where they performed poorly had high between-laboratory variation in %PNSP. Sentinel groups detected large changes in prevalence of nonsusceptibility over time but rarely detected emerging fluoroquinolone resistance. Characteristics of hospital-affiliated laboratories were not useful predictors of a laboratory's %PNSP. Sentinel surveillance for resistant pneumococci can detect important trends over time but rarely detects newly emerging resistance profiles.

Antibiotic-resistant infections are an emerging problem in community as well as nosocomial settings. *Streptococcus pneumoniae* infections are a leading cause of community-acquired respiratory illness in young children, the elderly, and persons with chronic medical conditions. Pneumococcal infections range from otitis media and bacteremia to pneumonia and meningitis. Although penicillin has traditionally been an effective treatment for pneumococcal infections, in recent years the increasing prevalence of drug-resistant pneumococci threatens the effectiveness of antibiotic therapy (1,2).

Surveillance for resistant pneumococci is an essential component of public health efforts to prevent the spread of drug resistance. In addition to increasing awareness of the public and health-care providers about resistance, surveillance data can be used to target high-prevalence areas for judicious use of antibiotics, pneumococcal vaccination campaigns, or both; identify newly emerging strains and resistance profiles; and assess trends in resistance. At the national level, surveillance data can contribute to the development of clinical guidelines for managing pneumococcal disease (3,4). Local surveillance data can in some instances guide patient care (4).

The prevalence of drug-resistant pneumococci varies geographically. Because national trends may not reflect trends within specific regions, local and state-specific data can motivate prevention efforts (5). Although invasive disease due to drug-resistant pneumococci was added to the National Notifiable Diseases List in 1994, mandatory reporting remains low (53% of states and territories in 1999) (6), in part because collecting antimicrobial susceptibility data can be difficult. Active, population-based surveillance for resistant pneumococci based on laboratory-confirmed invasive disease may be considered the most accurate method of estimating rates of drug-resistant pneumococcal disease in a defined area. Such systems, however, are often costly and labor-intensive for state or local health departments to maintain.

Sentinel surveillance, a system that collects information on drug-resistant pneumococci from a limited sample of hospital, clinic, and/or private laboratories, has been suggested as a feasible alternative method of collecting regional data, and some states are adopting this approach (7). Although sentinel systems are useful for monitoring trends in a number of diseases (8-10) and a sentinel hospital surveillance system in the 1980s first detected increases in the prevalence of penicillin-resistant pneumococci in the United States (11), observations that the prevalence of resistant pneumococcal isolates can vary dramatically from laboratory to laboratory within a state or area (12) raise the question of whether sentinel laboratories can accurately reflect an area's prevalence of pneumococcal resistance.

For pneumococcus, the most common approach to sentinel surveillance is to select a small number of clinical laboratories within an area and collect information on susceptibility of all invasive pneumococcal isolates at those facilities as a way of estimating the prevalence of resistance in the area as a whole. To evaluate the validity of this sentinel approach, we assessed how often small groups of laboratories in a given area accurately estimated the area's proportion of resistant invasive pneumococcal isolates, using population-based surveillance as the standard. We also evaluated whether such sentinel groups of laboratories accurately tracked changes in the proportion of drug-resistant pneumococci over time, and whether they could detect newly emerging resistance profiles. Finally, we

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explored whether hospital characteristics could be used to guide selection of hospital laboratories for inclusion in sentinel systems, in order to increase the system's representativeness and reliability.

Methods

Population-Based Data

Invasive pneumococcal surveillance was conducted from 1996 to 1998 as part of the Active Bacterial Core Surveillance/ Emerging Infections Program Network (ABCs) using previously described methods (1). Briefly, project personnel communicated at least twice each month with contacts in all participating microbiology laboratories serving acute-care hospitals in San Francisco County, California; Connecticut; eight counties in Georgia (Cobb, Clayton, De Kalb, Douglas, Fulton, Gwinnett, Newton, and Rockdale) with 12 additional Atlanta-area counties starting in 1997; six counties in Maryland (Anne Arundel, Baltimore, Baltimore City, Carroll, Harford, and Howard); seven counties in Minnesota (Anoka, Carver, Dakota, Hennepin, Ramsey, Scott, and Washington); seven counties in New York starting in 1997 (Genesee, Livingston, Monroe, Ontario, Orleans, Wayne, and Yates); three counties in Oregon (Clackamas, Multnomah, and Washington); and five counties in Tennessee (Davidson, Hamilton, Knox, Shelby, and Williamson).

A case was defined as the isolation of *Streptococcus pneumoniae* from a normally sterile site (e.g., blood or cerebrospinal fluid) from a resident of a surveillance area. Periodic audits were conducted in each area. Any cases newly identified by audits were included in the surveillance database.

All isolates were sent to one of two centralized laboratories for susceptibility testing by broth microdilution, with a panel of drugs that included (in 1998) penicillin, amoxicillin, cefotaxime, cefuroxime, meropenem, erythromycin, clindamycin, chloramphenicol, vancomycin, rifampin, levofloxacin, trovafloxacin, and quinupristin-dalfopristin (Synercid7). Nonsusceptibility (resistance and intermediate susceptibility) was determined according to criteria of the National Committee for Clinical Laboratory Standards (13).

Ability of Sentinel Laboratory Groups To Estimate Proportion of Resistant Isolates

In each surveillance area for 1998, we generated all possible simple random samples of three, four, and five laboratories, excluding laboratories with <10 isolates. We limited our selection to up to five laboratories because a central objective of sentinel surveillance is to reduce required resources by reducing the number of facilities participating in the surveillance system. We refer to these simple random samples as sentinel groups of laboratories. We then calculated the percent of penicillin-nonsusceptible (MIC $\geq$0.1 µg/mL) pneumococci (%PNSP) among isolates in each of these sentinel groups and compared these percentages to the area's actual %PNSP, as measured by ABCs. The %PNSP in sentinel groups was considered to be accurate if it was within 5 percentage points of the area's actual %PNSP. We chose this interval because variation in the %PNSP within this range is unlikely to influence public health decisions (12).

We used a finite population correction based on the total number of isolates in each surveillance area to assess the number of randomly sampled isolates that would be needed to estimate an area's actual %PNSP within 5 percentage points (14). We compared that number with the number of isolates in sentinel groups in each area.

Ability of Sentinel Groups To Track Changes in Prevalence of Drug-Resistant Pneumococci over Time

In each surveillance area, we subtracted the %PNSP in each possible group of five laboratories in 1996 from that measured for the group of five laboratories in 1998. We included only laboratories with $\geq$10 isolates in each of the 2-year periods. We then measured how often the change in %PNSP in sentinel groups was within 5 percentage points of the area's actual change in %PNSP during the same time periods, based on ABCs data. We performed a similar analysis using the percentage of erythromycin-nonsusceptible (MIC $\geq$0.5 µg/mL) isolates as the outcome measure.

Ability of Sentinel Goups To Detect Emerging Fluoroquinolone Resistance

Using data from 1998, we measured the proportion of all possible groups of five sentinel laboratories within each surveillance area that captured any pneumococcal isolates with fluoroquinolone (levofloxacin or trovafloxacin) nonsusceptibility. We then compared that proportion with area-specific data on the presence of pneumococcal fluoroquinolone resistance from ABCs in 1998.

Evaluation of Hospital Predictors of %PNSP

We merged ABCs data from 1997 and 1998 with purchased data on hospital characteristics collected by the American Hospital Association (AHA) as part of the AHA Annual Survey of Registered American Hospitals in 1997. We categorized each hospital that matched between the two datasets into the following PNSP classes: $\geq$5 percentage points above the surveillance area proportion PNSP (high PNSP), <5 percentage points above or below the surveillance area PNSP (average PNSP), or $\geq$5 percentage points below the surveillance area PNSP (low PNSP). We used logistic regression to perform univariate analyses. We compared hospital characteristics in the high group with those in the average group, separately comparing hospital characteristics in the low group with those in the average group. We categorized continuous variables according to their quartiles or medians based on their distributions. We limited our analysis to hospital characteristics that might plausibly influence a hospital's %PNSP based on findings of previous studies (15,16).

Results

Population-Based Data

The %PNSP across surveillance areas in 1998 varied from 15 (California and New York) to 35 (Tennessee) (Table 1). The number of laboratories that isolated invasive pneumococci and the total number of invasive pneumococcal isolates also varied by surveillance area (Table 1). Consistent with previous observations (12), each surveillance area had striking variation across laboratories in the %PNSP in invasive pneumococcal isolates (Figure).

Ability of Sentinel Laboratory Groups To Estimate %PNSP

In New York, California, and Oregon (areas with a relatively small number of laboratories with ≥10 invasive pneumococcal isolates), sentinel groups of three, four, or five laboratories all did well at estimating the area's actual %PNSP (Table 1). In the remaining areas, increasing the number of laboratories included in sentinel groups from three to five increased the probability that the sentinel %PNSP approached the area's actual %PNSP. However, in Georgia and Tennessee, the two areas with the highest actual %PNSP, sentinel groups of five laboratories still poorly estimated the area's actual percentage (Table 1).

In surveillance areas where most sentinel groups had an adequate sample size to estimate %PNSP accurately (i.e., the number of isolates met the sample size requirement), sentinel groups performed well compared with population-based surveillance (Table 2). In contrast, in Georgia and Tennessee, where sentinel groups performed poorly, a smaller proportion of sentinel groups met the minimum sample size requirements. However, in some states that failed to meet sample size requirements (e.g., Connecticut), sentinel groups performed well.

Ability of Sentinel Groups To Detect Trends in Prevalence of Nonsusceptible Pneumococci

The actual change in %PNSP in 1998 compared with that in 1996 varied across areas, ranging from Georgia's 2% decline to Maryland's 7% increase (Table 3). Because sentinel groups of five were the most accurate at predicting an area's actual %PNSP, we focused strictly on groups of five for this analysis. Laboratories participating in ABCs in 1998 were often not the same as those participating in 1996 because of hospital or laboratory mergers, closing or opening of microbiology facilities in the surveillance areas, and expansion of areas under surveillance. Consequently, only a subset of all possible sentinel groups in 1998 matched those in 1996.

Over two thirds of each area's sentinel groups of five accurately estimated changes in %PNSP, except in Tennessee, where only 45% correctly estimated a <5 percentage point change (Table 3). In the three areas with large changes in %PNSP (≥3 percentage points), >90% of sentinel groups in each area predicted the direction of the change (increases in each case).

Trends in the proportion of isolates that were erythromycin nonsusceptible also varied by area, and three areas showed large increases from 1996 to 1998 (Table 3). Similar to trends observed for penicillin nonsusceptibility, sentinel groups had a high probability of detecting these increases in erythromycin nonsusceptibility (Table 3).

Ability of Sentinel Groups To Detect Emerging Fluoroquinolone Resistance

In 1998, seven isolates submitted to ABCs were nonsusceptible to levofloxacin; five of these were also nonsusceptible to trovafloxacin. The isolates came from seven different hospitals, located in five of the eight surveillance areas (California, Connecticut, Maryland, Minnesota, and Oregon). One of these hospitals, the only hospital from Oregon, had only five invasive pneumococcal isolates in 1998 and thus was excluded from our analysis of sentinel groups. Approximately 40% of sentinel groups of five laboratories in these areas (range 37% in Connecticut to 45% in Maryland) included a laboratory with a fluoroquinolone-nonsusceptible isolate, except in California, where there was only one possible sentinel group of five laboratories and this group included the fluoroquinolone-nonsusceptible isolate.

Table 1. Ability of sentinel groups of three, four, and five laboratories to estimate accurately %PNSP, 1998[a]

Area	Labs with ≥10 isolates (total labs)	Actual %PNSP	Total isolates	% of sentinel groups within 5 percentage points of actual %PNSP		
				3 labs (no. of groups; overall range in %PNSP)	4 labs (no. of groups; overall range in %PNSP)	5 labs (no. of groups; overall range in %PNSP)
CA	5 (9)	15	181	100 (10; 12-17)	100 (5; 13-16)	100 (1; NA)
CT	25 (32)	18	681	73 (2,300; 2-31)	81 (12,650;4-30)	87 (53,130;6-30)
GA	18 (34)	33	860	45 (816; 19-51)	52 (3,060; 20-49)	58 (8,568; 21-48)
MD	20 (26)	22	579	60 (1,140; 8-40)	68 (4,845; 9-38)	74 (15,504; 10-37)
MN	12 (24)	20	470	78 (220; 11-30)	88 (495; 12-29)	94 (792; 14-28)
NY	5 (19)	15	191	80 (10; 9-15)	100 (5; 10-14)	100 (1; NA)
OR	6 (13)	21	228	80 (20; 14-25)	93 (15; 14-23)	100 (6; 17-21)
TN	20 (30)	35	419	37 (1,140; 11-62)	40 (4,845; 13-59)	44 (15,504;14-57)

[a]In Active Bacterial Core surveillance areas.
%PNSP, percent of penicillin-nonsusceptible invasive pneumococcal isolates.

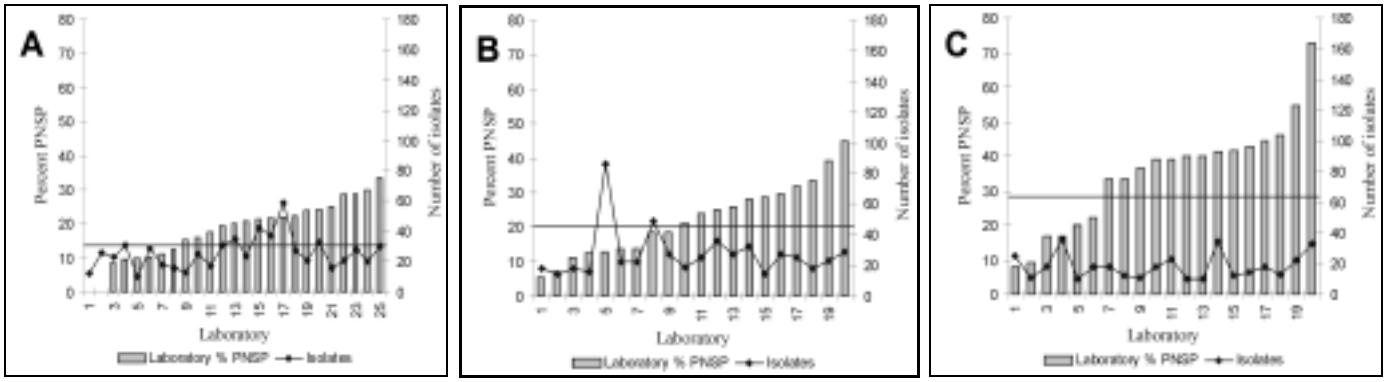

Figure. Between-laboratory variation in percent penicillin-nonsusceptible isolates (%PNSP) and number of invasive pneumococcal isolates in selected surveillance areas. A) Connecticut; B) Selected counties of Maryland; C) Selected counties of Tennessee. Solid line denotes the area's actual %PNSP from active, population-based surveillance.

Evaluation of Hospital Predictors of %PNSP

The merged dataset of ABCs and AHA hospitals contained 104 hospitals: 24 (23%) were in the high PNSP category, 52 (50%) were in the average PNSP category; and 28 (27%) were in the low PNSP category. Hospitals that admitted only children (four hospitals that matched between the two datasets) were significantly more likely to be in the high PNSP group than in the average group (all four hospitals fell in the high category; Fisher's exact test, p=0.008). Larger hospitals (measured by adjusted inpatient days, total beds, or total beds set up and staffed) were more likely to fall in the average category, but this trend was not consistent for all indicators capturing hospital size (Table 4). Additional variables tested by univariate analysis were not predictive of falling in the high or low category (Table 4). When we performed similar analyses using the percent of erythromycin-nonsusceptible isolates or of isolates with resistance to more than one drug class as the primary outcome measure, no additional predictors were identified.

Table 2. Number of isolates required to estimate accurately %PNSP in a given area and percentage of sentinel laboratory groups that met sample size requirements

Area	Actual %PNSP (target range)	No. of isolates needed to estimate %PNSP[a]	% of sentinel groups of 5 laboratories with ≥ no. of required isolates
CA	15 (10-20)	94	100
CT	18 (13-23)	172	3
GA	33 (28-38)	243	40
MD	22 (17-27)	183	12
MN	20 (15-25)	163	70
NY	15 (10-20)	97	100
OR	21 (16-26)	120	100
TN	35 (30-40)	191	0

[a] No. of isolates, n, required to estimate the area's actual %PNSP (P) within 5 percentage points (d=0.05) with 95% confidence (Z=1.96) is: $n= (Z^2 P(1-P))/d^2$, where d is the range of accepted variation around the actual %PNSP, and Z is the Z-score range within which values must fall. Because the total no. of isolates per area, N, was small, we corrected this estimate for finite population size: n=n/[1+(n-1)/N]. There is no power associated with this estimate (14).
%PNSP, percent of penicillin-nonsusceptible pneumocooccal isolates.

In areas where sentinel surveillance did not accurately estimate the %PSNP (Georgia and Tennessee), can hospital predictors be used to improve performance? When we limited sentinel groups of five to the laboratories with the largest number of isolates, the range in %PNSP narrowed, but accuracy was not guaranteed (range in Georgia 29%-34%; range in Tennessee 36%-44%). Additionally, consistent with the analysis above, hand-picking sentinel hospitals to include those with a high proportion of pediatric isolates was likely to overestimate the actual %PNSP; in Georgia the children's hospital had a %PNSP of 61%, whereas the area's true %PNSP was 33% (Table 1).

Table 3. Ability of sentinel groups of five laboratories to estimate an area's change in %PNSP and erythromycin-nonsusceptible pneumococci, 1996–1998

Outcome measure	Area[a]	Actual change in % NS pneumococci	% sentinel groups within 5 percentage points of the area's actual change in % NS pneumococci	% of sentinel groups detecting an increase or decrease in the actual % NS pneumococci[c]
Penicillin NS	CA	+3	100 (1)	100
	CT	+1	67 (15,504)	
	GA	-2	76 (2,002)	
	MD	+7	70 (15,504)	93
	MN	+6	97 (252)	99
	TN	0	45 (462)	
Erythromycin NS	CA	-2	100 (1)	
	CT	+2	95 (15,504)	
	GA	+6	80 (2,002)	86
	MD	+6	97 (15,504)	99
	MN	+7	83 (252)	99.6
	TN	+2.5	51 (462)	--

[a] NY joined ABCs in 1997; the only group of 5 laboratories in OR in 1996 did not match any of the groups in 1998.
[b]Groups that merged between the 2 years.
[c]We limited this analysis to areas with ≥3% change in either direction.
%PNSP, percent penicillin-nonsusceptible pneumococci; NS, nonsusceptible.

Table 4. Univariate analysis of characteristics of hospitals with a high or low %PNSP compared with hospitals with an average %PNSP[a]

	High vs. average %PNSP				Low vs. average %PNSP			
	No.				No.			
Hospital characteristic	High	Avg	Odds ratio	p value	Low	Avg	Odds ratio	p value
Adjusted inpatient days[b]				0.02				0.06
0-66,452	11	7	Ref[c]		8	7	Ref	
66,453-104,771	5	11	0.29	0.09	10	11	0.80	0.73
104,772-146,879	6	17	0.23	0.03	3	17	0.15	0.02
>146,879	3	16	0.12	0.007	7	16	0.38	0.17
Total beds set up and staffed				0.04				0.25
0-173	11	8	Ref		7	8	Ref	
174-300	6	11	0.40	0.18	10	11	1.04	0.96
301-413	4	16	0.19	0.02	5	16	0.36	0.16
>414	4	16	0.19	0.02	6	16	0.43	0.23
Adult medical/surgical and ICU beds								
0-16	15	19	Ref		17	19	Ref	
>16	7	26	0.31	0.05	9	29	0.39	0.06
Pediatric medical/surgical and ICU beds								
0-10	13	20	Ref		16	20	Ref	
>11	9	25	0.55	0.26	10	25	0.50	0.17
Hospital with a pediatric ICU								
No	18	35	Ref		22	35	Ref	
Yes	4	10	0.78	0.70	4	10	0.64	0.49
Medicaid inpatient days				0.10				0.36
0-3,730	9	10	Ref		7	10	Ref	
3,731-8,797	7	10	0.78		9	10	1.3	0.71
8,798-19,477	7	15	0.52		4	15	0.38	0.20
>19,477	2	16	0.14		8	16	0.71	0.61
Medicare inpatient days				0.04				0.02
0-18,246	10	6	Ref		10	6	Ref	
18,247-29,026	5	12	0.25	0.06	9	12	0.45	0.24
29,027-45,471	5	18	0.17	0.01	3	18	0.10	0.005
>45,471	5	15	0.20	0.03	6	15	0.24	0.04
Metropolitan statistical area size								
1 million population	5	10	Ref		5	10	Ref	
≥l million population	20	41	0.98	0.97	23	41	1.12	0.84

[a]High %PNSP was defined as ≥5 percentage points above the surveillance area % of penicillin-nonsusceptible pneumococci (PNSP) ; low as ≥5 percentage points below the surveillance area %PNSP; average as <5 percentage points above or below the surveillance area %PNSP.
[b]Adjusted inpatient days were calculated as Inpatient Days + (Inpatient Days * [Outpatient Revenue/Inpatient Revenue]).
[c]Ref=Referent group.
ICU, intensive-care unit.

Discussion

As the incidence of drug-resistant pneumococcal disease continues to increase, the need for local and state-specific data on the emergence of drug-resistant invasive pneumococcal strains also grows. Although active, population-based surveillance provides highly accurate data for tracking pneumococcal resistance trends, few states can afford to implement such labor-intensive and costly systems. Moreover, states may have a variety of objectives for their surveillance systems, ranging from increasing awareness of resistance in local communities and promoting appropriate antibiotic use activities to estimating directly the drug-resistant isolates and trends in drug resistance; some of these objectives require more accurate surveillance systems than others.

Our evaluation of the performance of sentinel laboratory groups suggests that sentinel surveillance is a viable alternative to population-based surveillance in situations where a high degree of accuracy is not required. In some cases, sentinel

surveillance may also be useful when accurate estimates of %PNSP trends are a primary objective. Sentinel laboratory groups were most reliable at detecting large increases or decreases in the proportion of nonsusceptible invasive isolates; the groups varied in their ability to predict an area's actual %PNSP; and they were poor at detecting newly emerging fluoroquinolone resistance. As a result, areas considering sentinel surveillance should design systems and interpret data with caution.

Baseline information on isolates processed annually per laboratory and between-laboratory variability in %PNSP can be used to predict how well sentinel systems will perform at estimating this percentage in a given area. Such information can often be collected retrospectively or prospectively from microbiology laboratories. Authorities in areas with high between-laboratory variability or with few isolates per laboratory may want to consider alternatives or complements to sentinel systems.

Reasons for high between-laboratory variability in the proportion of nonsusceptible invasive pneumococcal isolates, such as we observed in Tennessee (Figure), remain unclear. This variability likely reflected differences in the risk for nonsusceptible pneumococcal infections in communities served by different laboratories. Because health insurance policies in the United States often determine the hospitals and laboratories that patients use, these facilities rarely serve populations that are representative of the community as a whole or even the neighborhood where the hospital is located. Characterizing risk factors for nonsusceptible invasive pneumococcal disease in a hospital's patient population is difficult. Readily obtainable hospital characteristics such as those collected by AHA did not explain the between-laboratory variation we observed. Unfortunately, some known predictors of resistance in healthcare settings, such as suburban middle- and upper-class patient populations (15,16), were not available to link to our surveillance data.

Although most basic hospital characteristics were not a reliable guide to selecting laboratories to be included in sentinel systems, pediatric hospitals were significantly more likely than other hospitals in an area to have a high %PNSP. Because children are a primary reservoir of *S. pneumoniae* and the incidence of invasive pneumococcal disease is elevated in children and the elderly (1), states may sometimes choose to include children's hospitals in sentinel surveillance systems to increase their likelihood of identifying resistance problems. However, to track trends in resistance to drugs such as fluoroquinolones that are not indicated for use in children, children's hospitals may not be reliable indicators.

For states wishing to increase the reliability of sentinel systems, increasing the overall number of laboratories participating in sentinel systems improved the accuracy of systems, particularly in areas where the %PNSP approaches 50%. However, in areas with high between-laboratory variation in %PNSP, accuracy is difficult to achieve without including most laboratories in the system.

For states or regions with a primary objective of detecting rare, newly emerging resistance profiles, more than one surveillance approach may be necessary. For example, sentinel surveillance combined with universal reporting of fluoroquinolone- or vancomycin-resistant pneumococci will help detect important new strains before they become widespread. Additionally, authorities in such areas may consider collecting the isolates captured by sentinel facilities and conducting susceptibility testing by using a more diverse drug panel than is typically used in most clinical microbiology laboratories.

If used and interpreted appropriately, sentinel laboratory surveillance helps document pneumococcal resistance and improve prevention efforts. Evaluation of alternative surveillance methods such as analysis of hospital antibiograms (17) or direct electronic reporting of susceptibility results from hospital laboratories to a central network (M. Soriano-Gabarro, unpub. data) will further contribute to identifying low-cost, feasible methods of documenting trends in pneumococcal resistance.

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Research Studies: Articles should be 2,000 to 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.

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

Evaluation in Nonhuman Primates of Vaccines against Ebola Virus

Thomas W. Geisbert,* Peter Pushko,* Kevin Anderson,* Jonathan Smith,* Kelly J. Davis,* and Peter B. Jahrling*

Ebola virus (EBOV) causes acute hemorrhagic fever that is fatal in up to 90% of cases in both humans and nonhuman primates. No vaccines or treatments are available for human use. We evaluated the effects in nonhuman primates of vaccine strategies that had protected mice or guinea pigs from lethal EBOV infection. The following immunogens were used: RNA replicon particles derived from an attenuated strain of *Venezuelan equine encephalitis virus* (VEEV) expressing EBOV glycoprotein and nucleoprotein; recombinant *Vaccinia virus* expressing EBOV glycoprotein; liposomes containing lipid A and inactivated EBOV; and a concentrated, inactivated whole-virion preparation. None of these strategies successfully protected nonhuman primates from robust challenge with EBOV. The disease observed in primates differed from that in rodents, suggesting that rodent models of EBOV may not predict the efficacy of candidate vaccines in primates and that protection of primates may require different mechanisms.

Ebola virus (EBOV) and *Marburg virus* (MBGV), which make up the family *Filoviridae*, cause severe hemorrhagic disease in humans and nonhuman primates, killing up to 90% of those infected. EBOV was first recognized in the former Zaire in 1976. Subsequently, outbreaks have been documented in Sudan, Gabon, the former Zaire, Côte d'Ivoire, and Uganda (1-3). In addition to the African outbreaks, the species *Reston Ebola virus*, which may be less pathogenic for humans, was isolated from cynomolgus monkeys imported from the Philippines to the United States (4). Although outbreaks of EBOV have been self-limiting, the lack of an effective vaccine or therapy has raised public health concerns about these emerging pathogens.

In early attempts to develop a vaccine against EBOV, guinea pigs or nonhuman primates were vaccinated with formalin-fixed or heat-inactivated virion preparations. Results from these studies were inconsistent: Lupton et al. (5) partially protected guinea pigs against EBOV, while Mikhailov et al. (6) achieved complete protection of four of five hamadryad baboons by vaccinating them with an inactivated EBOV vaccine. However, other studies suggested that inactivated EBOV did not induce sufficient immunity to reliably protect hamadryl baboons against a lethal challenge (7). Conventional strategies of attenuating viruses for use as human vaccines have not been pursued for EBOV because of concerns about reversion to a wild-type form. However, the possibility of following this strategy by using newly developed infectious clones of EBOV may now be feasible (8).

Recent efforts have focused on the use of recombinant DNA techniques to stimulate cytotoxic T-lymphocyte responses. Vaccinating guinea pigs with plasmids against EBOV nucleoprotein (NP), soluble glycoprotein, or glycoprotein (GP) elicited humoral and cellular immune responses against these gene products but only partially protected them against lethal challenge (9). However, results of this study were difficult to interpret because all the guinea pigs were killed 10 days after EBOV challenge, which is within the expected survival time for untreated animals (8-14 days) (10). In 2000, Sullivan et al. (11) reported protection of cynomolgus monkeys from EBOV infection by injecting them with naked-DNA GP, followed by an adenovirus-expressing GP booster. Results of this study document the feasibility of vaccination against EBOV. However, these results require confirmation and further evaluation, as a low dose (6 PFU) was used for the challenge. Other studies reported a protective effect of EBOV vaccination with a low infective challenge dose (ten 50% lethal doses [LD_{50}]) (7); however, all vaccinated animals in these dosing studies died after receiving higher infective doses (100 and 1,000 LD_{50}), which may more accurately mimic natural or nosocomial exposures.

Our efforts to develop a vaccine against EBOV focused on several potential vaccine candidates. First, we used *Venezuelan equine encephalitis virus* (VEEV) replicon particles (VRP) expressing EBOV genes known to protect guinea pigs and mice from EBOV disease (10); VRP expressing MBGV genes also protected guinea pigs and cynomolgus monkeys against MBGV (12). Second, we used a recombinant *Vaccinia virus* (VACV) system expressing EBOV GP and demonstrated that this vector protected guinea pigs from EBOV hemorrhagic fever (13). A third strategy used encapsulated, gamma-irradiated EBOV particles in liposomes containing lipid A (14); and the fourth approach evaluated vaccination with a concentrated, gamma-irradiated whole-virion preparation. None of these approaches, which successfully protected rodents from lethal infection, were protective for cynomolgus or rhesus macaques challenged with EBOV.

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Materials and Methods

Cynomolgus macaques (*Macaca fascicularis*) or rhesus macaques (*M. mulatta*) weighing 4 to 6 kg were used. For vaccine studies with VEE replicons, EBOV GP or NP genes were introduced into the VEEV RNA as described (10). Groups of three cynomolgus macaques were vaccinated with VRP that expressed EBOV GP, EBOV NP, a mixture of EBOV GP and EBOV NP, or a control antigen (influenza hemagglutinin) that has no effect on EBOV immunity. Animals were vaccinated by subcutaneous injection of 10^7 focus-forming units of VRP in a total of 0.5 mL at one site. Vaccinations were repeated 28 days after the first injection and 28 days after the second.

In conducting research with animals, the investigators followed the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (1996). The animal facilities and animal care and use program of the U.S. Army Medical Research Institute of Infectious Diseases are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

For vaccine studies using primates, we adapted the optimal immunization regimens determined from the rodent studies. For the vaccine based on recombinant VACV, the EBOV GP gene was inserted into a VACV transfer vector plasmid, and recombinant VACV expressing EBOV GP were isolated as reported (13). Three cynomolgus macaques were injected subcutaneously with the EBOV GP-expressing VACV vector. Injections were repeated at 28 and 53 days after the first injection.

For vaccine studies with inactivated EBOV whole-virion preparation, viral particles were concentrated from Vero cell culture fluids by ultracentrifugation in a sucrose density gradient. Infectivity titer of the preparation was approximately 8.0 $\log_{10}$ PFU/mL. The preparation was inactivated by exposure to ^{60}Co gamma rays (6 x 10^6 rads). The absence of residual infectivity was proven by exhaustive testing for residual infectivity in assays in Vero cells (15,16). Two cynomolgus monkeys and two rhesus monkeys were injected subcutaneously with a 50-µg dose of the gamma-irradiated virion preparation in RIBI adjuvant (Corixa, Hamilton, MT). As a further check on complete viral inactivation, blood samples taken from the monkeys 3 and 5 days after they received the vaccine were free of infectious viremia. Injections were repeated at days 7 and 35 after the initial injection.

For vaccine studies using a liposome formulation, three cynomolgus monkeys were vaccinated with gamma-irradiated virus encapsulated in liposomes containing lipid A, as described for previous studies in mice (14). Animals received 1.0 mL of the liposome preparation by intravenous injections that were repeated at 28 and 55 days after the initial vaccination. Four macaques (two cynomolgus and two rhesus) served as unvaccinated controls for the VACV, gamma-inactivated virion, and liposome studies.

Anti-EBOV neutralizing antibody titers were monitored by measuring plaque reduction in a constant virus:serum dilution format (15). All macaques received intramuscular injections in the leg with 1,000 PFU of the Zaire subtype of EBOV, which was isolated from a human patient in 1995 (16). Blood was obtained from all monkeys under Telazol anesthesia (Fort Dodge Laboratories, Fort Dodge, IA) at 2- or 3-day intervals postinfection to determine infectious viremia, neutralizing antibody titers, and standard hematologic and clinical pathology parameters. All terminally ill monkeys were killed and necropsied for pathologic examination. Virus infectivity assays on plasma and tissue homogenates were done by forming plaques on Vero cell monolayers as described (15,16).

Tissues were immersion fixed in 10% neutral-buffered formalin and processed for histopathologic and immunohistochemical characteristics as described (17-19). Replicate sections of spleen were stained with phosphotungstic acid hematoxylin to demonstrate polymerized fibrin. Sections of spleen from five EBOV-infected guinea pigs and five mice from previous studies (20,21) were similarly stained for polymerized fibrin. Portions of selected tissues from 11 monkeys were also immersion fixed in 4% formaldehyde and 1% glutaraldehyde and processed for transmission electron microscopy according to conventional procedures (17-19).

Results

Serologic Response

Prechallenge EBOV neutralization titers were measured for the 26 nonhuman primates used in this study (Table 1). Although all vaccinated animals seroconverted by immunoglobulin G enzyme-linked immunosorbent assay, neutralizing antibody ($PRNT_{50}$) titers were very low. Only one macaque vaccinated with VRP-expressed EBOV GP had detectable

Table 1. Prechallenge neutralization titers of Ebola virus (EBOV)-vaccinated monkeys

Nonhuman primate species	No. of animals	Vector	Antigen	Neutralization titers[a]
Cynomolgus	3	Replicon	GP	0, 0, 0
Cynomolgus	3	Replicon	NP	0, 0, 0
Cynomolgus	3	Replicon	GP + NP	0, 0, 10
Cynomolgus	3	Replicon	Influenza HA	0, 0, 0
Cynomolgus	3	Vaccinia	GP	10, 20, 20
Cynomolgus	3	Liposome	Inactivated virion	20, 40, 80
Cynomolgus	2		Inactivated virion	10, 20
Rhesus	2		Inactivated virion	10,[b] 20
Cynomolgus	2	None		0, 0
Rhesus	2	None		0, 0

[a]Immunoglobulin G enzyme-linked immunosorbent assay, neutralizing antibody ($PRNT_{50}$) All vaccinated monkeys seroconverted by enzyme-linked immunosorbent assay before challenge.
[b]Animal survived challenge.
GP, glycoprotein; NP, nucleoprotein.

neutralizing antibody. The marginal PRNT did not preclude challenge of the monkeys; however, in previous studies, similar results were obtained when cynomolgus macaques were vaccinated with the VRP expressing MBGV genes, yet the animals were protected from lethal disease (12).

Challenge of Vaccinated Monkeys with EBOV

All animals, including the four untreated macaques, were challenged with 1,000 PFU of EBOV. Timing of challenge varied because of differences in the optimal immunization regimens determined by preliminary testing in rodents. VRP-vaccinated animals were challenged 49 days after the third vaccine dose. At postchallenge day 3, all animals became ill; two animals from each vaccination group (i.e., GP, NP, GP + NP, influenza HA) died on day 6, and the remaining animals died on day 7 (Table 2). VACV GP-inoculated macaques were challenged 45 days after the third vaccine dose, EBOV liposome-vaccinated animals 35 days after the third vaccine dose, and macaques vaccinated with the gamma-irradiated whole-virion preparation 43 days after the third vaccine dose. Again, all animals except one rhesus macaque, which received the gamma-irradiated virion preparation, became ill on the third day after challenge. Two cynomolgus macaques vaccinated with the gamma-irradiated virion preparation, one VACV-GP animal, and one untreated cynomolgus macaque died on postchallenge day 6 (Table 2). The two remaining VACV-GP animals died at day 7 after challenge, as did two of the animals vaccinated with the EBOV liposome preparation and the remaining untreated cynomolgus macaque. The untreated rhesus macaques died on days 8 and 9 postchallenge; one rhesus vaccinated with the gamma-irradiated virion preparation died on day 9, and the other survived challenge. The remaining animal vaccinated with the EBOV liposome preparation died 11 days after challenge. The rhesus macaque that survived challenge did not become ill during the study and had a $PRNT_{50}$ values >320 at day 26 postchallenge and 80 at days 26, 61, 99, and 902 postchallenge.

Table 2. Challenge of vaccinated monkeys with Ebola virus (EBOV)

NHP Species	Vector	Antigen	Survival/ total	Viremic/ total	Day of death[a]
Cynomolgus	Replicon	GP	0/3	3/3	6, 6, 7
Cynomolgus	Replicon	NP	0/3	3/3	6, 6, 7
Cynomolgus	Replicon	GP + NP	0/3	3/3	6, 6, 7
Cynomolgus	Replicon	Influenza HA	0/3	3/3	6, 6, 7
Cynomolgus	Vaccinia	GP	0/3	3/3	6, 7, 7
Cynomolgus	Liposome	Inactivated virion	0/3	3/3	7, 7, 11
Cynomolgus		Inactivated virion	0/2	2/2	6, 6
Rhesus		Inactivated virion	1/2	2/2	9
Cynomolgus	None		0/2	2/2	6, 7
Rhesus	None		0/2	2/2	8, 9

[a]Number of days after challenge with 1,000 PFU of EBOV.
NHP, nonhuman primate; GP, glycoprotein; NP, nucleoprotein.

Histopathologic Examination

Conventional histopathologic and electron microscopic examination of lymphatic tissues, liver, and gastrointestinal tract showed no differences in lesions between the vaccinated animals and the unvaccinated EBOV-infected controls. Depletion and necrosis or apoptosis were noted in all lymphoid germinal centers in spleen, peripheral, and mesenteric lymph nodes, as described in other studies (17-19). The spleen had copious deposits of fibrin throughout the red pulp, as well as abundant karyorrhectic cellular debris. By electron microscopy, widespread bystander lymphocyte apoptosis was a prominent feature in all the lymphatic tissues examined. Fibrin and fibrinocellular thrombi were also prominent in the submucosa of the gastrointestinal tract and in hepatic sinusoids, again consistent with well-documented findings (17,18).

We also evaluated retrospectively EBOV-infected rodent tissues in parallel. Although sites of infection and morphologic changes between guinea pigs, mice, and nonhuman primates had many similarities, the lack of fibrin thrombi in spleen and visceral vasculature was particularly striking in the EBOV-infected mice (Figure). Fibrin deposition was seen in guinea pigs as reported (20), but fibrin deposits and thrombi were considerably less prevalent compared with deposits in nonhuman primates (Figure). Lymphocyte apoptosis was also less frequently observed by electron microscopy in rodent lymphatic tissues than in nonhuman primates. EBOV was demonstrated in liver, spleen, kidney, lung, adrenal gland, and lymph nodes of all necropsied monkeys by immunohistochemistry, electron microscopy, or virus infectivity titration.

Discussion

Our results indicate that rodent models of EBOV hemorrhagic fever do not consistently predict efficacy of candidate vaccines in nonhuman primates, perhaps because the disease course in rodents differs from that reported in human and nonhuman primates (17-19,22,23). Mice do not have the hallmark disseminated intravascular coagulation (DIC) found in end-stage lesions of humans and nonhuman primates. Viremia and widespread tissue dissemination are much more apparent in nonhuman primates than in guinea pigs (20). In addition, guinea pigs have less DIC than do nonhuman primates. Lymphocyte apoptosis was not reported to be a prominent feature of EBOV infection in mice or guinea pigs (20,21) but was a consistent feature of disease in humans (24) and nonhuman primates (19). Clinical disease and related pathologic features in nonhuman primates infected with EBOV appear to more closely resemble those described in human EBOV hemorrhagic fever (22,23). Other studies have shown inconsistencies between rodent and nonhuman primate models of human hemorrhagic disease in the protective efficacy of candidate vaccines. For example, guinea pigs were protected from *Lassa virus* by VACV recombinants expressing the viral nucleopro-

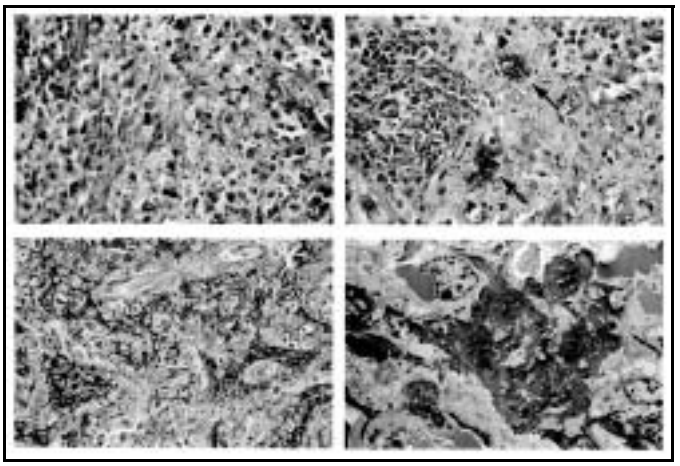

Figure. Sections of spleen from Ebola virus (EBOV)-infected animals. Top left, BALB/c mouse, note absence of polymerized fibrin (phosphotungstic acid [PTA] hematoxylin, original magnification X400). Field representative of five of five mice tested. Top right: guinea pig. Note discreet foci of polymerized fibrin (arrows) (PTA hematoxylin, original magnification X400). This field shows infrequent fibrin deposits; most fields in five of five animals examined showed no evidence of polymerized fibrin. Lower left: cynomolgus monkey. Note deposition of polymerized fibrin in red pulp (PTA hematoxylin, original magnification X400). Field representative of 25 of 25 monkeys. Lower right: cynomolgus monkey. Electron micrograph showing abundant fibrin deposits in pulp (original magnification X5,300). Field representative of 11 of 11 monkeys examined.

tein (25,26); however, this vaccination strategy failed to protect rhesus macaques (27).

The effort to develop an EBOV vaccine began after the initial identification of EBOV in 1976, but 25 years later the goal remains elusive. Attempts to develop killed-virus vaccines against EBOV hemorrhagic fever have had inconsistent results (5-7). Recent progress in genetic vaccination strategies has demonstrated that immunity can be achieved against a low dose of EBOV. While protection against any lethal challenge dose of EBOV is a remarkable achievement, we have set the bar somewhat higher than 6 PFU, since a laboratory exposure through a needlestick and infected blood would likely entail a dose of at least 1,000 PFU. Therefore, our priority is to empirically develop a vaccine that protects against at least 1,000 PFU rather than to initiate an exhaustive investigation of protective immune mechanisms. We were encouraged by the demonstrated success of the VEEV replicon vector expressing MBGV glycoprotein in protecting cynomolgus macaques from challenge with homologous MBGV (12). No MBGV-neutralizing activity was observed at $\geq$1:20 dilutions in prechallenge sera of any of the MBGV GP VRP-vaccinated macaques (12), yet these animals did not become viremic, showed no signs of disease, and survived challenge. Historically, *Filovirus*-neutralizing antibodies have been difficult to demonstrate in vitro (15); while the presence of neutralizing antibodies is desirable, it is neither sufficient nor necessary to clear viral infection (16). Unfortunately, the VEEV replicon strategy that was successfully employed for MBGV in cynomolgus macaques and for EBOV in mice and guinea pigs (10) did not protect cynomolgus macaques from EBOV disease. These differences observed between EBOV and MBGV may result from differences in the course of infection. Specifically, the mean day of death for untreated cynomolgus monkeys experimentally infected intramuscularly with 1,000 PFU of EBOV (Zaire subtype) is 6.3 (n=15; data not shown), while the mean day of death for cynomolgus monkeys infected intramuscularly with a comparable dose of MBGV (Musoke isolate) is 9.1 (n=8; data not shown). Thus, macaques infected with MGBV have nearly three more days to mount an effective immune response against the challenge virus than macaques infected with EBOV (Zaire). Clearly, other variables, including differences observed between EBOV (Zaire) and MBGV with respect to GP gene expression (28), tropism, and host cell responses, may contribute to differences in disease pathogenesis and outcome of infections.

The induction of humoral and cytotoxic T-lymphocyte responses to EBOV NP and GP has been demonstrated in guinea pigs, although the relative contributions of these responses to immune protection are unclear (9). Moreover, transfer of EBOV immune serum in rodent and nonhuman primate models provided inconsistent results. Passive transfer of immune serum from VRP-vaccinated animals did not protect guinea pigs or mice against lethal challenge (10); however, transfer of hyperimmune equine immune globulin (which had high EBOV neutralization titers) to guinea pigs protected them against disease (16,29). Passive treatment of cynomolgus monkeys with the equine immune globulin delayed death but did not ultimately protect the monkeys against lethal EBOV hemorrhagic fever (16,29). In contrast, hamadryl baboons were protected against lethal EBOV challenge by passive treatment with the equine immune globulin and the use of a lower challenge dose (30). These results suggest that cell-mediated effector mechanisms may play a more important role in protection than do humoral responses. Nonetheless, the role of humoral immunity is in fact supported by studies showing consistent delay in death or protection of primates therapeutically treated with EBOV-neutralizing antibodies (16,29,30).

We conclude that, although rodent models are useful as preliminary screens for candidate vaccines and therapeutic treatments, nonhuman primates likely provide a more useful and definitive model for EBOV hemorrhagic fever in humans. Furthermore, differences in disease pathology between rodent and nonhuman primate models of EBOV suggest that protection of primates may require different protective mechanisms.

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Perspectives. Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two.

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.

Typical and Atypical Enteropathogenic *Escherichia coli*

Luiz R. Trabulsi,* Rogéria Keller,* and Tânia A. Tardelli Gomes†

Typical and atypical enteropathogenic *Escherichia coli* (EPEC) strains differ in several characteristics. Typical EPEC, a leading cause of infantile diarrhea in developing countries, is rare in industrialized countries, where atypical EPEC seems to be a more important cause of diarrhea. For typical EPEC, the only reservoir is humans; for atypical EPEC, both animals and humans can be reservoirs. Typical and atypical EPEC also differ in genetic characteristics, serotypes, and virulence properties. Atypical EPEC is more closely related to Shiga toxin–producing *E. coli* (STEC), and like STEC these strains appear to be emerging pathogens.

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhea in developing countries. In industrialized countries, the frequency of these organisms has decreased, but they continue to be an important cause of diarrhea (1). The central mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E), which is characterized by microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at sites of bacterial attachment (Figure 1). The fluorescent actin staining test allows the identification of strains that produce A/E lesions, through detection of aggregated actin filaments beneath the attached bacteria (3). Ability to produce A/E lesions has also been detected in strains of Shiga toxin–producing *E. coli* (enterohemorrhagic *E. coli* [EHEC]) and in strains of other bacterial species (1).

The genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE) (4), a pathogenicity island that contains the genes encoding intimin, a type III secretion system, a number of secreted (Esp) proteins, and the translocated intimin receptor named Tir (1) (Figure 2). Two LEE insertion sites have been described on the *E. coli* chromosome, and a third unidentified insertion site has been reported (5).

Intimin, a 94-kDa outer membrane protein encoded by the *eae* gene, is responsible for the intimate adherence between bacteria and enterocyte membranes. Studies of antigenic variations in the 280-amino acid residues of the C-terminal portion of intimin (the receptor-binding domain of the protein) and the use of polymerase chain reaction analysis allow the classification of distinct intimin types or subtypes among EPEC and STEC strains (6). The Esp molecules (EspA, B, and D) are involved in the formation of a translocon that delivers effector molecules to the host cell and disrupts the cytoskeleton, subverting the host cell functions (7). Tir, which is one of the EPEC translocated proteins, is inserted into the host cell membrane, where it acts as a receptor to intimin (8).

Many EPEC strains produce a characteristic adherence pattern, called localized adherence, in tissue culture cells (9). In this pattern, bacteria bind to localized areas of the cell surface, forming compact microcolonies (bacterial clusters) that can be visualized after bacteria have been in contact with cells for 3 hours. This phenomenon is associated with the presence of the large EPEC adherence factor (EAF) plasmid, which carries the so-called EAF sequence (Figure 2) (1). Also present in the EAF plasmid is the cluster of genes that encode bundle-forming pili (BFP), which interconnect bacteria within microcolonies and thus promote their stabilization (1).

The EAF plasmid is not essential for the formation of A/E lesions, although its presence enhances their efficiency,

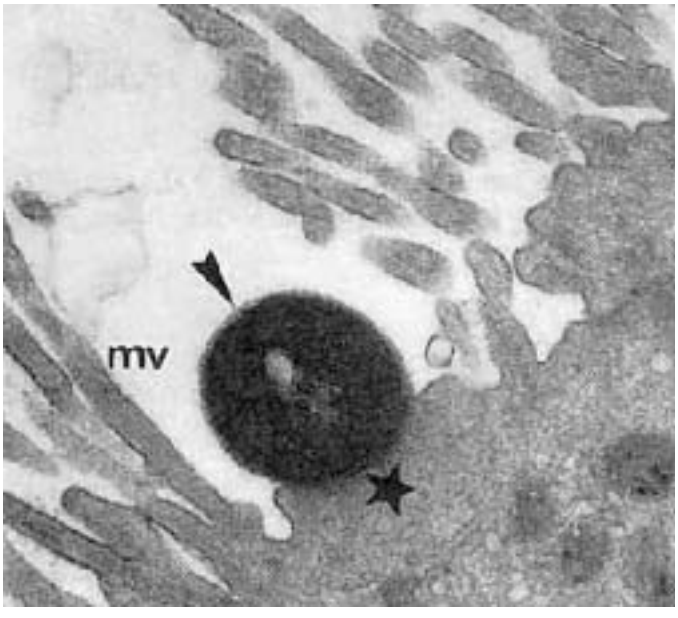

Figure 1. Attaching and effacing lesion showing effacement of microvilli (mv) and pedestal (star) with adherent enteropathogenic *Escherichia coli* (EPEC) (arrow). Reprinted from reference 2, with permission of the director of American Society of Microbiology journals.

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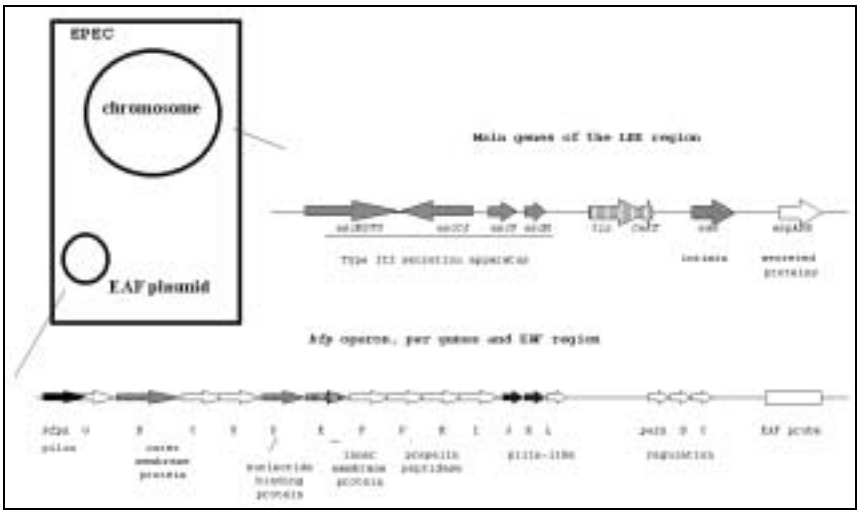

Figure 2. Diagram of the main genes of the locus of enterocyte effacement (LEE) region and the enteropathogenic *Escherichia coli* (EPEC) adherence factor (EAF) plasmid.

probably through the influence of a cluster of plasmid-borne regulatory genes (*per A, B, C*) that increase expression of the chromosomal LEE genes (1). Evidence also indicates that BFP plays a role in host cell adhesion that would similarly increase the efficiency of A/E lesion formation (7).

In 1995, during the Second International Symposium on EPEC in São Paulo, most participants accepted the following EPEC definition: "EPEC are diarrheogenic *Escherichia coli* that produce a characteristic histopathology known as attaching and effacing (A/E) on intestinal cells and that do not produce Shiga, Shiga-like, or verocytotoxins. Typical EPEC of human origin possess a virulence plasmid known as the EAF (EPEC adherence factor) plasmid that encodes localized adherence on cultured epithelial cells mediated by the . . . Bundle Forming Pilus (BFP), while atypical EPEC do not posses this plasmid. The majority of typical EPEC strains fall into certain well-recognized O:H serotypes" (10). According to this definition, the basic difference between typical and atypical EPEC is the presence of the EAF plasmid in the first group of organisms and its absence in the second.

The most studied EPEC strains belong to a series of O antigenic groups known as EPEC O serogroups. Twelve EPEC serogroups were recognized by the World Health Organization in 1987: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158. These serogroups include both typical and atypical EPEC strains, as well as other diarrheogenic *E. coli* categories, mainly enteroaggregative *E. coli* (EAEC) (11-14). Furthermore, most of the strains of each category correspond to specific serotypes in each O serogroup. The division of EPEC strains into typical and atypical has important implications that are not yet fully appreciated. EPEC can no longer be considered as a single group of enteropathogenic organisms. The aim of this article is to review the main differences between typical and atypical EPEC, which should be taken into account in studies involving these organisms.

Serotypes

Typical and atypical EPEC strains belong to two different sets of serotypes (Table 1). This table was constructed on the basis of similar studies carried out in São Paulo (11-15) and the United Kingdom (14) and on a smaller scale in Rio de Janeiro (16) and Italy (17). Most of the typical strains were isolated in São Paulo and Rio de Janeiro and most of the atypical ones in United Kingdom and in Italy. The serotypes isolated in São Paulo include motile and nonmotile strains (indicated by placing the H antigen in brackets). The H antigens of these nonmotile strains were inferred by restriction analysis of the *fliC* genes (B.A. Botelho et al., unpub. data). These serotypes may include both motile and nonmotile variants (Table 1).

Most of the serotypes in Table 1 may easily be classified as typical or atypical. However, some serotypes are not so readily classified, mainly those that include Stx-producing strains, of which the most frequent are serotypes O26:H- and H11, and O111ac:[H8] (considered by some authors as EHEC or STEC) (1). In fact, these serotypes and others with properties similar to those of O128:H2 are not true atypical EPEC or STEC serotypes but rather are heterogeneous serotypes that include different clones or genetic lineages. For example, we have recently shown by random amplified polymorphic DNA that O26:H11 Stx-producing strains isolated in Europe and North America are genetically different from Stx-negative strains of the same serotype isolated in Brazil (18). Although this kind of study has not been done with serotype O128:H2, this serotype is also heterogeneous since it includes different ribotypes with distinct virulence characteristics (L.R. Trabulsi et al., unpub. data). Certain Stx-producing clones have an irregular geographic distribution and so may be found in some countries but not in others. Other characteristics that may complicate distinguishing typical from atypical EPEC are related to the EAF

Table 1. Frequently isolated enteropathogenic *Escherichia coli* (EPEC) serotypes, including typical and atypical strains

Strains	Serotypes
Typical	O55:[H6], O86:H34, O111:[H2],[a] O114:H2, O119:[H6], O127:H6, O142:H6, O142:H34
Atypical	O26:H[11], O55:[H7], O55:H34, O86:H8, O111ac:[H8], O111:[H9], O111:H25, O119:H2, O125ac:H6, O128:H2

[a] Brackets denote the frequent occurrence of nonmotile strains.

plasmid markers. For example, serotypes O119:H2 and O128:H2 react with the *bfpA* probe but do not have a true EAF plasmid. These serotypes have a 100-MDa plasmid that does not contain the *bfp* operon and consequently does not produce BFP (19). In contrast, some O142:H6 strains do not react with the EAF probe but produce BFP and show a typical localized adherence (LA) pattern. These strains may have an EAF plasmid with a defect in the EAF region that does not interfere with the the plasmid's functions. Perhaps the best distinguishing characteristic for typical and atypical EPEC serotypes would be production or nonproduction of BFP.

Virulence Characteristics

In general, typical EPEC strains are more homogeneous in their virulence characteristics than the atypical ones. With few exceptions, typical strains produce only the virulence factors encoded by the LEE region and the EAF plasmid. The exceptions are the production of the cytolethal distending toxin (CDT) by all O86:H34 strains (L.R. Trabulsi et al., unpub. data) and the production of the enteroaggregative heat stable toxin (EAST1) by some strains of serotypes O55:H6 and O127:H6 (T.A.T.Gomez et al., unpub. data) that are potential virulence factors. In contrast, atypical EPEC strains frequently express EAST1 and other potential virulence factors not encoded in the LEE region (Table 2). Accordingly, there are two kinds of atypical EPEC strains: those that express only the LEE-encoded virulence factors and those that express both LEE and the non-LEE encoded virulence factors. Usually both kinds of strains belong to a single clone (11,12,15). All atypical EPEC serotypes, with exception of O125ac:H6, include both kinds of strains. All strains of this serotype examined thus far show the aggregative adherence pattern and the LEE region. The occurrence of more than one kind of strain in most atypical serotypes is another interesting difference between typical and atypical EPEC.

Typical and atypical EPEC strains also differ in adherence patterns. The typical strains show only the LA pattern, while atypical strains may show the LAL (localized-like adherence) pattern (12), the DA (diffuse adherence) pattern, or the AA (aggregative adherence) pattern (Figure 3). The LAL pattern is characteristic of the strains of most serotypes and is mediated mainly by intimin (20). The DA pattern is mediated by the Afa adhesin (R. Keller et al., unpub. data), and the AA is mediated by an aggregative adhesin. The *cdt* gene of serotype O86:H34 (L.R. Trabulsi et al., unpub. data) and the *afa* gene of serotype O55:H7 are located on the bacterial chromosome (R. Keller et al., unpub. data). Typical and atypical EPEC also have some interesting differences with regard to the intimin types (Table 3).

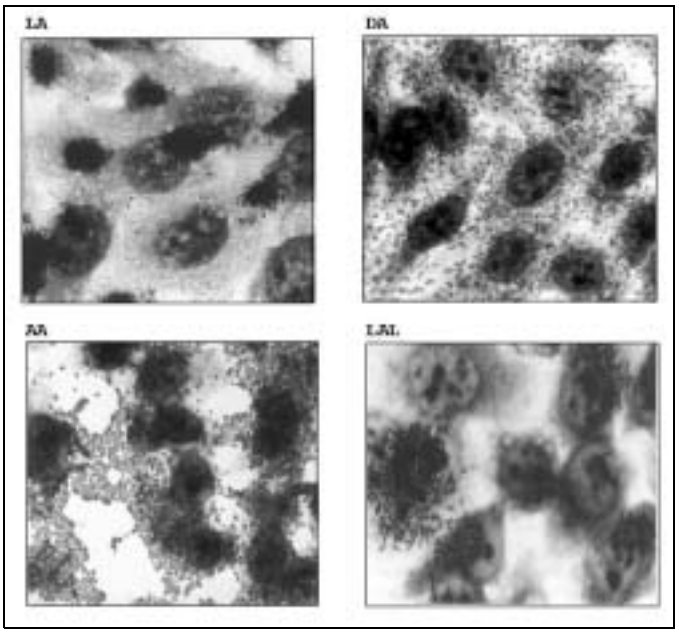

Figure 3. Adherence patterns of enteropathogenic *Escherichia coli* (EPEC) strains. Localized adherence (LA), diffuse adherence (DA), aggregative adherence (AA), and localized adherence-like (LAL). Magnification: X100.

Table 2. Virulence characteristics not encoded on the locus of enterocyte effacement (LEE) of atypical enteropathogenic *Escherichia coli* (EPEC) strains isolated in São Paulo, Brazil

Serotype	Characteristics
O26:[H11][a]	EAST1, E-hly[b]
O55:[H7]	EAST1, Afa
O111ac:[H8]	E-hly
O111:[H9]	E-hly
O119:H2	EAST1
O125ac:H6	AA
O128:H2	EAST1

[a] Brackets denote the frequent occurrence of nonmotile strains.
[b]EAST, heat-stable toxin 1 of EAEC; E-hly, EHEC hemolysin; AA, aggregative adherence; Afa, afimbrial adhesin.

Genetic Relationships

To investigate the genetic relationships between typical and atypical EPEC strains, we used random amplified polymorphic DNA to study our collection of strains, which includes most of the serotypes shown in Table 1. The dendrogram derived from these data (Figure 4) shows that most typical and atypical strains belong to different genetic groups and that the atypical strains are closer to the serotype O157:H7 strains (EHEC), which were included in the study for comparison purposes (S.Y. Bando et al., unpub. data). The only exceptions were the typical and atypical H2 strains that did not separate and formed a subgroup in the atypical/STEC group. The overall results of this study resemble those reported by Whittam et al. (21), who used multilocus enzyme electrophoresis to study a similar collection of strains and distinguished four genetic groups: EPEC 1 (H6/H34 strains), EPEC 2 (H2 strains), EHEC 1 (O55:H7 and O157:H7 strains), and EHEC 2 (O26:H11 and O111ac:H- strains). The EPEC 2 group was also closer to the EHEC groups. For this article, we have not used the division of EPEC into EPEC 1 and EPEC 2, but it may be important in the future. Several other differences exist

Table 3. Intimin types of typical and atypical enteropathogenic *Escherichia coli* (EPEC) serotypes

Intimin types	Typical	Atypical
Alpha	O55:[H6],[a] O127:H6, O142:H6, O142:H34	O111:[H9], O125ac:H6
Beta	O111:[H2], O114:H2, O119:[H6]	O26:H[11], O119:H2, O128:H2
Gamma		O55:[H7], O111ac:[H8]
Delta	O86:H34	

[a] Brackets denote the frequent occurrence of nonmotile strains.

between the two clonal groups (R. Keller et al., unpub. data). With regard to epidemiology, an EPEC 2 serotype (O111:H2) is strongly associated with nosocomial infection, while an EPEC 1 serotype (O119:H6) is more strongly associated with infection in the community (22).

Pathogenicity

The pathogenicity of most typical EPEC serotypes has been confirmed by volunteer studies (1). For atypical EPEC we are aware of only one volunteer study, which was performed by Levine et al. (23) with an O128:H2 strain. This strain was administered in differing doses to 15 adult volunteers, none of whom became ill. Although this study was carefully conducted, its results are difficult to evaluate because the virulence characteristics of the strain were not known and serotype O128:H2 may include nonvirulent strains (24).

The atypical EPEC strains may be less virulent than the typical ones. One reason may be the lack of the EAF plasmid; Levine et al. (25) have shown that an O127:H6 strain without plasmid was less virulent for adult volunteers than the wild-type strain. However, atypical EPEC strains have not been proven to be less pathogenic, and these organisms have other virulence factors that may compensate for the absence of the EAF plasmid. More studies are necessary to resolve this issue.

Association with Diarrhea

Typical EPEC serotypes are strongly associated with diarrhea in children <1 year of age. In this age group, these serotypes have been found to be the main cause of endemic diarrhea in several well-controlled studies carried out in Brazil (26,27). The frequency of typical EPEC serotypes in children >1 year of age is lower and similar to the frequency in controls (2%-4%). Adult infections are rare and usually associated with other conditions (1). The increased resistance in older children and adults may be associated with the development of immunity or the loss of receptors for some specific adhesin (1).

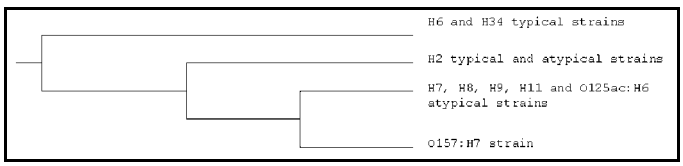

Figure 4. Dendogram to illustrate genetic differences between typical and atypical enteropathogenic *Escherichia coli* (EPEC) strains and *E. coli* O157:H7 strains.

Regarding immunity, several studies carried out in Brazil (28) and more recently in Mexico (29) have shown that children develop high levels of antibody against the main EPEC virulence factors. In addition, the colostrum of mothers living in endemic areas is very rich in immunoglobulin A antibodies against the EPEC virulence factors (28-30). Much less is known about the association of atypical serotypes with diarrhea, but usually these serotypes are isolated from children with diarrhea who are not carriers of other enteropathogenic agents. A strong association of atypical EPEC serotypes with endemic diarrhea has not yet been demonstrated. However, a large outbreak of diarrhea caused by serotype O111:H9 has been described in Finland (31).

Prevalence in Developing and Industrialized Countries

A remarkable epidemiologic difference between typical and atypical EPEC serotypes is their geographic distribution. Typical EPEC serotypes have traditionally been associated with outbreaks of infantile diarrhea, and, in fact, the first EPEC strains isolated in different countries were of serotypes O55:H6 and O111:H2 (32). In the past, these epidemic serotypes were frequently identified in industrialized countries as a cause of outbreaks and sporadic cases of diarrhea, but at present they are very rare (1). In these countries today, serotypes without the EAF plasmid predominate (14,33). In the United Kingdom, for example, EAF-positive strains represent only 10% of all EPEC strains (14). The situation in developing countries is not well defined, but several studies in Brazil in the 1980s and early 1990s showed a high frequency of typical serotypes (34). However, some recent studies have shown a very low frequency of typical EPEC and a relatively high frequency of atypical EPEC (L.C. Campos, pers. commun. and unpub. data). This finding coincides with a decline in the number of diarrheal cases in several regions in Brazil, suggesting that the changes that have occurred in industrialized countries are likely already under way in Brazil. The reason for these changes is not clear, but the decline in the frequency of the EAF-positive serotypes that has occurred in Europe and the United States and is beginning to occur in Brazil may be due to improvements in therapy, sanitary conditions, and control of hospital infections. On the other hand, the emergence and rise in frequency of atypical EPEC strains may have origins similar to those that led to the emergence and increase in frequency of O157:H7 and other STEC serotypes (35).

Reservoir

Typical EPEC serotypes have not been found in animals (1), suggesting that humans are the only living reservoir for these organisms. In contrast, most atypical EPEC serotypes have been isolated from different animal species. The association between serotype O26:H11 and calves is well known (36). Recent studies have emphasized the isolation of Stx-producing strains because of their role in hemolytic uremic syndrome, but *eae*-positive, Stx-negative strains have been isolated from

cattle (37). This kind of strain should be considered atypical EPEC. A similar situation exists in regard to serotype O111ac, and the 69 O111ac strains reported by Ewing et al. in 1963 were all isolated from monkeys (38).

Serotype O128:H2 is rather frequent in rabbits and dogs and, like the human strains isolated in Brazil, is EAF negative (Pestana de Castro, pers. commun.). In a recent study by Pestana de Castro's group, serotypes O119:H2 and O111:H25 (an EAF-negative serotype rare in Brazil but frequent in the United Kingdom) were isolated frequently from dogs. More studies of the prevalence of atypical EPEC serotypes in animals are needed, but available data strongly suggest that the primary reservoir for these organisms is different animal species, as is the case with STEC strains.

Stx-Negative and *eae*-Positive *E. coli* Strains in Non-EPEC O Serogroups

Both stx-negative and *eae*-positive *E. coli* strains are found in many non-EPEC O serogroups (39). We have detected such strains in more than 30 *E. coli* O serogroups, and a large proportion of strains do not agglutinate in the usual set of *E. coli* O antisera. Some strains react with the EAF probe (*eae*+, EAF+ strains), but most do not react with this probe (*eae*+, EAF- strains). With a few exceptions, only one or two strains of each of these serotypes have been reported (40).

The additional virulence characteristics of the *eae*+, EAF+ strains have not been studied, but recently we investigated the virulence profile of 49 different *eae*+, EAF- strains isolated from children with diarrhea in São Paulo. The profile was similar to that of atypical EPEC: many strains were EAST1+ and *E-hly*+, and a few expressed either the AA or the DA adherence pattern. Some strains had the gamma intimin sequence, and in many of the strains the intimin type could not be identified.

Some of these strains do correspond to typical or atypical EPEC, and more studies are necessary to establish a precise concept for them, especially for the EAF-negative strains. Some are likely STEC strains that have lost the *stx* genes; we cannot exclude the possibility that the DA and AA strains are not true EAEC or DAEC that have received the LEE pathogenicity island by horizontal transfer. The situation is quite different for atypical EPEC, since a larger number of strains have been studied and most of them belong to well-characterized serotypes.

The role played by these EAF+ and EAF- strains outside the EPEC serogroups in endemic diarrhea has not been established. In general, the strains are rarely isolated from diarrheal cases and controls, and the global difference is not statistically significant. However, some *eae*+, EAF+ serotypes as well as some *eae*+, EAF- strains with specific virulence profiles seem to be associated with endemic diarrhea (2,33,40). With regard to outbreaks, an *eae*+, EAF- serotype (O39:H-) was responsible for a foodborne diarrheal outbreak in 1991, involving 100 adults in Minnesota (41).

Conclusion

Typical and atypical EPEC seem to constitute two groups of distinct organisms that have in common the LEE pathogenicity island. Atypical EPEC are closer to STEC in genetic characteristics, serotypes, production of toxins, reservoir, and other epidemiologic aspects. As STEC, they resemble emerging pathogens. In industrialized countries, they have become a more frequent cause of diarrhea than typical EPEC, and the same shift may be occurring in Brazil. A large number of Stx-negative, *eae*-positive typical and atypical EPEC-like strains outside the EPEC O serogroups, as well as atypical EPEC strains, require further study in regard to their virulence and epidemiologic significance.

Acknowledgments

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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.

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Increasing Quinolone Resistance in *Salmonella enterica* Serotype Enteritidis

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Until recently, *Salmonella enterica* serotype Enteritidis has remained sensitive to most antibiotics. However, national surveillance data from Denmark show that quinolone resistance in *S.* Enteritidis has increased from 0.8% in 1995 to 8.5% in 2000. These data support concerns that the current use of quinolone in food animals leads to increasing resistance in *S.* Enteritidis and that action should be taken to limit such use.

Salmonella enterica serotype Enteritidis is the most common cause of foodborne salmonellosis worldwide. Historically, this serotype has remained sensitive to most antibiotics, unlike other common serotypes such as Typhimurium, Hadar, Virchow, and Infantis, in which resistance to a wide range of antimicrobial agents is common (1). Recently in Denmark, we have recorded increasing resistance to quinolones in *S.* Enteritidis from human infections. This finding is cause for concern because fluoroquinolones are first-line drugs for treatment of human salmonellosis.

The Study

From 1995 to 2000, 13,334 *S.* Enteritidis infections were recorded in Denmark, accounting for 62% of all zoonotic salmonella infections. To monitor drug resistance (2), we examined a random sample of 2,546 isolates, of which 82 (3.2%) were resistant to the quinolone nalidixic acid. These data showed that quinolone resistance increased from 0.8% (3 of 384 isolates) in 1995 to 8.5% (31 of 366) in 2000 (Figure). Resistance to other antimicrobial agents was infrequent, and quinolone resistance was mainly present as a single resistance.

Quinolone resistance was related to foreign travel as well as *S.* Enteritidis phage type (PT). In isolates from patients with a known history of foreign travel, 8.9% were resistant, compared with 2.4% in domestically acquired infections ($p<0.0001$; Table). In 157 patients who had returned from a European destination (excluding Scandinavia), 18 (11.5%) had resistant isolates. Resistance was highest in patients returning from Spain: 12 (19.7%) of 61 isolates were resistant. Five (7.5%) resistant strains were found in 67 isolates from Asia (mainly Turkey and Thailand), but no resistant strains were recovered from 25 persons who had traveled to Africa. Five patients had visited other countries (1 resistant strain), and we had no information about the destination for the remaining 48 patients (3 resistant strains).

The major sources of domestically acquired *S.* Enteritidis infections are raw or undercooked eggs produced in Denmark (3, unpub. data). The most common phage types in Danish layer hens are PT 6 and PT 8, which accounted for 65.1% of the domestically acquired infections in our study. Resistance in these two phage types remained low (Table), as were the rates of resistance in PT 13A, PT 25, and PT 34. These types also originate from layer hens. In contrast, the proportion of resistant isolates was highest in phage types PT 1, PT 4, PT 6A, PT 14B, and PT 21, which are often associated with infections from imported poultry products, including imported broiler chickens.

From 1994 to 1997 in England and Wales, quinolone resistance in *S.* Enteritidis increased from 0.4 % to 1.3%. As in our study, resistance was highest in PT 1 (19%) and PT 6A (14%) (4). These types were mainly associated with foreign travel. In a recent study from Spain, 31% of 385 *S.* Enteritidis isolates overall but 80% of PT 1 isolates were reported to be quinolone resistant (5).

Conclusions

The emergence of quinolone resistance in the most common salmonella serotype worldwide is a serious public health concern. Resistance to nalidixic acid has been associated with reduced efficacy of fluoroquinolones such as ciprofloxacin (6,7). The use of nalidixic acid or fluoroquinolones in humans is unlikely to contribute substantially to the increase in resistance, for the following reasons: an antibiotic prescribed in connection with a physician's request for a fecal specimen is unlikely to have affected the resistance pattern because treatment is usually initiated after the specimen is collected. Fluoroquinolones are potent bactericidal drugs and are not likely to select for resistance when therapeutic concentrations are obtained (8). Nalidixic acid is used in some developing countries for the treatment of dysentery, but the this practice is unlikely to select for quinolone-resistant *S.* Enteritidis in the

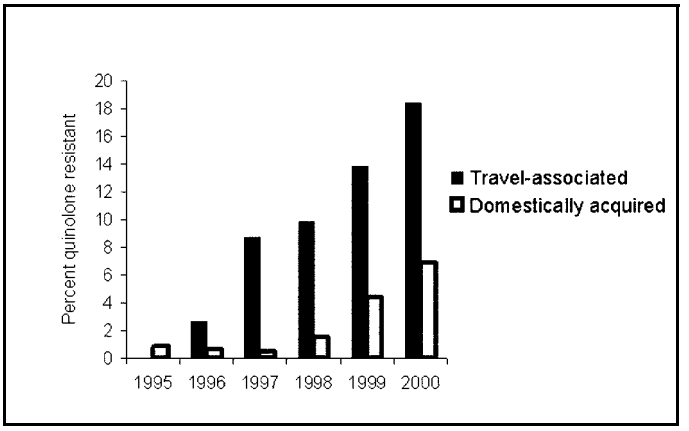

Figure. Annual proportion of quinolone resistance in isolates of *Salmonella* Enteritidis, Denmark, 1995–2000.

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Table. Prevalence of quinolone resistance in human isolates of *Salmonella enterica* serotype Enteritidis, by phage type and history of foreign travel. Denmark, 1995–2000.

	No history of foreign travel		Foreign travel	
Phage type	No. resistant (%)	Total	No. resistant (%)	Total
1	22 (23.4)	94	11 (29.7)	37
4	7 (2.4)	292	5 (3.8)	131
6	4 (0.5)	765	0 (-)	28
6A	1 (5.9)	17	4 (22.2)	18
8	8 (1.3)	627	0	31
13A	0 (-)	9	0	4
14B	1 (20.0)	5	1 (14.3)	7
21	3 (4.9)	61	0	4
21B	0 (-)	16	0	0
25	0 (-)	19	0	0
34	0 (-)	81	0	2
Others and nontypeable	6 (3.9)	152	4 (13.8)	29
Not typed	2 (1.9)	105	2 (18.2)	11
Total	54 (2.4)	2,243	27 (8.9)	302

zoonotic reservoir. The prevalence of resistance in *S.* Enteritidis was, in our study, highest in patients returning from developed countries. Furthermore, fluoroquinolones are not used to treat children. In children <15 years of age, the prevalence of quinolone-resistant strains was 9.5% (4/42) among patients with a history of foreign travel and 1.4% (7/499) in domestically acquired cases. The corresponding figures for adults were 8.9% (23/260) and 2.7% (47/1,744). Finally, the use of quinolones in humans could not conceivably be responsible for the large variation in the prevalence of resistance by phage type. If the use of quinolones in human medicine contributed to the emergence of quinolone resistance in *S.* Enteritidis, resistance would be found independently of phage type.

Increasing quinolone resistance is not confined to foodborne salmonella but also includes campylobacters; resistance is primarily driven by the use of fluoroquinolones in the livestock production (8,9). Limited quantities of fluoroquinolones are currently used in food production in Scandinavia. During 1997-1998, the annual use of the liquid formulation of fluoroquinolones for 130 million to 140 million poultry was <150 kg; during 1999-2000, usage decreased to <100 kg (2). Unfortunately, quantitative data on the use of fluoroquinolones are not available from most areas. Several fluoroquinolones are licensed and used in other countries of Europe, Southeast Asia, and the Americas for treatment of food animals, particularly for mass medication in the poultry industry, mainly for broiler chickens (2,8-10). Our data support concerns that the current pattern of quinolone use in food animals leads to increasing quinolone resistance in *S.* Enteritidis and that action should be taken to limit this use.

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Molecular Characterization of *Corynebacterium diphtheriae* Isolates, Russia, 1957–1987

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In the 1990s, the Newly Independent and Baltic States of the former Soviet Union experienced the largest diphtheria outbreak since the 1960s; it was caused by *Corynebacterium diphtheriae* strains of a unique clonal group. To address its origin, we studied 47 clinical isolates from Russia and demonstrated that this clonal group was an integral part of the endemic reservoir that existed in Russia at least 5 years before the epidemic began.

In the pre-vaccine era, diphtheria was a major cause of childhood illness and death worldwide. After the diphtheria toxoid vaccine was introduced, a decline in diphtheria cases was seen where the vaccine was used. In some areas of the Soviet Union, diphtheria vaccination started as early as the 1920s, but it was not included in the general immunization program for children until 1958 (1). After 1958, reported diphtheria cases declined steadily except for a small increase in incidence during the 1980s and the epidemic that started in 1990. In 1991, after the breakup of the Soviet Union, routine childhood vaccination programs were disrupted due to interruption of vaccine supplies to countries in Central Asia, the Caucasus, and the Baltic region. A major diphtheria epidemic began in Russia in 1990; during the next 4 years, it reached all the Newly Independent States and Baltic States of the former Soviet Union (FSU) (1,2). The European Regional Office of the World Health Organization (WHO) now considers this diphtheria outbreak, which resulted in more than 150,000 cases and 4,000 deaths, to be nearly under control (1). Several factors, such as an increased proportion of susceptibles in the population, migration, and a deteriorating health infrastructure, are suspected to be major catalysts for this outbreak (2). However, the role of biological factors of the causative organism is not clear.

To assess the genetic diversity and structure of the bacteria and its toxin, different molecular typing methods have been used successfully as a complement to traditional characterization (3-6). Popovic et al. and de Zoysa et al. identified a particular epidemic clonal group, characterized by ribotyping, multilocus enzyme electrophoreses (MEE), and pulsed-field gel electrophoresis (PFGE), associated with the appearance and spread of this outbreak (7,8). Our study focuses on the origin of this epidemic clonal group and is the first to include a limited number of archival isolates collected more than 30 years before this outbreak began.

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The Study

A convenience sample of 47 *Corynebacterium diphtheriae* isolates was available for analysis from a collection of isolates obtained during 1957-1987, before the onset of the recent diphtheria outbreak. These isolates were collected from both carriers (n=37) and patients (n=10) in different regions of Russia. All isolates were kept freeze-dried at the G. N. Gabrichevsky Institute for Epidemiology and Microbiology, Moscow, Russia, and were transported on silica gel packages to the Centers for Disease Control and Prevention, Atlanta, Georgia, for molecular characterization.

All isolates were biotyped by using the commercial API Coryne kit (Biomerieux, Lyon, France). Toxigenicity status was determined by the Elek test, as recommended by WHO (9), and by the polymerase chain reaction (PCR), which targeted both A and B subunits of the *tox* gene (10).

All the strains were characterized by ribotyping as previously described (11). The hybridization was done by using five oligonucleotide probes according to Regnault et al. (12). Ribotyping pattern designations were based on the scheme established by Popovic et al. (7). A difference in one band was defined as an individual ribotype (RT).

MEE was carried out as previously described (7,11). The electromorphs of the same enzyme were visualized in a starch gel matrix as bands of different migration rates. Each electromorph was considered to represent a distinct allele of the same enzyme. By testing 27 different enzymes, a profile of electromorphs, defining the electrophoretic type (ET) of each strain, was obtained. The genetic relatedness of ETs was illustrated as a dendrogram, which was generated by the average-linkage method of clustering ETs described by Selander et al. (13).

We examined 47 *C. diphtheriae* isolates collected in the pre-epidemic period (1957-1987) from 10 patients and 37 carriers in different areas of Russia. Thirty-nine strains were of the gravis biotype, 7 were the mitis biotype, and 1 was of the intermedius biotype. All the mitis biotype strains were toxigenic. Among the gravis biotype strains, 36 were toxigenic, and 3 were nontoxigenic. No discrepancies between the results obtained by traditional identification, the API Coryne test, or toxigenicity testing by the Elek test and PCR were detected.

In the 47 isolates, 12 different RTs were identified (Figure 1). Twenty-two (47%) were of the M11e RT; all were toxigenic and of the gravis biotype. They were collected from 1957 to 1985. RT G4, characteristically seen in the recent epidemic clonal group, was identified in 6 (13%) isolates, all of which were collected from 1984 through 1987. Four isolates

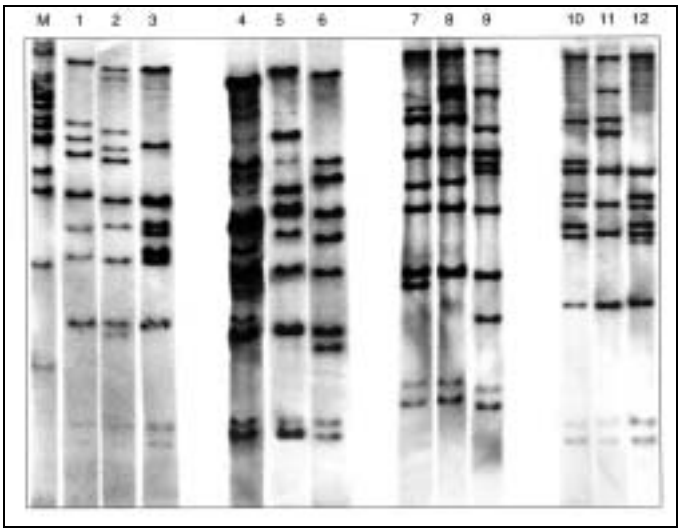

Figure 1.Twelve *Bst*EII ribotypes identified in 47 C*orynebacterium diphtheriae* isolates collected in the Russian Federation between 1957 and 1987. The figure is composed of ribotype gels exemplifying the different patterns observed in the strain collection. Lane M, molecular weight marker; lane 1, ribotype M11e; lane 2, M11f; lane 3, M13a; lane 4, M7a; lane 5, unique; lane 6, G4; lane 7, unique; lane 8, M11g; lane 9, M3; lane 10, M1b; lane 11, M6; lane 12, M13b.

had two new ribotype patterns, not previously described. They were collected from 1977 through 1981.

Sixteen (6 isolates of RT pattern G4 and 10 isolates of different RT patterns) of the 47 isolates were analyzed by MEE; 13 different ETs were identified. Of the six isolates with the G4 patterns, four also belonged to the ET8 complex (Figure 2). An additional isolate (strain designation B533 in Table) collected in 1957 belonged to the ET8 complex but had a different RT.

Conclusions

In the pre- and early vaccine era, diphtheria incidence was high in the Soviet Union. After the diphtheria vaccine was introduced, a decrease in incidence was seen in the 1950s. During the mid-1970s, immunization programs resulted in control of diphtheria throughout the country. However, an increase in incidence was noted at the end of 1970 and during the 1980s, and a peak was observed in 1983. This resurgence was associated with a change in the biotype of the circulating *C. diphtheriae* strains from gravis, which had been dominating for several decades, to mitis (14).

To allow better monitoring of the global spread of diphtheria, the WHO ribotyping database for *C. diphtheriae* was established at the Pasteur Institute in Paris, France. The institute demonstrated that *C. diphtheriae* RTs are quite diverse worldwide but remain stable over time (15). Both ribotyping and MEE have provided a significant level of differentiation and reliability and subsequently have been accepted as the standard for molecular subtyping of *C. diphtheriae*. Thus, we used these molecular methods to characterize our archival isolates.

Twelve different RTs were found in our 47 isolates. Our data show that nine *C. diphtheriae* isolates from the 1950s and 1960s had an RT pattern (M11e) that was very similar to ribotype M11, which was only seen occasionally in the FSU in the 1990s. Epidemic RT G4 was seen in six toxigenic *C. diphtheriae* isolates collected from 1984 through 1987 in four distant regions of Russia (Moscow and Moscow region, Anapa, Smolensk, and Sverdlovsk) from both diphtheria patients and carriers; four of these isolates were also members of the ET8 complex.

Our investigation of the origin of the epidemic clonal group determined that, in our strain collection, the earliest reported strain of this clonal group was identified in Smolensk in 1985, and that strains of this clonal group were simultaneously present in several geographically distant areas in Russia from 1985 through 1987. These findings suggest that the current epidemic clone was an integral part of the endemic reservoir that existed in the FSU at least 5 years before the epidemic began. Further studies that would include a large number of gravis biotype strains from throughout the Soviet Union isolated from 1980 through 1985 might unveil where and when strains of the epidemic clone were first associated with disease or carriage.

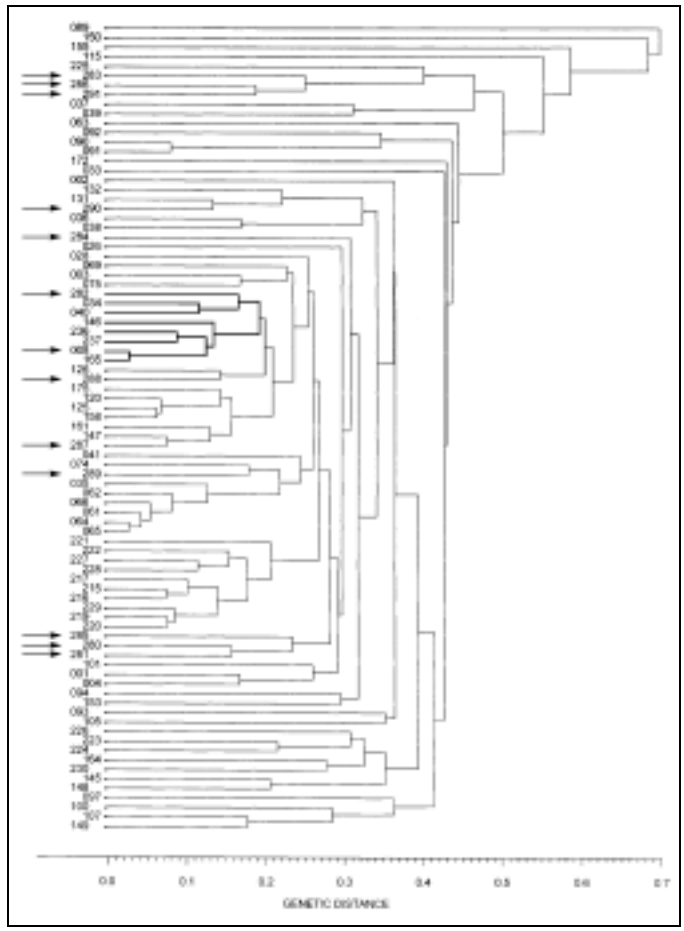

Figure 2. Dendrogram showing the genetic relatedness of 85 electrophoretic types (ETs) of *Corynebacterium diphtheriae* isolates collected in different countries around the world. Arrows indicate the different ETs identified among the 47 *C. diphtheriae* isolates. The ET8 complex is marked with thicker lines.

Table. Designations and characteristics of 47 *Corynebacterium diphtheriae* strains collected in Russia, 1957–1987

Ribotype	Geographic area of isolation	No. isolates	Year of isolation	Biotype[a]	ET[b]
G4	Anapa	1	1984	G	286
	Moscow	2	1985, 1987	G	291, 8
	Smolensk	1	1985	G	8
	Sverdlovsk	2	1987	G	8
M1b	Anapa	1	1984	M	287
M3	Krasnoyarsk	2	1979	M	290, ND
	Ivanov	1	1976	M	ND
M6	Moscow	1	1981	G	ND
M7a	Moscow	2	1972, 1973	G	ND
M11e	Moscow	13	1964-1977	G	ND
		1	1964	G	283
	Vladivostok	2	1957	G	280,
		1	1957	G	281
					ND
	Buryatiya	1	1976	G	285
	Groznyi	1	1985	G	ND
	Vladimir	1	1977	G	ND
	Tatarstan	1	1977	G	ND
	Omsk	1	1976	G	ND
M11f	Vladivostok	1	1957	G	ND
	Omsk	2	1977	G[e]	ND
M11g	Kirov	1	1978	G	ND
M13a	Vladivostok	1	1957	G[e]	282
	Vladimir	1	1976	G	284
	Krasnoyarsk	1	1979	G	289
M13b	Moscow	1	1981	I[d]	ND
New[c]	Vladivostok	1	1981	M	ND
	Moscow	1	1977	M	ND
		1	1977	G[e]	288
	Krasnoyarsk	1	1979	M	ND

[a]G, biotype gravis; M, biotype mitis; I, biotype intermedius.
[b]ET, electrophoretic type.
[c]New ribotype, pattern has not been previously observed.
[d]ND, not done.
[e]Nontoxigenic by the Elek test and polymerase chain reaction.

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Outbreak of *Neisseria meningitidis*, in Edmonton, Alberta, Canada

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From December 1999 to April 2001, the greater Edmonton region had 61 cases of invasive meningococcal infection, two fatal. The outbreak was due to *Neisseria meningitidis* serogroup C, electrophoretic type 15, serotype 2a. Analysis of the strains showed that 50 of 56 culture-confirmed cases were due to a single clone and close relatives of this clone. This strain had not been previously identified in the province of Alberta dating back to January 1997.

Neisseria meningitidis causes outbreaks of disease resulting in severe illness and death. These outbreaks occur in persons in their teens and early twenties; however, in some outbreaks, the very young (<2 years of age) are also severely affected (1). Persons >25 years of age appear to be less affected. North American outbreaks are confined primarily to serogroup C strains and less commonly to Y and W135 (2-5).

We report an outbreak of a serogroup C clone of *N. meningitidis* in the Edmonton region of Alberta, Canada; the serogroup had a unique restriction fragment length polymorphism (RFLP) pattern as determined by pulsed-field gel electrophoresis (PFGE).

The Outbreak

The Edmonton region has a mixed metropolitan and rural population totaling 827,507 (6). From January 1997 to November 1999 (35 months), this region had 13 cases of culture-confirmed invasive *N. meningitidis* disease (5 from blood, 6 from cerebrospinal fluid [CSF], and 2 from joints) (Figure 1). Serogroup determination, by the antiserum agar method previously described, showed that these included two cases of serogroup B, seven of serogroup C, two of serogroup W135, and two of serogroup Y (7,8). During this period, the incidence of culture-confirmed meningococcal disease did not exceed two cases per month (Figure 1). However, from December 1999 to April 2001, 61 cases of invasive *N. meningitidis* disease occurred; 57 of these were confirmed by culture and 4 on the basis of clinical findings, positive results from an antigen detection assay, or both. The culture-confirmed cases were from blood (51 cases) and CSF (6 cases). Of the 57 culture-confirmed cases, 56 were serogroup C, and 1 was serogroup B (blood isolate).

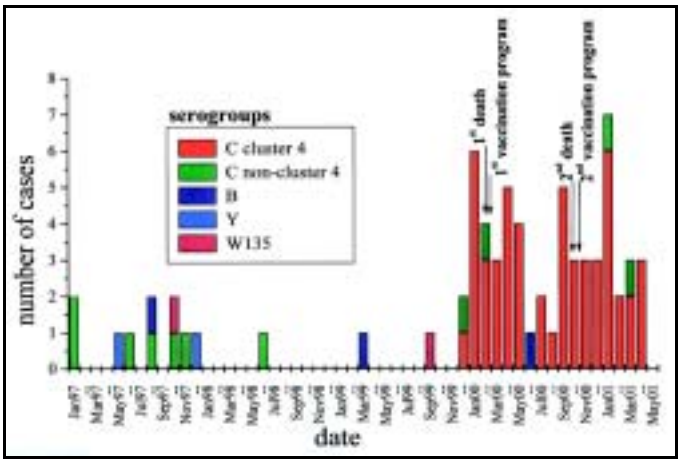

Figure 1. *Neisseria meningitidis* cases, Edmonton, Alberta, January 1997 to May 2001.*
*Cluster 4 refers to clusters derived from restriction fragment length polymorphism patterns, designated in Figure 2.

In relation to clinical outcome, 43 (70.5%) of the 61 patients fully recovered; 2 (3.3%) died (a 16-year-old female and a 19-year-old male, both infected with serogroup C) (Figure 1); 4 (6.6%) required amputations; 7 (11.5%) had severe scars; and 9 (14.8%) had other sequelae such as knee pain, neurologic sequelae, decreased hearing, decreased sensation at the extremities, and stiffness in hands. The ages affected during the outbreak period ranged from 5 weeks to 77 years. Outbreak-associated patients were primarily <24 years of age. Age breakdown showed that 10 (17.9%) of 56 confirmed serogroup C strains were in the birth- to 1-year age group (Table). The conjugate vaccine for use in this age group was licensed in Canada in May 2001 and was therefore not available during the outbreak. The high number of cases in this age group translates into an incidence rate of 50 per 100,000 (Table). In comparison, the most recently published national data show the rate for the group <1 year of age to be 12.9/100,000 for 1997 and 6.5/100,000 for 1998 (9). Also, age groups 15-19 and 20-24 showed unusually high incidences of disease in this outbreak (Table). Thirty patients with culture-confirmed disease were female, and 27 were male. Patients were scattered geographically throughout the region, with no more than one case a close contact of another. All contacts of patients were treated with rifampin. Except for age group, no particular populations were determined to be at greater risk for infection than other.

A vaccination campaign that targeted persons ages 2 to 19 was undertaken in the region from February 14 to 28, 2000, using polysaccharide quadravalent meningococcal vaccine; 168,000 children were immunized. Because of a continuing higher-than-expected number of cases, the vaccine was again

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Table. Rates of *Neisseria meningitidis* disease in the Edmonton, Alberta Canada, region (per 100,000)[a]

	Age (cases)								
Rate	≤1	2-4	5-9	10-14	15-19	20-24	25-34	35-59	≥60
17-month period[b]	50.0 (9)	9.7 (3)	5.3 (3)	6.8 (4)	35.7 (20)	10.6 (6)	4.0 (5)	2.3 (7)	1.7 (3)
Annualized	37.5	7.3	4.0	5.1	26.8	8.0	3.0	1.8	1.3

[a] Population=827,507 (6).
[b] December 1999 through April 2001.

offered in October 2000 (Figure 1). This time, the vaccine was offered to all previously unimmunized 2- to 24-year-olds (61,900 doses delivered in 6 days). In April 2001, vaccine was again offered to those 2-year-olds not previously eligible in October 2000. Overall, 87% of people in the targeted age group were vaccinated. After the vaccine campaigns, nine cases of invasive meningococcal disease occurred in those eligible for immunization but not immunized (total population of 2- to 24-year-olds 265,300). Nine cases also occurred in the immunized population, for a calculated vaccine effectiveness of 84%.

Conclusions

Electrophoretic typing, serotyping, and serosubtyping performed by the National Microbiology Laboratory, Population and Public Health Branch of Health Canada, Winnipeg, Canada, showed that all serogroup C strains belonged to electrophoretic type (ET)15, serotype 2a (10). ET15 entered the Canadian population as early as 1986 in Ontario and has since been demonstrated to be responsible for a number of outbreaks in this country (9,11). The most recent data for ETs in Canada date to 1997 and 1998 (9). During 1997, ET15 accounted for 83.1% of strains analyzed. This proportion increased in 1998 to 93.7%, indicating that ET is the predominant type causing invasive disease in Canada. Serosubtyping of serogroup C isolates in this outbreak showed variation in the class 1 outer membrane protein (OMP1). Strains were either P1.2,5 (27 isolates), P1.2 (24 isolates), P1.15 (2 isolates), or P1.- (3 isolates). The two fatal cases were both P1.2,5.

Serogrouping, ET, and serosubtyping provided accurate characterization of the circulating strains in the Edmonton region; however, they failed to determine if the outbreak was clonal or if the increased cases were due to unrelated *N. meningitidis* strains. To determine this, RFLP analysis via PFGE was used with minor modifications (12). Bacterial cells were grown on sheep blood agar. The cells were scraped off and placed in 10% formalin for 15 minutes for bacterial inactivation. The cell number was standardized to an optical density of 1.4 at A_{610}. Lysozyme was added to 100 µL of cell suspension at a final concentration of 0.2 mg/mL, mixed gently, and added to 1 mL of 1.6% (W/V) low-melting agarose. The mixture was then transferred to the gel plug mold. The gel plug was sus-

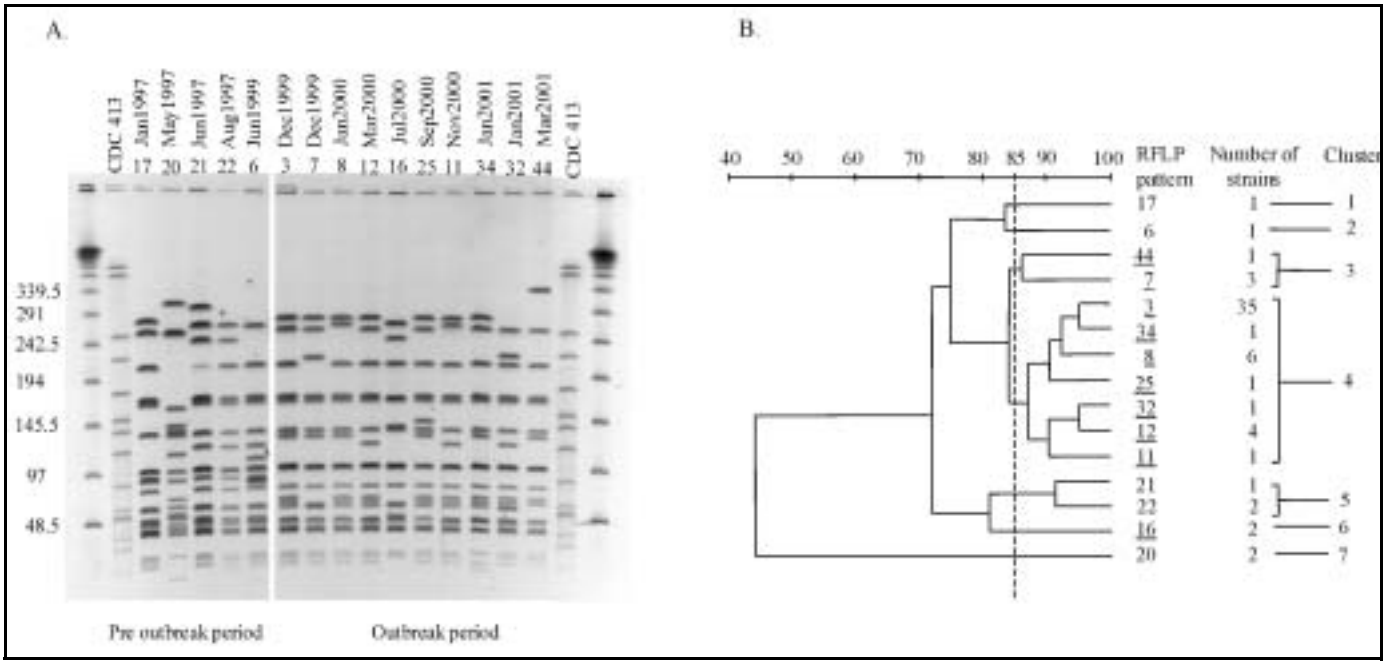

Figure 2. A, restriction fragment length polymorphism (RFLP) analysis of *Neisseria meningitidis* serogroup C strains generated by pulsed-field gel electrophoresis (PFGE) by using the *Spe* I restriction endonuclease. The strain CDC-413 was used as a control for the PFGE.
B, dendogram analysis generated from "A." Percent identity is shown at the top. The RFLP pattern designation is shown on the right. RFLP patterns not underlined were seen from January 1997 to November 1999. Underlined RFLP patterns were seen from December 1999 to April 2001. The dashed line indicates 85% identity. A 1% tolerance was used to generate the dendogram.

pended in Lysis II solution (1 mg/mL lysozyme, 0.5% Brij 35, 0.2% sodium deoxycholate, and 0.5% sodium lauryl sarcosine in Tris-EDTA buffer) for 1 hour at 37°C, replaced with ESP buffer (0.25 M EDTA, pH 8.0, 1% sodium lauryl sarcosine, 0.5 mg/mL Proteinase K), and incubated at 50°C for 2 hours. Slices of plug (1X5 mm) were digested by using 30 U of the restriction endonuclease enzyme *Spe*I (GIBCO BRL, Burlington, Ontario, Canada) for 2.0 hours. The restricted DNA was resolved by PFGE with the following running conditions: initial switch time: 5.0 seconds, final switch time: 25 seconds at 6 V/cm with included angle at 120°C for 19 hours. The gel was stained with ethidium bromide. Analysis was performed by using the BioRad Gel Doc System (Bio-Rad Laboratories, Mississauga, Ontario) and Molecular Analyst Software (Bio-Rad). RFLP pattern numbers were assigned to each isolate with one band difference in the RFLP profile.

RFLP analysis showed 15 distinguishable patterns (Figure 2A). Five of these RFLP patterns were seen only from January 1997 to November 1999 (patterns 6, 17, 20, 21, and 22). These patterns were identified by retrospectively determining their RFLP profile from archived strains. The remaining 10 were present only from December 1999 to April 2001 (patterns 3, 7, 8, 11, 12, 16, 25, 32, 34, and 44). Figure 2B shows a dendrogram analysis of the 15 *Spe* I-generated RFLP profiles generated by using a 1% tolerance. We used an 85% breakpoint to determine relatedness, as reported by Popovic et al. (13). At the 85% relatedness breakpoint, the RFLP profiles formed seven clusters. RFLP patterns for the largest cluster (49 isolates-cluster 4) were only seen during the outbreak period (December 1999 to April 2001). Both deaths were associated with RFLP pattern 3 strains. Interestingly, pattern 7 in cluster 3 was similar on visual examination to pattern 3 (Figure 2A). The first RFLP pattern (first case) detected was pattern 3 (December 24, 1999) followed by pattern 7 (second case, December 29, 1999). Pattern 7 was not detected before December 1999. These data suggest that patterns 3 and 7 arose concomitantly. Even though these patterns appear close in time, pattern 3 and its relatives resulted in 51 cases, whereas pattern 7 was isolated from only 3 cases. Whether pattern 3 strains are more virulent than pattern 7 strains remains to be determined. We have also received reports that this clone has caused disease in other regions of the province of Alberta and in one other Western Canadian province in the same period reported for our outbreak.

In conclusion, the Edmonton region in the province of Alberta, Canada, had an outbreak of *N. meningitidis* caused by a clone unique to this region. This clone was associated with increased deaths and can readily spread beyond defined geographic boundaries. Other provincial and state laboratories need to be able to recognize this clone should it appear in their area.

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Cefepime MIC as a Predictor of the Extended-Spectrum β-Lactamase Type in *Klebsiella pneumoniae*, Taiwan

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To guide selection of carbapenems or fourth-generation cephalosporins as therapy, 110 *Klebsiella pneumoniae* isolates with extended-spectrum β-lactamases from Taiwan were characterized by phenotypic (MICs), molecular, and chemical methods. MIC patterns of ceftazidime and cefepime clearly differentiate strains treatable by cefepime and those capable of efficiently hydrolyzing available cephalosporins (CTX-M series and SHV-types). Continued use of cefepime appears to be a treatment option in cases for which MIC results are available and interpreted by the criteria presented.

In recent years, extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* (ESBL-KP) strains of the TEM, SHV, and CTX-M types have been discovered worldwide. Reference broth microdilution susceptibility rates (MIC ≤8 mg/L) for cefepime among ESBL-KP in various geographic regions show a wide range: Canada 94.4%, United States 87.6%, Western Pacific 76.1%, Europe 63.6%, and Latin America 49.6% (1). In Taiwan, the in vitro cefepime susceptibilities of ESBL-KP ranged from 37% to 100% (2,3). The gene encoding SHV-5 (pI 8.2) has been reported to be the most common ESBL in klebsiellae in Taiwan (2,4). The CTX-M-3 (pI 8.4) enzyme has also been discovered in *Escherichia coli* isolates in southern Taiwan (5). For our study, we focused on the mechanisms of cefepime resistance among ESBL-KP isolates in Taiwan and attempted to predict cephalosporin therapeutic potentials by simple phenotypic patterns.

The Study

We initially conducted reference broth microdilution tests (6,7) for 211 isolates of endemic and epidemic ESBL-KP from Taiwan; 53% of isolates had a cefepime MIC of ≤8 mg/L (susceptible). Isoelectric focusing (IEF) was then performed by the method of Matthew et al. (8). Approximately 40% of isolates had an enzyme with a pI of 8.2 (SHV-5); 40% of isolates produced enzymes with a pI of 7.9, 8.4, or 8.8 (CTX-M-type); an additional 20% of isolates contained both an SHV-5 plus a CTX-M enzyme.

The IEF results of 110 geographically representative isolates of ESBL-KP were categorized by cefepime MIC level (Table). The enzymes with pIs of 7.6 and 5.4 were SHV-1 and TEM-1, respectively, which have been reported previously in Taiwan hospitals (2,4). All the enzymes with pIs of 5.4, 7.6, and 8.2 were evenly distributed among the isolates regardless of cefepime MIC values, indicating no association with resistance to this fourth-generation cephalosporin. All 23 isolates with pI 8.2 enzymes and a nonsusceptible cefepime MIC (≥16 mg/L) contained enzymes with pIs of 7.9, 8.4, or 8.8. In the absence of these CTX-M enzymes, isolates with pI 8.2 enzymes remained susceptible to cefepime. Thus, the high MIC level for cefepime was attributed to enzymes with pIs of 7.9, 8.4, and 8.8. This finding is supported by the fact that those isolates with a single CTX-M enzyme (10 with pI 7.9 enzymes [CTX-M-14] and 8 with pI 8.4 enzymes [CTX-M-3]) had very elevated cefepime MIC results in the absence of a pI 8.2 enzyme (9). Two isolates with pI 8.4 enzymes remained susceptible to cefepime (MIC 2 μg/mL) and probably produced low levels of CTX-M-3.

These data indicate that cefepime resistance in ESBL-KP isolates from Taiwan may result from either the cumulative effect of pI 7.9, 8.4, 8.8, or 8.2 enzymes or hyperproduction of any of the enzymes with the CTX-M phenotype (pI 7.9, 8.4, or 8.8). The enzyme with a pI of 8.8 is a novel CTX-M β-lactamase most similar to CTX-M-3 (9).

Several CTX-M enzymes have been shown to confer high MIC levels for cefepime (10-12). Bauernfeind et al. reported an isolate of *Salmonella Typhimurium* that had a CTX-M-2 enzyme (pI 7.9) and a cefepime MIC of 64 mg/L (10). Outbreaks have also been reported of isolates producing CTX-M enzymes (pI 8.4), including *K. pneumoniae* (cefepime MIC 4-8 mg/L), *E. coli* (cefepime MIC 8-32 mg/L), and *Serratia marcescens* (cefepime MIC 16-64 mg/L) (11).

Szabo et al. reported an outbreak of 14 ESBL-KP strains (pI 8.2, probably SHV-5) that had high-level resistance to cefepime (MIC_{90} >256 mg/L) (12). Tzouvelekis et al. also noticed seven isolates of ESBL-KP (SHV-5) with cefepime MICs ranging from 32 mg/L to 64 mg/L. These researchers described the elevated cefepime MIC as being due to the combined effect of SHV-5 hyperproduction and decreased outer membrane permeability (loss of 36-kDa outer membrane protein [OMP]) (13). The cefepime MIC for isolates hyperproducing SHV-5 without loss of the 36-kDa OMP remained <16 mg/L, a susceptible level (13). Loss of the 36-kDa OMP also conferred cefoxitin resistance, and introduction of a plasmid carrying the 36-kDa OMP gene markedly reduced the MIC of cefoxitin, from 128 mg/L to 16 mg/L (13). Whether the isolates reported by Szabo et al. also had concomitant outer membrane defects is unknown, but these authors later recommended that cefepime not be considered the treatment of choice against SHV-5-producing ESBL-KP (14). Whether our cefepime-resistant isolates had a concomitant OMP defect is

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Table. Distribution of pI[a] values in 110 isolates of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae,* stratified by cefepime MIC level,[b] Taiwan

Cefepime MIC (mg/L) (n=110)	pI 5.4 (n=89)	pI 6.3 (n=7)	pI 7.6 (n=92)	pI 7.8 (n=4)	pI 7.9 (n=40)	pI 8.2 (n=62)	pI 8.4 (n=43)	pI 8.8 (n=3)
≥32 (n=38)	38	0	31	0	29[c]	20[d]	18[e]	2
16 (n=6)	6	0	5	0	5[c]	3[d]	5[e]	0
8 (n=26)	20	2[f]	22	0	6[g]	8[h]	12[g]	0
4 (n=19)	9	4[f]	17	1[f]	0	12[h]	6[g]	1[g]
2 (n=10)	7	1[f]	8	3[f]	0	8[h]	2[g]	0
≤1 (n=11)	9	0	9	0	0	11[h]	0	0

[a]pI, Isoelectric point. Each strain may have multiple pIs.
[b]MIC test, reference broth microdilution method according to National Committee for Clinical Laboratory Standards.
[c]Ten of 34 isolates having a cefepime MIC ≥16 mg/L did not have enzymes with pI values of 8.2 or 8.4.
[d]All 23 isolates were simultaneously coexistent with pI 7.9, 8.4, or 8.8 (12 with pI 7.9 plus 8.4; 9 with pI 7.9; and 2 with pI 8.8).
[e]Eight of 23 isolates (cefepime MIC ≥16 mg/L) were not coexistent with pI 8.2 or 7.9.
[f]All the 11 isolates (pI 7.8 or 6.3) were coexistent with pI 8.2.
[g]Ceftazidime MIC ≤8 mg/L; ceftriaxone MIC ≥32 mg/L.
[h]Ceftazidime MIC ≥16 mg/L.

similarly unknown. However, in 44 isolates with cefepime MICs ≥16 mg/L, only 7 were resistant to cefoxitin. Furthermore, for isolates with high cefepime MIC values resulting from single CTX-M enzymes (10 with pI 7.9 and 8 with pIs of 8.4), only two (one each with pIs of 7.9 and 8.4) were resistant to cefoxitin. The relatively low rates of cefoxitin coresistance provide indirect evidence that 36-kDa OMP loss may not play an important role in the expression of cefepime resistance in ESBL-KP strains in Taiwan.

Conclusions

Alternative therapy using cefepime against ESBL-KP strains in Taiwan could be reliable if appropriately guided by cefepime and ceftazidime MIC results. The cefepime MIC is useful for predicting the presence of CTX-M enzymes, which usually confer resistance to this fourth-generation cephalosporin. Cefepime cannot be used if the MIC exceeds 8 mg/L, which predicts the presence of CTX-M β-lactamases. Cefepime may reasonably be used clinically if the MIC is consistently ≤1 mg/L, which indicates the absence of a CTX-M enzyme. For isolates with cefepime MICs ≥2 to ≤8 mg/L, use of cefepime should be further guided by the ceftazidime MIC. If the ceftazidime MIC remains in the susceptible range (≤8 mg/L, predicting enzymes of pI 7.9, 8.4, or 8.8), cefepime should not be used. If the ceftazidime MIC is >8 mg/L (predicting enzymes of pI 8.2), cefepime at appropriate doses has a potential therapeutic role because most pI 8.2 enzymes rarely elevate the cefepime MIC to >8 mg/L.

In conclusion, outer membrane defects and the inoculum effects (13) that may adversely elevate MIC values must still be considered if cefepime is chosen as an alternative therapy against ESBL-KP strains. This strategy of focused utilization of a newer cephalosporin could reduce some selective pressures of carbapenem use among ESBL-KP and thus minimize the development of carbapenem-resistant strains. In addition, phenotypic characteristics appear to accurately differentiate two important endemic and epidemic groups of ESBL types (CTX-M series and SHV-like) in *K. pneumoniae* strains in Taiwan.

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Deer Meat as the Source for a Sporadic Case of *Escherichia coli* O157:H7 Infection, Connecticut[1]

Terry Rabatsky-Ehr,* Douglas Dingman,† Ruthanne Marcus,* Robert Howard,‡ Aristea Kinney,‡ and Patricia Mshar,‡

We report a case of *Escherichia coli* O157:H7, which was acquired by eating wild White-Tailed deer (*Odocoileus virginianus*). DNA fingerprint analysis verified venison as the source of infection. This pediatric case emphasizes the need for dissemination of information to hunters regarding the safe handling and processing of venison.

Escherichia coli O157:H7 (O157) is a cause of acute infectious diarrhea in humans and the leading cause of hemolytic uremic syndrome, especially among children in the United States (1). Many animals, including cattle, sheep, and goats, are known to harbor O157; however, cattle are most often implicated as the zoonotic source of human infection (2). Transmission is usually attributed to contaminated foods. In recent years, meats, other than beef, from which O157 has been isolated include pork, lamb, and poultry (2,3). Although several reports document the presence of O157 in deer (4-6), only one report (4) has shown evidence of an O157 infection from eating venison. This report was specific to the Black-Tailed deer (*Odocoileus hemionus*). To our knowledge, this is the first case of O157 infection linked with eating wild White-Tailed deer (*Odocoileus virginianus*) meat.

Case Report

A previously healthy 7-year-old boy was seen at a Connecticut emergency room with a 3-day history of gastrointestinal illness. Symptoms included bloody diarrhea, abdominal cramps, and nausea. The child was treated with antibiotics as an outpatient; diarrhea resolved after 6 days. The child's stool sample was positive for O157. Stool samples were not obtained from other family members.

Two days before the child's onset of illness, his father butchered and grilled freshly killed venison for the family. The child ate a large quantity of undercooked (red), gamey-tasting grilled venison tenderloin. His father ate a few bites of the venison; his mother and sister ate none. The only other family member to report symptoms of illness was the father, who reported having an "unsettled stomach" without diarrhea the same day as his son's onset of illness. Four weeks later, O157 was recovered from a frozen sample of uncooked venison obtained from the same carcass as the fresh, grilled tenderloin.

As part of routine disease surveillance, all patients with O157 infections who are reported to the Connecticut Department of Public Health (CDPH) are interviewed by telephone, using a standardized questionnaire. Parents of the 7-year-old boy were interviewed 2 weeks after the onset of symptoms, and information about his clinical illness and potential exposures was collected. A second interview, conducted 2 weeks later, sought additional information on illness in other family members and on deer handling and processing practices. Permission to collect samples of uncooked deer meat stored in the family freezer was also obtained.

The O157 patient isolate was sent to the CDPH laboratory for confirmation, H antigen determination, and subtyping by DNA fingerprinting using pulsed-field gel electrophoresis (PFGE). The isolate was cultured on sorbitol-MacConkey agar; sorbitol negative colonies were identified as O157 by standard methods (7) and subtyped by PFGE as described by Barrett et al. (8).

Three separate packages of White-Tailed deer meat, frozen for 25 days, were obtained from the child's family and processed at the Connecticut Agricultural Experiment Station. For all three packages (steak, butterfly cut, and sausage pieces), a combined weight of 25 g frozen meat shavings were macerated, incubated in enrichment medium, and immunomagnetically separated (IMS), according to the manufacturers' instructions (Dynal, Inc., Lake Success, NY). Magnetic beads were washed during the IMS extraction procedure as reported by Tomoyasu (9). Final suspensions of the magnetic beads were plated on cefixime-tellurite sorbitol MacConkey agar, sorbitol-negative colonies were confirmed using API20E (bioMérieux Vitek, Inc., Hazelwood, MO), serotyped using the RIM *E. coli* O157:H7 latex test (Remel, Lenexa, KS), and subtyped by DNA fingerprinting using PFGE (8). To verify uniqueness and confirm indistinguishable PFGE patterns, repeat subtyping by PFGE of patient and venison isolates was done at the CDPH laboratory. Restriction-fragment banding patterns were matched digitally using a Gel doc 1000 System (Bio-Rad, Hercules, CA) and compared using the Molecular Analyst Plus software (Bio-Rad). Molecular Analyst Fingerprinting DST version 1.6 software (Bio-Rad) for screening DNA patterns permitted a 3% molecular weight matching-tolerance; all matches were confirmed visually.

Both the clinical and venison O157 isolates were confirmed biochemically as *E. coli* and serotyped as O157:H7. DNA fingerprint analysis using PFGE demonstrated a pattern from the uncooked venison O157 isolate that was indistin-

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guishable from the pattern of the clinical O157 isolate (Figure) and unique (occurring only once) among 26 patterns previously described.

Interviews with the child's parents found no history of traditional exposures for O157 infection. The child's father provided information regarding the deer hunt and venison processing. The deer was shot (but not immediately killed) at noon in mid-November in Vermont. After tracking the wounded animal for 2 hours, the hunters located and field dressed the dead animal. An abdominal gunshot wound had resulted in intestinal rupture; no intact internal organs were visible when the deer was eviscerated in the field. No rinsing of the intestinal cavity occurred, as is general practice among deer hunters. The deer was dragged to a truck, brought back to camp, and hung outside overnight before being transported from southern Vermont to Connecticut. The deer was again hung outdoors overnight. Ambient air temperature ranged from 0°C-13°C during this period. The following morning the deer was skinned and cut into large sections. Individual sections were further cut, trimmed, and rinsed under running water before being packaged and stored in a home freezer. The tenderloin was rinsed, placed on a clean plate, refrigerated, and grilled outdoors that evening.

Discussion

This investigation implicates venison from White-Tailed deer (*O. virginianus*) as the source of human O157 infection. We speculate that the deer acquired O157 from cows grazing on dairy farms in Vermont. The prevalence of O157 in White-Tailed deer sharing rangeland with cattle has been well documented (4-6). In addition, a field prevalence study in Georgia found that 3 (4%) of 77 hunter-killed White-Tailed deer carried O157 (5). Deer, like cattle, are transient carriers of O157 and are more likely to be colonized with O157 in the fall and winter (6). Thus, deer are most likely to carry O157 during the time of greatest human exposure, the fall hunting season.

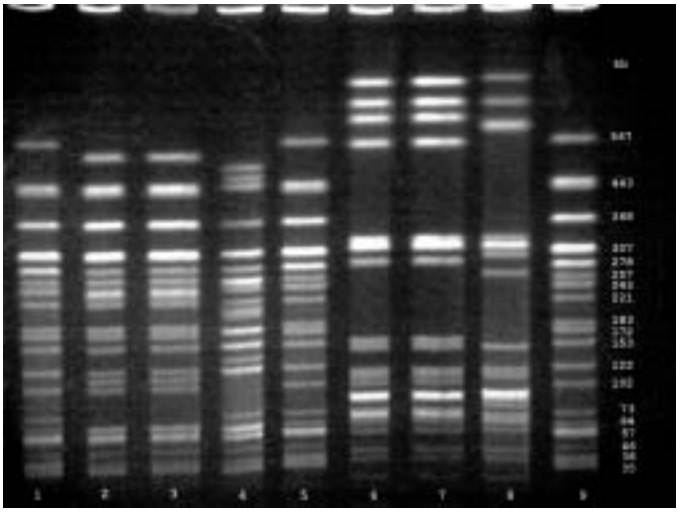

Figure. Pulsed-field gel electrophoresis of O157 isolates from the Connecticut child and the deer meat showing *Xba*I and *Bln*I-digested genomic DNA. Lanes 1, 5, and 9 are *Escherichia coli* G5244, a standard strain used to characterize molecular size; lanes 2 (*Xba*I) and 6 (*Bln*I) are digests from the child's O157 isolate, lanes 3 (*Xba*I) and 7 (*Bln*I) are digests from the deer meat O157 isolate, and lanes 4 (*Xba*I) and 8 (*Bln*I) are digests from an unrelated O157 patient. Numbers at right are molecular sizes (in base pairs).

An estimated 11.3 million Americans hunt big game such as deer or elk each year (10). Nationwide, the annual big game hunting prevalence rate is 7%; regional rates vary from a low of 4% in the Pacific States (Alaska, California, Hawaii, Oregon, and Washington) to a high of 14% in the North Central States (Kansas, Iowa, Minnesota, Missouri, Nebraska, North Dakota, and South Dakota). Relatively few cases of O157 infection have been associated with eating venison over the many years of deer hunting. In a 1995 report, a cluster of household cases was linked with eating jerky made from Black-Tailed deer meat, and a sporadic case in 1987 of O157 infection in Washington State was linked to venison (4).

Routine molecular subtyping of O157 isolates by the CDPH laboratory allowed us to link the sporadic case of O157 with eating venison. Fifty-five patient isolates were subtyped during that same year; 26 distinct patterns were identified. Twenty of these patterns (77%) were unique. This marked heterogeneity of isolates is not limited to Connecticut and emphasizes that many O157 infections are sporadic and caused by contamination of raw foods as well as food preparation and hygiene behaviors.

Multiple factors contributed to the contamination of the deer meat that was eaten by the Connecticut child. The abdominal gunshot wound increased the likelihood that intestinal contents initially contaminated the deer carcass. In addition, the extended time it took the deer to die, fecal contamination of the abdominal cavity, the warm day and mild evening temperatures, and the 2-day interval between deer kill and processing likely supported the dissemination and growth of O157 throughout the carcass. Lastly, a large quantity of undercooked venison tenderloin was eaten.

Hunters who handle wild game in the field are sometimes unaware of the risk of contaminating the meat with foodborne pathogens while dressing, handling, and transporting it. Contamination of game is usually related to the manner in which the animal is killed, dressed, handled, or processed. Improper temperature control, preservation, and cooking may also contribute to contaminated game. Proper handling of deer carcasses begins in the field with a clean shot to the neck or torso (lungs, heart, liver) and quick removal of the intestines/entrails (field dressing) from the abdominal cavity. If any of the internal organs smell offensive, or exhibit discharge, or blood is seen in the muscle, the flesh is unfit for consumption. The abdominal cavity should be cleaned, dried, and cooled to <5°C until the meat is processed.

This case study provides direct evidence for O157 in White-Tailed deer and is the first report to link human illness to the presence of O157 in this species of deer. Our findings contribute to the body of evidence that eating venison may be a source of human infection and highlight the need to provide hunters with guidelines for the proper handling and processing of deer carcasses.

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Phylogenetic Analysis of a Human Isolate from the 2000 Israel *West Nile virus* Epidemic

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Specimens from a patient of the 2000 Israel *West Nile virus* epidemic were analyzed by reverse transcription-polymerase chain reaction. Products corresponding to E, NS3, and NS5 sequences were amplified from cerebellar but not from cortical samples. Phylogenetic analyses indicated a closer relationship of this isolate to 1996 Romanian and 1999 Russian than to 1998-99 Israeli or 1999 New York isolates.

West Nile fever is typically a mild febrile illness characterized by headache, myalgias, lymphadenopathy, and a maculopapular rash; West Nile fever occurs sporadically throughout endemic areas of northeastern Africa and tropical Asia (1-5). The causative agent, *West Nile virus* (WNV), is a member of the genus *Flavivirus* (family *Flaviviridae*), which is grouped together with Alfuy virus, *Cacipacore virus*, *Koutango virus*, *Japanese encephalitis virus*, Kunjin virus, *Murray Valley encephalitis virus*, *St. Louis encephalitis virus*, *Usutu virus*, and *Yaounde virus* in the *Japanese encephalitis virus* antigenic complex (6,7). Two different lineages (I and II) of WNV are characterized genetically (8). Whereas lineage II viruses are isolated in endemic areas, lineage I viruses are isolated during epidemic outbreaks of WNV infection and may cause severe encephalitis in the elderly or immunosuppressed persons. Epidemics of West Nile fever were first reported in the 1950s in Israel (2) and the 1970s in South Africa (9). Sites of notable recent outbreaks include Romania (1996, 1997), Italy (1998), Russia (1999), France (2000), United States (1999, 2000) and Israel (2000) (5,10). Sequence analysis of the WNV responsible for the United States outbreak in 1999 (WNV-NY1999) showed a close phylogenetic relationship to a WNV isolated from a goose in Israel in 1998 (WNV-ISR1998) (11,12).

WNV is transmitted mainly by mosquito vectors, although it has also been isolated in several tick species (3,5,13). Birds are an important WNV reservoir. In several avian species, virus replication generates serum titers sufficient to sustain arthropod transmission (4,5). Birds, during seasonal migrations, are also believed to be instrumental in the geographic spread of WNV (3-5,14). The virus is only occasionally transmitted to humans or other mammals. Viremia in mammals is low level; thus, mammals are considered to be dead-end hosts.

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The Study

From July through November 2000, a WNV epidemic occurred in central and northern Israel. More than 430 people were diagnosed with WNV infection; 29 of these patients had fatal encephalitis. We report phylogenetic analysis of WNV sequences isolated from the brain of an encephalitis patient from the 2000 Israel epidemic.

A 72-year-old woman with a history of recurrent meningioma of the sphenoidal ridge, dementia, and depression was hospitalized because of fever and general deterioration of 5 days' duration. On admission, the patient was responsive only to painful stimuli and had generalized muscle stiffness and limb tremors. Clinical and laboratory values were consistent with viral encephalitis; thus, the patient was initially treated with intravenous acyclovir for presumptive herpes simplex encephalitis. When polymerase chain reaction (PCR) analysis of cerebrospinal fluid (CSF) showed no evidence of herpes simplex virus infection, and WNV antibodies were detected in serum and CSF, acyclovir was discontinued and ribavirin was initiated at an oral dosage of 2.4 g per day. The patient's clinical status continued to deteriorate with aspiration pneumonia and intermittent generalized seizures. Intravenous immunoglobulin was added (35 g/d for 2 days) without improvement. Approximately one month after onset of illness, the patient died of respiratory failure.

Postmortem examination of the brain showed multiple meningiomas, generalized atrophy, and surgical resection of the right parietal lobe. Histology was remarkable for neurofibrillary plaques consistent with Alzheimer's disease, and scattered microglial nodules and perivascular lymphocytic inflammation in the medulla, pons, and midbrain were consistent with viral encephalitis.

RNA was extracted from frontal cortex and cerebellum with TRI-Reagent (Molecular Research Center, Cincinnati, OH). Four micrograms of total RNA from each brain region were used as a template for reverse transcription-polymerase chain reaction (RT-PCR) with primer sets representing three regions of sequence conservation in flavivirus genomes: NS3-1 (EDL/Fla-U5004, 5´- GGA ACD TCM GGH TCN CCH AT and EDL/Fla-L5457, 5´- GTG AAR TGD GCY TCR TCC AT), NS5-1.1 (EDL/Fla-U9093, 5´- AGY MGR GCH ATH TGG TWY ATG TGG and EDL/Fla-L9279, 5´- TCC CAV CCD GCK GTR TCA TC), and NS5-2 (EDL/Fla-U9954, 5´- GSS AAA KCH TAY GCN CAV ATG TGG and EDL/Fla-L10098 5´- AGC ATR TCT TCH GTN GTC ATC CA) (15,16). Amplification products were obtained with RNA derived from the cerebellum in reactions with all three primer sets; no amplification products were obtained with RNA from the cortex. These amplification products were cloned into the

pGEM-Teasy vector (Promega, Madison, WI) and subjected to automated dideoxy sequencing (ABI Prism Model 377, Foster City, CA). Sequences were submitted to GenBank (NS3, GenBank accession no. AF394218; NS5, GenBank accession nos. AF394219 and AF394220).

Signal of cerebellar amplification products in ethidium bromide-stained gels was reduced in comparison with similar studies performed with brain materials from four patients of the 1999 New York City outbreak (data not shown; New York patients were 75 years to 80 years of age, 3 male, 1 female, who died of severe WNV encephalitis during the 1999 outbreak [16]). The 5´-nuclease real-time RT-PCR analysis (17) indicated a relative virus load of 140 copies/200 ng RNA in the Israeli sample, compared with a range of 20 to 7000 copies/200 ng RNA in specimens analyzed from the New York City outbreak (Table). However, since the virus load of the sample from Israel was within the range of virus loads observed with the New York samples, this result for a single Israeli sample may not indicate a strain difference. Quantitative analysis was restricted to the NS5 target because no signal was obtained with primer/probe set prNS3 (fwd, 5´- GCa CTG AGA GGA CTG CCc AT; probe, 5´-6FAM-TAc CAG ACA TCc GCA GTG cCC AGA-T-TAMRA; rev, 5´- TGg GTG AGG GTa GCA TGa CA), because of point mutations in WNV-ISR2000 sequence that prevented efficient hybridization with the primer and probe oligonucleotides (fwd - 2 mismatches, probe – 3 mismatches, rev - 3 mismatches; given above in lower case). Sensitivity was not substantially reduced in assays with primer/probe set prNS5, which had one mismatch in the 3′-terminal sequence of the probe oligonucleotide (Table).

Sequence analysis of the cloned NS3 and NS5 gene fragments indicated similarity to completely sequenced Romanian and Russian WNV isolates WNV-RO97-50-1996 and WNV-RUS-VLG4-1999, respectively; thus, to facilitate detailed phylogenetic analysis, E gene sequence from the Israel human brain sample was amplified. An E gene sequence of 1,509 nucleotides (GenBank accession number AF394217) was amplified from total RNA by using GeneChoice UNIPOL polymerase (PGC Scientific, Gaithersburg, MD) and primers EDL/E-U1006 (5´- GGA GTG TCT GGA GCA ACA TGG GT) or EDL/E-U1476 (5´- TCC TGC GGC GCC TTC AT) and EDL/E-L2244 (5´- CCC CTC CAA CTG ATC CAA AGT CC) or EDL/E-L2538 (5´- TCC ATC CAA GCC TCC ACA TCA), respectively. Sequence analysis of this fragment confirmed data from NS3 and NS5 sequence analyses, indicating a closer relationship of WNV-ISR-hISR2000 sequence to Romanian and Russian isolates than to the 1997/98/99 Israeli and the WNV-NY1999 isolates (Figure).

The extent to which this WNV genotype contributed to human disease in the 2000 epidemic remains to be determined. WNV-ISR-hISR2000 may have been carried into Israel by migrating birds from reservoirs in southeastern Europe or reservoirs in northeastern Africa, where a highly related virus was isolated in 1998 (WNV-KEN-KN3829-1998) (18). The 2000 Israel isolates in birds (and pigs, strains ISR-00GooMaS and ISR-00PigC) were different from the previous Israeli isolates (1997/98/99; strains ISR-97Goo1, ISR-98Goo1, ISR-IS98St1, ISR-99Goo, and ISR-99Gull [Figure]), but similar to the human isolate. Nonetheless, precedent exists for implicating more than one genotypic variant in a WNV outbreak.

Table. Real Time reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA extracts from 2000 Israel West Nile patient specimens using primer set NY1999-NS5

NS5 standard NY1999		Armored RNA		Armored RNA extract		NY1999 specimens			NS5 standard ISR2000		ISR2000 specimens		
Amount[a]	C_T[b]	Dil.[c]	Amount[d]	Dil.[e]	Amount[d]	Patient no.	C_T	Amount[d]	Amount[f]	C_T	Sample	C_T	Amount[g]
$2.5x10^6$	16.4	$1:10^1$	$4.5x10^6$	$1:10^1$	$4.4x10^5$	1	29.3	$6.9x10^2$	$2.5x10^6$	16.5	cereb.	31.1	$1.4x10^2$
$2.5x10^5$	20.0	$1:10^2$	$3.4x10^5$	$1:10^2$	$4.0x10^4$	2	25.6	$7.3x10^3$	$2.5x10^5$	20.0	cortex	36.4[h]	$1.9x10^0$
$2.5x10^4$	23.6	$1:10^3$	$3.1x10^4$	$1:10^3$	$4.3x10^3$	3	30.0	$4.6x10^2$	$2.5x10^4$	23.1			
$2.5x10^3$	27.2	$1:10^4$	$3.1x10^3$	$1:10^4$	$6.0x10^2$	5	34.8	$2.3\ x10^1$	$2.5x10^3$	26.7			
$2.5x10^2$	31.1	$1:10^5$	$3.4x10^2$	$1:10^5$	$9.8x10^1$				$2.5x10^2$	30.3			
$2.5x10^1$	34.9	$1:10^6$	$3.1x10^1$	1:10	$2.1x10^0$				$2.5x10^1$	34.1			
$2.5x10^0$	36.8[h]	$1:10^7$	$3.0x10^0$ [h]	$1:10^7$	n.d[i]				$2.5x10^0$	37.3 [h]			
0	>45	0	0	0	0				0	>45			

[a] Plasmid DNA p88-D-21 was quantitated spectrophotometrically, and dilutions containing the indicated copy number of target sequence were added to each polymerase chain reaction (PCR) assay.
[b] C_T , Cycle number at which signal crosses threshold.
[c] Armored RNA *West Nile virus* (HNY1999) standard (Ambion, Austin, TX) was diluted 1:10, boiled, reverse transcribed, and then diluted to result in amounts per PCR assay equivalent to the indicated dilution of the stock (5 µL).
[d] Amount calculated based on calibration curve obtained with NS5 Standard NY1999 (column 1).
[e] Dilutions of Armored RNA *West Nile virus* (HNY1999) standard (Ambion) were extracted with TRI-Reagent (Molecular Research Center, Cincinnati, OH) and then subjected to RT-PCR to result in amounts per assay equivalent to the indicated dilution of the stock (5 µL).
[f] Plasmid DNA pISR-Dfrag-D6 was quantitated spectrophotometrically, and dilutions containing the indicated copy number of target sequence were added to each PCR assay.
[g] Amount calculated based on calibration curve obtained with NS5 Standard ISR2000 (column 5).
[h] Poisson effects take place at low template concentration; duplicate assay deviations: 36.2 / 37.4, NY1999; $6.0\ x10^0$ / 0, armored RNA; 37.4 / 37.1, ISR2000; and 36.4 / >45, cortex.
[i] n.d. , not determined.

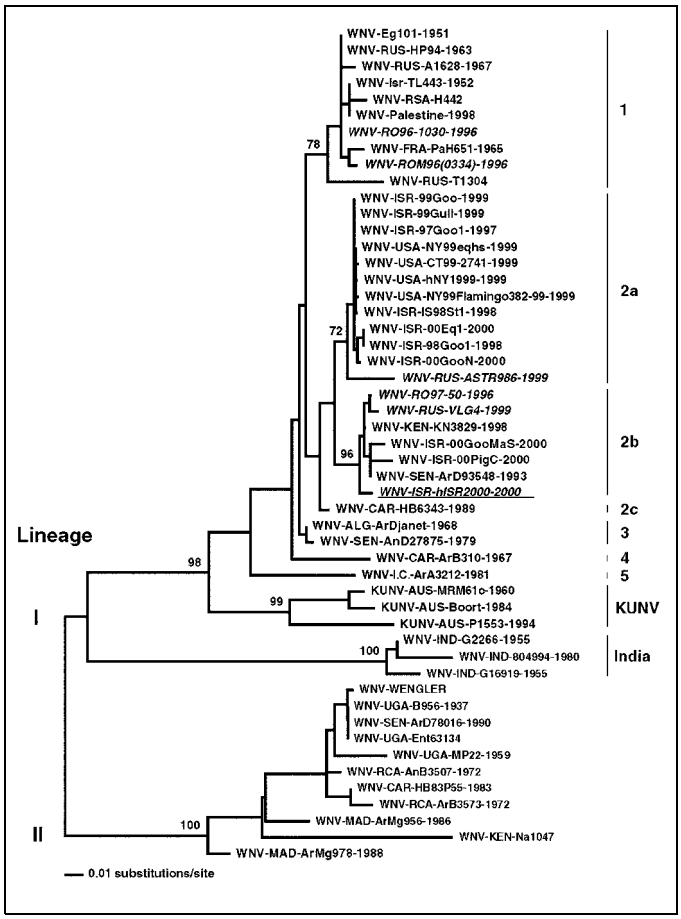

Figure. Phylogenetic analysis of WNV-hISR2000 E gene sequence. Phylogenetic analysis of the sequences listed below was performed with PAUP (Phylogenetic analysis using parsimony) 4.0b8 (Sinaur Associates, Sunderland, MA). A neighbor-joining tree was constructed using maximum likelihood distances with the HKY85 model of substitution and allowing different rates of substitution at each codon position. Bootstrap values are the result of 1000 neighbor-joining replicates under this same model. Only relevant bootstrap values are shown. WNV-Eg101-1951 (human, H), AF260968; WNV-RUS-HP94-1963, AF237565; WNV-RUS-A1628-1967 (bird, B), AF237563; WNV-ISR-TL443-1952 (H), AF205881; WNV-RSA-H442, AF205880; WNV-Palestine-1998, V. Deubel unpub.data; WNV-RO96-1030-1996 (H), AF130363; WNV-FRA-PaH651-1965 (H), AF001560; WNV-ROM96(0334)-1996, AF208579; WNV-RUS-T1304; AF237566; WNV-ISR-99Goo-1999 (B), AY033391; WNV-ISR-99Gull-1999 (B), AY033390; WNV-ISR-97Goo1-1997 (B), AF380663; WNV-USA-NY99eqhs-1999 (equus, E), AF260967; WNV-USA-CT99-2741-1999 (mosquito, M), AF206518; WNV-USA-hNY1999-1999 (H), AF202541; WNV-USA-NY99Flamingo382-99-1999 (B), AF196835; WNV-ISR-IS98ST1-1998 (B), AY033389; WNV-ISR-00Eq1-2000 (E), AF380669; WNV-ISR-98Goo1-1998 (B), AF205882; WNV-ISR-00GooN-2000 (B), AF380665; WNV-RUS-ASTR986-1999 (H), AF237562; WNV-RO97-50-1996 (M), AF260969; WNV-RUS-VLG4-1999 (H), AF317203; WNV-KEN-KN3829-1998 (M), AF146082; WNV-ISR-00GooMaS-2000 (B), AF380667; WNV-ISR-00PigC-2000 (pig, P); WNV-SEN-ArD93548-1993 (M), AF001570; WNV-ISR-hISR2000-2000 (H), AF394217; WNV-CAR-HB6343-1989 (H), AF001558; WNV-ALG-ArDjanet-1968 (M), AF001567; WNV-SEN-AnD27875-1979 (primate, P), AF001569; WNV-CAR-ArB310-1967 (M), AF001566; WNV-I.C.-ArA3212-1981 (M), AF001561; KUNV-AUS-MRM61c-1960 (M), D00246; KUNV-AUS-Boort-1984 (E), AF196519; KUNV-AUS-P1553-1994 (M), AF196495; WNV-IND-G2266-1955 (M), AF196525; WNV-IND-804994-1980 (H), AF196526; WNV-IND-G16919-1955, AF205885; WNV-WENGLER, M12294; WNV-UGA-B956-1937 (H), AF394221; WNV-SEN-ArD78016-1990 (M), AF001556; WNV-UGA-Ent63134, AF001573; WNV-UGA-MP22-1959 (M), AF001562; WNV-RCA-AnB3507-1972 (B), AF001563; WNV-CAR-HB83P55-1983 (H), AF001557; WNV-RCA-ArB3573-1972 (M), AF001565; WNV-MAD-ArMg956-1986 (M), AF001564; WNV-KEN-Na1047 (M), AF001571; WNV-MAD-ArMg978-1988 (M), and AF001574.

During the 1999 outbreak in Volgograd, Russia, two different genotypes were isolated: WNV-RUS-ASTR986-1999 (similar to 1997/98/99 Israeli and the WNV-NY1999 isolates, genotype lineage I subtype 2a) and WNV-VLG22889/WNV-RUS-VLG4-1999 (similar to WNV-ISR-hISR2000, subtype 2b [Figure]) (19). Indeed, even more divergent genotypes were identified during the 1996-97 WNV outbreak in Romania (WNV-RO97-50-1996 similar to WNV-ISR-hISR2000, genotype lineage I subtype 2b; WNV-RO96-1030-1996 and WNV-ROM96(0334)-1996, belonging to a different subtype, subtype 1 [Figure]) (20). The fact that no such divergence of genotypes of WNV isolates was observed during the 1999 New York epidemic (Figure) was interpreted as being compatible with a single, new introduction of this virus to the Western Hemisphere. While this manuscript was under review, another group reported WNV sequences from four patients of the 2000 Israel outbreak: two isolates most closely related to WNV-R097-50-1996 and two identical to the WNV-NY1999 isolates (21). Analysis of additional isolates from the Israel 2000 and other outbreaks, including isolates obtained in 2000, 2001, and subsequent years in the USA, will be required to establish the extent to which avian migration and viral mutation contribute to the epidemiology of WNV-related disease.

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Knowledge of Bat Rabies and Human Exposure among United States Cavers

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We surveyed cavers who attended the National Speleological Society convention in June 2000. Fifteen percent of respondents did not consider a bat bite a risk for acquiring rabies; only 20% had received preexposure prophylaxis against the disease. An under-appreciation of the risk for rabies from bat bites may explain the preponderance of human rabies viruses caused by variant strains associated with bats in the United States.

Over the past century, human rabies has become exceedingly rare in the United States. The decreasing incidence of human rabies has followed the decline of rabies in domestic dogs. From 1946 to 1965, 236 human *Rabies virus* (RABV) infections were reported in the United States. From 1946 through 1949, the number of human RABV infections averaged 24/year, declining to 1.5/year from 1962 through 1965. Ninety percent of RABV infections were caused by dog bites from 1946 through 1949, decreasing to 67% from 1962 through 1965 (1). As canine rabies declined, the relative importance of other reservoirs in the United States increased. From 1970 to 1989, human infections averaged 3.3/year. Of these infections, 45% were caused by canine RABV variants (all but one was acquired outside the United States), 30% were caused by bat RABV variants, and one was caused by a corneal transplant from an unsuspected rabies patient; (2,3). From 1990 through 2000, bat RABV variants have emerged as the predominant cause of human rabies in the United States (4). In the past 11 years, total human rabies deaths have averaged 2.9/year, and 24 (75%) of 32 deaths were due to bat RABV variants. If the six cases caused by foreign canine RABV variants are excluded, then 24 (92%) of the 26 human rabies deaths acquired domestically were caused by bat RABV variants. The other two cases were due to a dog/coyote RABV variant found in Texas (4).

Confusion remains about potential exposures to rabies from bats. Only 2 (8%) of the 24 patients with human rabies caused by bat RABV variants had a definitive history of a bat bite. Nine patients (38%) had a history of direct physical contact with bats, 5 (21%) had a history of a bat inside the living area, and 8 (33%) had no history of proximity to bats (4). Because of the paucity of bat (or other animal) bite histories, could these human rabies cases have been acquired through aerosol transmission? The diagnosis of rabies in two people who had no known history of a bite, but who worked extensively in caves inhabited by bats, received considerable attention in 1953 (1,2). Although the aerosol route is considered a possible mechanism of RABV acquisition, few data support such transmission under typical field conditions. A more plausible hypothesis is that many people may not be aware that a bat bite is a risk for rabies transmission and fail to report it.

Because of the potential contact with bats, cavers are considered at a higher risk for rabies exposure than the general population. Since the 1960s, the recommendation has been that cavers receive rabies preexposure prophylaxis (PreEP) (5). The objectives of this study were to learn about cavers' knowledge of the risks for bat-to-human rabies transmission and to quantify cavers' use of rabies PreEP prophylaxis and postexposure prophylaxis (PostEP).

The Study

We administered a survey to cavers attending the National Speleological Society Convention in Elkins, West Virginia, USA, in June 2000. The survey was included in the convention registration packet. Verbal reminders to return the survey were given, and collection boxes were located at several sites at the convention.

The survey asked respondents about demographic information, how long and how many times they had been caving, how often they encountered bats when caving, if they had been advised to receive the rabies PreEP and if they had received it, if they considered specific scenarios (bat bite, bat scratch, bat on skin, bat on clothing, indirect contact with bats) as a potential risk for rabies, if they had ever had a potential exposure to rabies, and if they had ever received rabies PostEP.

Categorical variables were compared using the chi-square test or the Fisher's exact test (2-tailed), as appropriate. Continuous variables were analyzed with the Wilcoxon rank-sum test (6). Multivariate logistic regression was used for multivariate analysis.

Questionnaires were returned from 392 (26%) of 1,508 cavers attending the convention. The respondents' mean age was 47 (range 12-84) years, 68% were male, and 76% were college graduates. The respondents caved a mean of 23 (range 1-58) years and a mean of 16 (range 0-150) times in the past year. When asked how often they see bats on their caving trips, 1% responded never, 29% sometimes, 22% about half the time, 43% often, and 5% always. Respondents were asked to address whether specific scenarios with bat(s) were considered a risk for rabies (Table 1).

The respondents who thought a bat bite was not a risk for rabies were younger (43 versus 48 years, p=0.009) and less educated (43% versus 21% were not college graduates, p=0.005) but did not differ significantly by gender, number of years caving, or number of times caving in the past year. The respondents who thought that indirect contact with bats was a risk for rabies were older (52 versus 46 years, p<0.001), and

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Table 1. Number of cavers who considered the scenario as a risk for rabies

Scenario	≥College degree	No college degree	Total (%)
	(n=298)	(n=94)	
Bat bite	262/294 (89)	69/93 (74)[a]	331/387 (86)
Bat scratch	191/290 (66)	42/92 (46)[a]	233/382 (61)
Bat on skin	42/292 (14)	9/93 (10)	51/385 (13)
Bat on clothing	10/293 (3.4)	1/93 (1.1)	11/386 (2.9)
Being around bats[b]	37/293 (13)	8/93 (8.6)	45/386 (12)

[a] For having ≥college degree compared to no college degree, p<0.001.
[b] Indirect contact with bats.

caved more years (28 versus 22; p<0.001). They did not significantly differ by gender, education, or number of times caving in the past year. Seventy-six (20%) respondents received PreEP (Table 2). In multivariate analysis, having been advised to receive the vaccine was independently associated with having received it (odd ratio = 31; 95% confidence interval 15 to 61).

Eighty-eight (23%) respondents had been advised to receive PreEP. Those who caved more years (25 versus 22, p=0.05), and more times in the last year (25 versus 15, p<0.001) were more likely to have been advised to have PreEP. College graduates were more likely to be advised to have PreEP, but statistical significance was not found (24% versus 17%, p=0.14). Those advised to get PreEP did not differ by age or gender. Of the 66 respondents advised to get PreEP because of caving, 37 (57%) had done so; of the 20 advised to get PreEP for other reasons, 17 (85%) had done so. Twenty-four (1.6%) respondents felt they had been potentially exposed to rabies. Of the 24, only 5 involved exposures to bats (3 from bites), and only 1 indicated this exposure was directly associated with caving.

Conclusions

Despite the cavers' education level and their familiarity with bats, 14% of the cavers did not consider a bat bite risk for rabies. When only the cavers without a college degree were considered, 26% did not think a bat bite was a risk for rabies.

Table 2. Number of cavers who had/had not received preexposure prophylaxis (PreEP)

Characteristic	Received (n = 76)	Not received (n = 313)	p value
College graduate	63/76 (83)	231/311 (74)	0.12
Advised to get PreEP[a]	56/75 (75)	31/315 (10)	<0.001
Male gender	55/76 (72)	210/312 (67)	ns
See bats ≥ half of the time	57/71 (80)	211/311 (68)	0.04
Mean age	49 yrs	46 yrs	ns
Mean yrs caving	26 yrs	22 ys	0.01
Mean times caving per yr	34/yr	13/yr	< 0.001

[a]The only variable independently associated with receiving PreEP.

Given the general public's assumed education level and overall lack of familiarity with bats, the percentage of the public who do not consider a bat bite a risk for rabies is probably higher than (or closer to) 26%, than 14%. If so, this would support the hypothesis that people may lack the knowledge to seek medical care if a bat bites them. Unlike bites from larger mammalian carnivores, lesions resulting from a bat bite probably will not warrant seeking medical care. In addition, 39% of cavers did not think a bat scratch was a risk for rabies. Technically, a scratch contaminated with saliva is an exposure, but scratches alone are less likely to transmit rabies than a bite. The practical problem arises in the consideration of scratches from bats. Does the patient know if the scratch is contaminated with saliva? And more importantly, can a patient discern a scratch from a bite, particularly under the darkened and tight recesses of a cave?

Eleven percent of cavers felt that indirect contact with bats was risk for rabies. Some cavers (especially older, more experienced members) may possess knowledge of those rare cases of human rabies that are attributed to aerosol transmission. Two infections in the 1950s, commonly attributed to aerosol transmission in crowded bat caves (in a bat researcher and a mining engineer), had other possible mechanisms of infection (7,8), and no other infections have been reported in cavers. Interestingly, the lack of rabies cases in cavers is evidence against the occurrence of aerosol transmission, except under extraordinary circumstances. The respondents in our study, if projected to only cavers who are members of the NSS, represent over 4 million caving episodes; nearly 60% involved cavers with no PreEP. Of course, the expected prevalence of rabies in freeranging bats is low, probably <1% (9).

This survey is limited by a low response rate and may be subject to selection bias. Those who did respond may be more or less familiar with rabies than the average caver. In addition, the survey may be subject to response bias. Relationships demonstrated are associations; cause and effect cannot be definitively determined.

Nevertheless, our study suggests that, despite longstanding guidelines for cavers to receive PreEP for rabies, only 20% have done so. The increase is modest when compared to a survey conducted in 1970 of 239 cavers, which found that only 14% had received PreEP (CDC, unpub. data). Increasing the cavers' awareness about the recommendation may increase compliance, as 64% of those advised to receive PreEP had done so, compared to 6% (n=19) of those not advised to do so. In fact, this was the only independent predictor of receiving PreEP. A future survey of the general public is indicated to explore their knowledge and attitudes towards bats, rabies, and the risk for acquisition.

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Dispatches. Articles should be 1,000 to 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 10), 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.

First Shiga Toxin-Producing *Escherichia coli* Isolate from a Patient with Hemolytic Uremic Syndrome, Brazil

To the Editor: Infection by Shiga toxin (Stx)-producing *Escherichia coli* (STEC), particularly strains of serotype O157:H7, can cause sporadic cases and outbreaks of diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) (1). Some other serotypes (e.g., O26:H11, O111:H8, O111:NM, and O113:H21) share a similar pathogenic potential. STEC are distributed worldwide, but most of the HC and HUS cases were reported from industrialized nations of the Northern and Southern Hemispheres (2). In South America, HUS is a major cause of acute renal failure in infants in Argentina (3) and Chile (4). However, in Brazil human STEC infections have been restricted to sporadic cases of nonbloody diarrhea (5,6). Although a high frequency of STEC strains was recently found in foods and animal reservoirs (7,8), only some of the serotypes identified in animals (8) were recognized as causes of human illness (e.g., O157:H7, O22:H16, O82:H8, and NT:H21). Moreover, there is currently no nationwide surveillance system for HUS in Brazil, and STEC-associated HUS has not been previously reported in our country.

We describe the case of an 8-month-old boy from a northeastern state in Brazil, who was admitted to the emergency room of Hospital São Paulo, São Paulo, on March 17, 2001; the boy had anemia, oliguria, and edema of lower extremities. He had an acute diarrheal prodromal illness 3 weeks before hospital admission. On the same day as admission, respiratory failure developed, and the child was transferred to the pediatric intensive-care unit of the hospital. The boy had hemolytic anemia (hemoglobin level 11.9 g/dL at admission, and 9.1 g/dL several days later), renal failure (blood urea nitrogen 43.8 mg/dL and serum creatinine 1.5 mg/dL), and thrombocytopenia (platelet count of 70,000/mm^3), leading to a diagnosis of HUS. The patient received treatment with fresh frozen plasma and needed renal support (peritoneal dialysis) for 7 days. Once renal function was reestablished, the patient's outcome was good.

Feces were collected as soon as HUS was suspected and plated onto MacConkey Sorbitol Agar (Difco, Becton Dickinson Microbiology Systems, Sparks, MD). Only sorbitol-positive colonies grew and were biochemically identified as *E. coli* by standard procedures. The *E. coli* isolates expressed Stx1, as identified by cytotoxicity and neutralization assays on Vero cells (5). Presence of *stx1* and intimin (*eae*) gene sequences was confirmed by polymerase chain reaction (9,10). The *E. coli* strain belonged to serotype O26:H11 and produced enterohemorragic *E. coli* hemolysin (enterohemolysin).

This report is the first on the isolation of an STEC strain in a HUS patient in Brazil. The serotype O26:H11 has been described as an agent of HC and HUS in other countries and was the second most frequent serotype found in STEC strains isolated from diarrheal cases in our settings (6). Moreover, expression of Stx1 and enterohemolysin and the presence of *eae* are virulent characteristics usually found in the human STEC strains isolated so far in Brazil. These findings show the importance of looking for non-O157 STEC strains besides O157:H7 in patients with HC and HUS in Brazil. Surveillance for HUS, either nationally or in sentinel population-based studies, should be performed in Brazil, and studies on the occurrence of HUS and its association with STEC infections are under investigation in our laboratory.

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Acknowledgments

We thank Dr. Tânia A.T. Gomes for encouragement on this subject.

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Emergence of Vancomycin-Intermediate *Staphylococcus aureus* and *S. sciuri*, Greece

To the Editor: Staphylococcal isolates with reduced susceptibility to glycopeptides, such as vancomycin and teicoplanin, are a serious public health problem because staphylococci frequently show multidrug resistance, and glycopeptides are the only remaining effective drugs. Since the early reports of glycopeptide-resistant staphylococci, teicoplanin resistance has become more common than vancomycin resistance, particularly among coagulase-negative staphylococcal species (1-3). In cases of staphylococci with reduced susceptibility to vancomycin (vancomycin-intermediate staphylococci), an increasing number of strains showing heteroresistance are reported (strains that contain subpopulations of cells at frequencies $\geq 10^{-6}$ for which the vancomycin MICs are 8 µg/mL to 16 µg/mL); homogeneous resistance still appears to be rare (2,4-7). In northern Greece, resistance to teicoplanin has recently been documented in *S. haemolyticus* strains isolated from clinical infections (8). We report the first bloodstream infections in Greece associated with *S. aureus* and *S. sciuri* strains that have homogeneous intermediate-resistance to vancomycin (MIC = 8 µg/mL).

In our department, all clinically significant staphylococcal isolates are screened for reduced susceptibility to vancomycin and teicoplanin by an agar incorporation method (9), which has been routinely performed since January 1999. An inoculum of 10^4 CFU/spot from a log-phase broth culture was spread on Mueller-Hinton agar plates containing appropriate antibiotic concentrations. The strains were incubated for a full 24 hours before the MICs were read. When a reduced susceptibility to vancomycin was observed (MIC 8 to 16 µg/mL), the test was repeated for confirmation of the result and the strains were also tested by National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution (9) and E-test (AB Biodisk, Solna, Sweden) with BHI agar (Oxoid, Ltd., Basingstoke, Hampshire, UK) and an inoculum density adjusted to 0.5 McFarland value. *S. aureus* ATCC 29213, which had MICs for vancomycin of 1 µg/mL and for teicoplanin of 0.5 µg/mL, was used as a control for the estimation of the MICs. Two vancomycin-intermediate staphylococcal isolates (one *S. aureus* and one *S. sciuri*) were recovered in our hospital during December 2000 and April 2001, respectively. The organisms were identified with the Vitek system (bioMerieux Vitek, La Balme les Grottes, France). Slide-coagulase test and Staph ID 32 API system (API system, bioMerieux) confirmed identification. Susceptibility to 18 antimicrobial agents was evaluated with the Vitek system according to the recommendations of the manufacturer, and carriage of the *mecA* gene was confirmed with a polymerase chain reaction (PCR) that amplifies a 449-bp product.

The first strain (*S. aureus*) was recovered from a 52-year-old man who was hospitalized after a severe traffic accident. The patient had multiple injuries, including an external laryngeal trauma, pelvic ring disruption, and various fractures of the extremities. He underwent immediate tracheotomy, and a neurosurgical operation was performed to evacuate an extracerebral hematoma. Ceftazidime, clindamycin, ciprofloxacin, metronidazole, teicoplanin, and vancomycin were periodically administered as prophylaxis. An oxacillin-resistant *S. aureus* isolate was recovered from two blood cultures 4 weeks after the patient's admission. The strain was also resistant to tobramycin, macrolides, tetracyclines, rifampicin, and fusidic acid, and had intermediate resistance to vancomycin (MIC 8 µg/mL) and teicoplanin (MIC 16 µg/mL) by all tested methods (agar dilution, broth microdilution, and E-test). The strain was susceptible to chloramphenicol, cotrimoxazole, fosfomycin, gentamicin, kanamycin, nitrofurantoin, and ofloxacin. The removal of an intravenous catheter and treatment with gentamicin and vancomycin eradicated the infection.

The second strain (*S. sciuri*) was recovered from a 35-year-old man who was an intravenous drug user. He was admitted with renal failure, electrolyte disturbances, and acute respiratory distress, which necessitated intubation and mechanical ventilation. The patient became febrile, and multiple courses of antibiotics (amikacin, cefepime, ciprofloxacin, metronidazole, and vancomycin, alone or in combinations) were administered before the *S. sciuri* strain was isolated. Seven weeks after his admission, an oxacillin-resistant *S. scuiri* strain that had cross-resistance to aminoglycosides, macrolides, quinolones, rifampicin, and tetracycline was found in subsequent blood cultures. The MIC of the strain for vancomycin was 8 µg/mL and for teicoplanin 16 µg/mL by the agar dilution method, and the result was confirmed by the E-test and the broth microdilution method. The strain was susceptible only to cotrimoxazole, fosfomycin, and nitrofurantoin. The patient improved clinically and was subsequently discharged on cotrimoxazole and vancomycin therapy.

In both cases, the MICs of vancomycin remained stable after repeated subcultures in a drug-free medium. PCR amplification showed that both staphylococcal strains carried the *mecA* gene. However, the *vanA, vanB,* and *vanC* genes were not amplified in any strain.

Vancomycin-intermediate staphylococci have been sporadically reported from clinical infections after prolonged exposure to vancomycin or preexisting infection with methicillin-resistant staphylococci (5,7). In our hospital, high rates of methicillin-resistant staphylococci are detected, and vancomycin has been the only treatment uniformly effective against staphylococcal infections. However, this is the first report of infection caused by vancomycin-intermediate *S. aureus* in Greece. In addition, *S. sciuri*, a species considered taxonomically the most primitive among staphylococci and found primarily in rodents and primitive mammals, has not been implicated previously in human infections caused by vancomycin-intermediate strains in our region or elsewhere.

Although various studies have described staphylococci with reduced susceptibility to vancomycin, the existence of isolates that have the homogeneous vancomycin-intermediate phenotype is rather limited (4-7). In this report, the vancomycin MIC for both staphylococcal isolates was repeatedly 8 µg/mL, and a confluent growth was observed after 24 hours on Mueller Hinton agar containing vancomycin at a concentration of 4 µg/mL. Discrete colonies were detected only in plates containing 6 µg/mL of vancomycin but not in plates containing 8 µ/mL of the drug even when a prolonged incubation of 48 hours and an inoculum of 10^6 CFU/spot were used. The Vitek system recorded correctly both isolates as having intermediate level of resistance to glycopeptides; this result was particularly important given the wide use of this commercial system in many hospital laboratories. However, as was reported for previous glycopeptide-intermediate staphylococci (2,5), both isolates appeared to be susceptible to vancomycin when tested by the disk diffusion method, with zones of 15 mm and 16 mm for the *S. aureus* and the *S. sciuri* isolates, respectively.

Vancomycin-resistant staphylococci had not been detected in our hospital until December 2000. Therefore, the emergence of vancomycin-resistant staphylococci is a recent development, suggesting a potential for wider dissemination. Since the NCCLS agar dilution method we used is not sensitive for the detection of heterogeneous resistance phenotypes (7), screening for vancomycin-resistant subpopulations in the vancomycin-susceptible isolates (mainly those with MIC for vancomycin of 4 µg/mL) is important. Whether the mechanisms responsible for homogeneous intermediate resistance to vancomycin in our staphylococci are similar to those described in isolates from Japan and elsewhere still remains to be answered.

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Guidelines for Letters. Letters discussing a recent Emerging Infectious Diseases article (400-500 words, 5-10 references) should be received within 4 weeks of the article's publication. Letters reporting preliminary data (500-1,000 words, 10 references) should not duplicate other material published or submitted for publication, should not be divided into sections, and should avoid figures or tables. All letters have the same authorship, financial disclosure, and acknowledgment requirements as full articles and should include a word count. For more guidance on manuscript preparation, see Emerging Infectious Diseases Instructions to Authors. Send letters to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA, or e-mail: eideditor@cdc.gov .

Letters Editor: Patricia M. Quinlisk, Des Moines, Iowa, USA

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

CD Review

Acute Respiratory Infection CD Module

Publishers, The Wellcome Trust, London, United Kingdom

The Acute Respiratory Infection (ARI) CD Module is the latest educational offering by The Wellcome Trust, a British-based charity. The module is part of the Topics in International Health CD-ROM series. The entire program is designed to provide accessible, current, high-quality information on tropical and international health. The material is directed towards students, teachers, health-care professionals, academics, and researchers in medicine and the life sciences. Each tutorial has been reviewed by at least two international subject experts.

The ARI module includes 11 separate tutorials ranging from etiology and risk factors to epidemiology and program management. An additional image collection of over 600 screens with accompanying text provides a useful bank of visual aids.

While the module covers all major ARIs, the focus is on conditions causing substantial illness and deaths in the developing world. Each tutorial averages 40 screens, with each individual screen featuring a variety of additional interactive tools, such as pop-up boxes and animated features. On average, tutorial post-tests follow every 6-10 screens. Although the content and information differ, each tutorial averages 2-4 hours to complete. Completing all 11 tutorials could take up to 50 hours.

Overall, the module succeeds in consolidating broad-reaching material and providing an authoritative presentation on ARI. Module strengths include the successful presentation of clinically current and well-researched information. (One possible exception is the management of ear problems. The module recommends irrigation of acute otitis media. This practice is highly questionable, and the accompanying illustrations minimize the major risks associated with this procedure in cases where the tympanic membrane has ruptured. The World Health Organization recommends "wicking.") Each tutorial provides ample references, and the repetition of important themes helps reinforce important clinical and public health concepts. The graphic features and video inserts are equally useful. For example, the integration of animated graphics in the pathology tutorial to demonstrate the major steps of viral multiplication offers an innovative visual tool for mastering complex information.

On the other hand, the module suffers in its attempt to cover a large quantity of material in a similar manner. Not all the tutorials are of comparable complexity. While 40 screens may be adequate to discuss ARI prevention and control measures, other sections, such as pathology and respiratory defenses, would have benefited from more discussion. By trying to maintain uniform format of comparable length, parts of these tutorials are superficially summarized at the expense of a clear and in-depth discussion. Another weakness pertains to the varying levels of difficulty in the post-tests interspersed throughout the module. Some of the questions are challenging, whereas other post-tests could be completed correctly without ever having taken the tutorial. Minor typographical errors were noted throughout; however, they did not detract from the overall presentation.

More important issues pertain to the module's intended audience. Although the material and clinical examples are designed to address ARIs in the developing world, they are not aimed at professionals from those countries. Rather, the module is better suited for health professionals from the industrialized world who have an interest in global health. The module would be far more practical had it been field-tested in those countries from which both clinical and public health examples were drawn.

The module requires additional software for accessing video clips, such as QuickTime or RealPlayer. The cost of the CD-ROM is also relatively high: $195.00 for institutions and $55 for students and individual purchasers. These two features limit the module's practicality as a teaching device for health professionals in the developing world. Additionally, the module does not have the capability of multitasking with other software applications. The user must exit the module to use any other software, which limits the ability to access other references at the same time.

In summary, the Acute Respiratory Infection CD Module serves as a useful adjunctive teaching tool for both clinical and public health practitioners serving the developing world. It is not intended to replace the clinical component of provider training. While the material is comprehensive in scope, this aspect conversely leads to an uneven presentation in places. Given the required sophisticated software and cost of the CD, its usefulness for health professionals from the developing world is questionable.

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Book Reviews. Short reviews (250 to 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.

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.

Correction, Vol. 8, No. 1

In the article "Antimicrobial Sensitivity in Enterobacteria from AIDS Patients, Zambia," by James Mwansa et al., errors were made in calculations for the table on page 93. The corrected table appears below and online at http://www.cdc.gov/ncidod/eid/vol8no1/01-0018.htm. We regret any confusion these errors may have caused.

Table. Antibiotic sensitivity patterns for three enterobacteria isolated from patients with HIV-related persistent diarrhea in Zambia

Antimicrobial agent[a]	Nontyphoidal salmonellae	*Shigella flexneri*	*Shigella dysenteriae*
	No. sensitive (%)	No. sensitive (%)	No. sensitive (%)
Tetracycline	37 (23)	2 (6)	3 (16)
Chloramphenicol	36 (23)	7 (23)	8 (42)
Gentamicin	119 (75)	24 (77)	18 (95)
Sulphamethoxazole-trimethoprim	25 (16)	3 (10)	0 (0)
Amoxycillin	74 (47)	9 (29)	7 (37)
Amoxycillin-clavulanic acid	95 (60)	27 (87)	12 (63)
Cephalexin	105 (66)	23 (74)	17 (89)
Cefuroxime	93 (59)	11 (35)	16 (84)
Cefotaxime	149 (94)	28 (90)	19 (100)
Nalidixic acid	107 (68)	31 (100)	19 (100)
Ciprofloxacin	157 (99)	30 (97)	18 (95)
Erythromycin	22 (14)	0 (0)	4 (21)
Azithromycin	64 (93)	9 (100)	19 (100)

[a]One hundred fifty-eight isolates of nontyphoidal salmonellae, 31 of *S. flexneri*, and 19 of *S. dysenteriae* were tested against all these antimicrobial agents, except for azithromycin, against which 69, 9, and 19 isolates were tested, respectively.

Correction, Vol. 8, No. 4

In "Antimicrobial Use and Antimicrobial Resistance: A Population Perspective," by M. Lipsitch and M.H. Samore, the following references appeared out of order: nos. 10, 14, and 19. The corrected version appears online at http://www.cdc.gov/ncidod/EID/vol8no4/01-0312.htm . We regret any confusion this error may have caused.

Correction, Vol. 8, No. 4

In the Letter to the Editor "O157:H7 Shiga Toxin-Producing *Escherichia coli* Strains Associated with Sporadic Cases of Diarrhea in São Paulo, Brazil," by Kinue Irino et al., reference no. 1 was inadvertently omitted. That reference is

Griffin P, Tauxe RV. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol Rev 1991;13:60-98.

References in the text should be renumbered accordingly. The corrected version appears online at http://www.cdc/gov/ncidod/EID/vol8no4/01-0490.htm. We regret any confusion this error may have caused.

Correction, Vol. 8, No. 2

In the article "Epidemiology of *Burkholderia cepacia* Complex in Patients with Cystic Fibrosis, Canada" by David P. Speert et al., an error was made in calculations for Table 2 on page 184. The corrected table appears below and online at http://www.cdc.gov/ncidod/eid/vol8no2/01-0163.htm.

In addition, the corrected percentages appear in two sentences from the results section on page 183, as follows: Most isolates (82.5%) were from genomovar III and included all strains that clustered in individual centers and appeared to be transmitted from patient to patient. Approximately 10% of infected patients were infected with *B. multivorans* (genomovar II), but there was little evidence among these isolates of genotypic clustering as determined by RAPD and PFGE.

We regret any confusion this error may have caused.

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

Species or genomovar	No. of patients infected with species or genomovar[a]	Percentage of patients (%)
Genomovar I	1	0.2
Burkholderia multivorans (genomovar II)	43	9.6
Genomovar III	369	82.5
Burkholderia stabilis (genomovar IV)	17	3.8
Burkholderia vietnamiensis (genomovar V)	7	1.6
Burkholderia cepacia complex (not genomovar I-VII)	8	1.8
Burkholderia fungorum	1	0.2
Burkholderia gladioli	5	1.1
Ralstonia pickettii	5	1.1
Pandoraea spp.	5	1.1
Total	461[a]	

[a]Some patients were counted twice if two or more different strains were recovered; therefore, the percentage of patients is based on a denominator of 447.

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.

About the Cover

Fable, c. 1600 (oil on canvas, 50 cm x 64 cm)

Domenikos Theodokopoulos (known as El Greco, 1541–1614). Courtesy of The Prado, Madrid, Spain.

The painting. El Greco's Fable is an enigmatic work of art whose meaning and date of origin have provoked much speculation among art historians.

In the center of the painting, a young boy (or perhaps a girl?) is blowing at a fire held with the left hand, trying to revive the flame to light a small candle held in the right hand. To the left, behind the youth, a chained monkey with an intelligent expression gazes attentively at the fire and also seems to be blowing at it. And to the right, the painting closes with the profile of a man wearing a bright red cap and a mocking grin on his bearded face.

El Greco painted more than one version of "the blower." An earlier version depicts only a young boy lighting a candle; a later version shows three figures (as seen here).

During his stay in Rome, El Greco moved in intellectual circles and studied literary sources, among them work by Pliny the Elder, a Roman writer from the first century A.D. Pliny's writings refer to a painter from antiquity remembered for his painting of a young boy blowing at a fire and for the light reflected from the fire on the boy's face and in the room. This text may have served as inspiration to El Greco.

Even though an occult humanistic meaning is possible, interpreting the version of the painting with only one central figure is not too much of a challenge; it could even be a study by the painter of the effects of artificial light on colors. On the other hand, in the version with the three figures, the expressions of the man and the monkey imply that El Greco may have intended to illustrate a fable or allegory, the meaning of which remains elusive.

About the painter. El Greco (meaning "the Greek") was born in Crete. Details of his early life and training are sketchy, but he probably studied painting in his youth. Although his early work has not survived, he probably painted in the late Byzantine style popular in Crete in his time. Reminders of the style are seen in his later works, which are all icons.

Around 1566, El Greco went to Venice, where he studied under Titian and was strongly influenced by Tintoretto, both masters of the High Renaissance. Further Italian inspiration came during the years the artist spent in Rome (1570–1576), where he met several Spaniards associated with the church in Toledo. The Christian doctrines of Spain had a great influence on his approach to painting—his art is filled with passion and restraint, religious fervor and Neoplatonism, as well as the mysticism of Counter-Reformation.

He arrived in Spain in 1577, possibly attracted by the chance of working on the decorations at Escorial. Although he did not produce any paintings there, his "Martyrdom of St. Maurice" is at the monastery. In the intellectual, passionate, and somewhat pessimistic milieu of Toledo, El Greco created a highly personal pictorial world in the Mannerist style, for which he was acclaimed as on the of the most original artists in his adopted country.

El Greco's later paintings (of which Fable is an example) exude a cultivated spirituality and feverish intensity and seem to pulsate with an eerie light generated by the figures themselves. He moved toward unusual colors, groupings, and figure proportions. The figures became increasingly elongated, and the canvases manifest "horror vacui," (dread of unfilled spaces). Subjects of classical mythology attest to El Greco's humanistic learning and his brilliantly personal and novel approach to traditional themes. The artist died in Toledo in 1614 and was buried there in Santo Domingo el Antiguo.

Sources: The Prado Museum, Madrid, Spain
http://sunsite.dk/cgfa/greco/greco_bio.htm
http://www.artchive.com/artchive/E/el_greco.html

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.

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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.

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Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

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Types of Articles

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.

Commentary. 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.

News and Notes. 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.) In this section, we also include summaries (500–1,000 words) of emerging infectious disease conferences. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.