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About the Cover

Landscape Transformation and Disease Emergence
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As of January 2005, summaries of emerging infectious disease conferences are published online only.
To understand the molecular basis of observed regional shifts in the genome types of adenovirus type 7 (Ad7) isolated in Korea during nationwide outbreaks from 1995 to 2000, the genetic variabilities of Ad7d and Ad7l were studied by sequence analysis of hexon, fiber, E3, and E4 open reading frame (ORF) 6/7 peptides. One amino acid change in the receptor-binding domain of fiber and 6 amino acid variations in E4 ORF 6/7 were identified between 2 genome types, while no variations were found in hexon and E3. Phylogenetic trees based on hexon, fiber, and E4 suggested that the Ad7 epidemic was probably caused by the introduction of the Japanese Ad7d strains. Our data also provide evidence that the rapid divergence of Ad7d to a novel genome type Ad7l could have been due to viral strategies involving multiple sequence changes in E4. This result suggests fiber and E4 ORF 6/7 peptides participate in the evolution of Ad7.

Adenovirus Type 7 Peptide Diversity during Outbreak, Korea, 1995–2000

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Korea. Genetic variations between the Ad7d and Ad7l genome types have been studied by analyzing the sequences of hexon, E3, fiber, and E4 ORF 6/7 peptides. Fiber and E4 ORF 6/7 peptides were chosen for study because of close proximity to a mutation site at 0.93 map units, and hexon and E3 peptides were chosen because of their reported genetic heterogeneities.

Methods

Virus Analysis

Twelve of the 98 Ad7 isolates obtained from Korean children with pneumonia from 1995 to 2000 were subjected to genetic analysis. Seven strains of genome type Ad7d and 5 strains of Ad7l were selected from various places and different times to represent epidemiologically unrelated strains (Table 1). Full-length adenoviral DNA was purified from infected HEP-2 cell lysates by using a modified Hirt procedure, as previously described (15). Genome types were assigned by restriction fragment analysis with 12 enzymes as in the previous study (9).

Sequence Analysis

The entire sequences of hexon, E3, fiber, and E4 ORF 6/7 peptides were determined. Adenoviral DNA (1–2 µg) was used as a template for sequencing. Sequencing primers were designed from GenBank reference sequences (GenBank accession no. AF053086 for the hexon gene; AF104383 for the E3, fiber, and E4 ORF 6/7 peptides). Nucleotide sequences were confirmed by duplicate reactions by using the primers shown in Figure 1.

Sequence analysis was performed by using a dideoxy chain termination reaction with BigDye Terminator (Applied Biosystems, Inc, Foster City, CA, USA), and run on an ABI 377 or ABI 3700 automated sequencer (Applied Biosystems, Inc). Data were analyzed with Sequencing Analysis v. 3.3 (Applied Biosystems, Inc). Chromatograms were imported into Sequencher 4.1.1 (Gene Codes Co, Ann Arbor, MI, USA) for assembly into contigs and for variation identifications. Both nucleotide and predicted amino acid sequences were aligned by using the ClustalW 1.4 method (http://www.ebi.ac.uk/clustalw/).

Nucleotide Sequence Accession Numbers

The sequences of hexon, E3, fiber, and E4 ORF 6/7 peptides of Korean strains were compared to those previously reported for Ad7 strains in GenBank. The sequences obtained during this study were registered with the GenBank database under the following accession numbers: the hexon gene, AY769945 for Ad7d and AY769946 for Ad7l; fiber and E4 ORF 6/7 peptides, AY921615 for Ad7p (Gomen strain), AY921616 for Ad7a (strain S-1058), AY921618 for Ad7d (strain 383), AY921620 for Ad7d (strain Bal), AY921621 for Ad7d (strain 95081), AY921622 for Ad7d (strain 98330), and AY921617 for Ad7l (strain 99095).

Phylogenetic Analysis

To understand the evolutionary process underlying the regional shift in the genome type of Ad7 in Korea, phylogenetic relationships were analyzed by using 1,428 bp making up nucleotides (nt) 297–1725 of the hexon gene. Another phylogenetic tree was generated based on 2,150 bp of fiber and E4 ORF 6/7 peptides. The phylogenetic trees constructed included sequences of hexon in 17 strains of Ad7 with diverse genome types and those of the fiber with E4 for only 7 strains, as available sequences were limited. Analysis was conducted by using MEGA version 2.0 (16). Kimura 2-parameters were used for the distance method, by using the neighbor-joining algorithm. Five hundred additional bootstrap analyses were performed on each phylogenetic tree.

Results

The complete sequence of the hexon (2,805 bp, 934 amino acids [aa]), E3 peptides (4,387 bp), the fiber gene (1,175 bp, 325 aa), and E4 ORF 6/7 peptides was successfully determined for 12 strains of Ad7.

Hexon Gene

No variations were found in the complete sequence of the hexon gene between the 7 strains of Ad7d and 5 of Ad7l isolated in Korea. Moreover, their sequences were identical to the previously published sequences of the Japanese Ad7d (AF053086, strain 383) and Ad7d2 (AF053087, strain Bal) strains. The Korean Ad7 isolates belonged to genome type cluster 2 based on the 2 hypervariable regions (14). Substitution of Leu with Gln at aa 440 of loop 2, which dramatically affects the hydrophobic character of this region, was also observed in 12 Korean
Ad7 strains. In terms of the 473 aa from position 100 to 572 on the hexon gene, 8 mutation sites were observed on comparing Korean Ad7 strains obtained from 1995 to 1999 and Chinese Ad7d isolates from 1981 to 1984 (Table 2). Ad7 strains with different genome types recently isolated from Korea and Japan shared identical sequences, whereas 4 Chinese Ad7d strains showed 8 aa changes within 1 genome type.

E3 Region

The sequencing of 4,387 nt of the E3 region demonstrated no nucleotide substitutions within 12 Korean Ad7 strains. Thus, no sequence differences associated with amino acid changes were found in the E3 region of Ad7d and Ad7l. In particular, the amino acid sequence of Ad7d and Ad7l was conserved at codon 89 in the ORF encoding a 14.9-kDa segment of the E3 region, where Gly was substituted with Ser in the Japanese strains of Ad7d2.

Fiber Gene

We analyzed 975 bp of the fiber gene. Two variations were detected in the amino acid sequences of fiber of Korean Ad7 versus previously reported Japanese Ad7 strains. These were located in the receptor-binding domain, the so-called knob region. Compared to the Japanese isolates of Ad7d and Ad7d2, all Korean Ad7 strains showed substitution of Ala to Val at codon 112. Within Korean Ad7, a change of Arg to Lys at codon 280 was observed in 4 strains of Ad7d and all Ad7l strains (Table 3). However, this variation was not correlated with genome type. Analysis of the hydrophobicity of this mutation site showed minimal influence on hydrophobic characters.

ORF 6/7 Peptides of E4

Nucleotide sequences were determined for the 33.2-kDa and 9.4-kDa peptides of the E4 ORF 6/7. On comparing 7 strains of Ad7d, 5 strains of Ad7l showed nucleotide substitutions at 18 sites, including a mutation site at 0.93 map units. Of these 18 sites, 6 resulted in amino acid changes as shown in Table 3. Nucleotide identity between Ad7d and Ad7l was 98.4%. Comparisons of the hydrophobicity plots of these 6 aa variations demonstrated small hydrophobic changes.

To exclude possibility that only the 12 isolates examined in the present study differed with respect to the residues of E4 ORF 6/7 peptides, an additional 28 isolates (15 of Ad7d and 13 of Ad7l) were also sequenced for this region. The sequencing results consistently showed identical nucleotide changes at the 18 sites in the Ad7d and Ad7l genome types.

Phylogenetic Relationships

To understand the distinct pattern of evolutionary relationship between Ad7 strains, phylogenetic relationships were inferred based on the sequences of hexon and fiber with E4 ORF 6/7 peptides (Figure 2). A phylogenetic tree based on the hexon sequence showed 2 distinct clusters, 1 of which subdivided into 2 lineages. Ad7 isolates from both Korea and Japan were clustered into the same lineage, regardless of genome type, since the sequences were identical. This cluster was distinct from other lineages, including the Chinese Ad7d and other genome types such as Ad7p, Ad7b, Ad7c, Ad7g, and Ad7h. Phylogenetic trees based on the fiber and E4 ORF 6/7 peptides demonstrated that genome type Ad7l showed remarkable changes within this region compared to the related genome types, Ad7d and Ad7d2.

Discussion

We identified previously unrecognized variations in the fiber gene and E4 ORF 6/7 peptides among the various genome types of Ad7. In particular, multiple amino acid changes at E4 ORF 6/7 peptides showed genome type–specific differences between Ad7d and a novel genome type Ad7l. However, no genetic divergence was identified in the hexon gene or in E3 peptides among the 12 Korean strains of Ad7. This result indicates that the nucleotide
structures of the fiber gene and of E4 ORF 6/7 peptides might have contributed to the genetic heterogeneity during the Ad7 epidemics in Korea, whereas those of the hexon gene and E3 were highly conserved.

So far, the fiber gene and E4 have rarely been addressed with regard to the evolution and molecular epidemiology of Ad7 strains (17). Restriction analysis of genomic DNA has been the most popular method of describing the molecular epidemiology of the adenoviruses (18). However, little is known about sequence variations with regard to the genome type within an epidemic or between the epidemics. Previous observations indicate that sequence variations of the hexon gene and of the E3 ORF encoding 14.9-kDa protein may contribute to genetic heterogeneity and the evolutionary process of Ad7 strains (6,14,19,20).

Alignment of the amino acid sequences of the hexon gene demonstrated 2 genetic clusters, GTC1 (Ad7p and Ad7p1) and GTC2 (Ad7a, Ad7b, Ad7c, Ad7d, Ad7d2, Ad7g, and Ad7h), based on variations in the hypervariable regions (14). Variations between 2 clusters have been observed in variable region 1 because of 3-aa length differences and in the variable region 2 because of a substitution from 440 Leu to Gln, the latter of which dramatically affected the hydropathic character of this region. Moreover, mutations of hexon of Ad could play an important role in new outbreaks of adenoviral infection (14). Compared to the Chinese Ad7d strains of the early 1980s, 1 aa substitution from Cys (or Tyr) to Phe at position 100 was consistently observed in all Korean isolates among 8 mutation sites. However, the Ad7d and Ad7l Korean strains shared the same hexon gene sequence as Japanese Ad7d and Ad7d2 in 1992 and 1995. Phylogenetic tree analysis based on the hexon sequences suggested that the Ad7 isolates from Korea and Japan cluster into the same lineage and that this cluster is distinct from those of Chinese Ad7d and other strains. Therefore, the Korean Ad7d epidemic that began in 1995 may have been caused by the introduction of isolates that were prevalent in Japan from 1987 to 1992.

Fiber is a major constituent of adenovirus outer capsid (21). Fiber protein consists of a trimeric projection terminated by a knob (head) (21,22), and it plays a crucial role in adenoviral infection by allowing, possibly by direct interaction, the virus to attach to specific receptors on the host cell surface (23). In addition, variability in this region is expected to account for the observed serologic difference between serotype 3 and 7 fibers (24). Two variation sites observed in Ad7 strains are located at the knob region of the fiber gene near its carboxy-terminal end. All Korean Ad7 strains showed a substitution of Ala to Val at codon

<table>
<thead>
<tr>
<th>Genome type</th>
<th>Strain name</th>
<th>Place of origin (y)</th>
<th>Accession no.</th>
<th>100 (166)</th>
<th>197 (200)</th>
<th>220 (233)</th>
<th>265 (268)</th>
<th>284 (287)</th>
<th>348 (351)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7p</td>
<td>Gomen</td>
<td>USA (1954)</td>
<td>Z48571†</td>
<td>Phe</td>
<td>Ile</td>
<td>Thr</td>
<td>Gly</td>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>7a</td>
<td>S-1058</td>
<td>USA (1955)</td>
<td>AFO53085</td>
<td>Phe</td>
<td>Ile</td>
<td>Thr</td>
<td>Gly</td>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>7d</td>
<td>BC3655</td>
<td>China (1981)</td>
<td>U77392</td>
<td>Cys</td>
<td>Ile</td>
<td>Thr</td>
<td>Gly</td>
<td>Ser</td>
<td>Gln</td>
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<tr>
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<td>BC4492</td>
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<td>Gly</td>
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<td>Gln</td>
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<tr>
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<td>Gly</td>
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<td>Gln</td>
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<td>BC8488</td>
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<td>Arg</td>
<td>Ala</td>
<td>Ser</td>
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<tr>
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<td>Japan (1992)</td>
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<td>Ile</td>
<td>Gly</td>
<td>Ser</td>
<td>Gln</td>
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<td>Gly</td>
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<tr>
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<td>Ile</td>
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<tr>
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<td>Korea (1999)</td>
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<td>Gly</td>
<td>Ser</td>
<td>Gln</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the amino acid positions of Ad7p, Gomen strain. The remaining 12 amino acid changes, which are specific to Gomen strain only, are not provided in this table.

†Complete genome of the Gomen strain is available at GenBank (accession no. AY594255).

Table 3. Comparison of amino acid sequences of fiber and E4 open reading frame (ORF) 6/7 peptides among adenovirus type 7 strains

<table>
<thead>
<tr>
<th>Genome type</th>
<th>Strain name</th>
<th>Accession no.</th>
<th>Amino acid position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber</td>
<td></td>
<td></td>
<td>104</td>
</tr>
<tr>
<td>33.2 KDa of E4</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>9.4 KDa of E4</td>
<td></td>
<td></td>
<td>12</td>
</tr>
</tbody>
</table>

*The remaining 5 amino acid changes, which are specific to Gomen strain only, are not provided in this table.

†Complete genome of the Gomen strain is available at GenBank (accession no. AY594255).
with or without a change of Arg to Lys at codon 280, unlike the Japanese strains. Therefore, the Korean Ad7d strains are thought to be closely related to the Japanese strains with changes in the receptor domain of the fiber gene. Changes in the receptor-binding domain could influence the interaction between fiber and the host cell and possibly influence genetic heterogeneity in different geographic locations.

The E4 region of human adenoviruses encodes a set of proteins that can regulate early gene expression for viral RNA export and stabilization (21,25). The functions of E4 ORF 6/7 peptides of Ad7 are unknown, but the corresponding protein of adenovirus type 5 has the ability to induce the binding of cellular transcription factor E2F to the viral E2a promoter region (26). E2F induced by ORF 6/7 peptides might facilitate adenoviral infection under more stringent environments, such as in the absence of the E1A gene products (27,28). Furthermore, the 33.2-kDa ORF 6 protein is known to promote cell cycle–independent adenovirus growth (29).

Nucleotide changes specific to a novel genome type Ad7l were observed at 18 sites in E4 ORF 6/7 peptides. These substitutions have not been previously reported in published E4 sequences of Ad7 isolates from other parts of the world. Although available sequences are limited, a phylogenetic tree based on the sequences of fiber and E4 ORF 6/7 peptides showed evidence that genome type Ad7l has experienced substantially more changes than Ad7d strains. This finding strongly suggests that the E4 region can be important to molecular diversity and evolution of the Ad7 strains. Interplay between the host cell and the E4 of Ad7 may contribute to the observed distinctive features of genome types.

A total of 270 adenoviruses were identified from childhood pneumonia patients in our children’s hospital during 10 years, 1990–2000. Of these 270 strains, Ad7d was not isolated until 1995, when explosive outbreaks occurred. Although we do not know the level of herd immunity to Ad7 at the beginning of epidemics, Ad7 had rarely circulated in Korea before the outbreak described in this study. Therefore, we assume that low immunity to Ad7 was critical to causing a large outbreak of Ad7. However, Noda et al. also reported that the mutation in the E3 of Ad7d strains could contribute to rapid spread during nationwide outbreaks in Japan (6).

As noted earlier, the nationwide outbreak of Ad7d in Korea in 1995 may have started with the introduction of the Japanese Ad7d strains, likely several years before 1995. To achieve a large outbreak, new Ad7d strains presumably required time to acquire greater access to hosts or the increased virulence acquired by fiber amino acid changes. However, a strain of novel genome type Ad7l was first detected in 1996, a year after the start of the Ad7d nationwide epidemic, spread rapidly during subsequent years, and then became the predominant genome type from 1998 to 2000. As reflected by their capacity to displace Ad7d, Ad7l might be more efficient than Ad7d for the dissemination in nonimmune hosts when a certain level of immunity was maintained. Whether the variations influence viral capacity for virulence and transmission during the epidemics is not currently understood; however, data shown in this study suggest that Ad7l was rapidly spread by multiple amino acid changes at fiber and E4 ORF 6/7 peptides during Ad7 outbreaks in Korea. This process might have been a consequence of viral adaptive strategies that allowed the virus to spread throughout Korea before the population had developed immunity.

In conclusion, the results presented here emphasize that fiber and E4 ORF 6/7 peptides may have roles in the evolutionary process and pathogenesis of Ad7 in Korea. The emergence of new genome types after the disappearance of a previously predominant type may be the result of type-specific host immune response or type-specific virulence.
perhaps mediated by amino acid variations in fiber or E4. However, the mechanisms underlying viral adaptive processes and interactions between virus and host have to be established by future study to allow us to more effectively counteract highly virulent genome types.

This research was supported in part by the Korean Ministry of Health and Welfare (grant HMP-99-M-04-002).

Dr. Eun Hwa Choi is a pediatric infectious diseases specialist at the Seoul National University Bundang Hospital in Korea. She is also an assistant professor at the Seoul National University College of Medicine. Her research interests focus on respiratory viruses with a particular emphasis on host genetic susceptibility to viral diseases.

References

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Risk Factors for Kala-Azar in Bangladesh

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Since 1990, South Asia has experienced a resurgence of kala-azar (visceral leishmaniasis). To determine risk factors for kala-azar, we performed cross-sectional surveys over a 3-year period in a Bangladeshi community. By history, active case detection, and serologic screening, 155 of 2,356 residents had kala-azar with onset from 2000 to 2003. Risk was highest for persons 3–45 years of age, and no significant difference by sex was seen. In age-adjusted multivariable models, 3 factors were identified: proximity to a previous kala-azar patient (odds ratio [OR] 25.4, 95% confidence interval [CI] 15–44 within household; OR 3.2 95% CI 1.7–6.1 within 50 m), bed net use in summer (OR 0.7, 95% CI 0.53–0.93), and cattle per 1,000 m² (OR 0.8, 95% CI 0.70–0.94). No difference was seen by income, education, or occupation; land ownership or other assets; housing materials and condition; or keeping goats or chickens inside bedrooms. Our data confirm strong clustering and suggest that insecticide-treated nets could be effective in preventing kala-azar.

Leishmania donovani is transmitted by the female sand fly, and humans are the only reservoir in South Asia (6). Blanket residual insecticide spraying decreased the incidence of kala-azar below detectable levels in India and Bangladesh by the 1960s (3), which suggests that sustained vector control could substantially reduce disease prevalence today. Efforts to control this neglected disease have recently gained momentum from the government of India’s commitment to eliminate kala-azar by the year 2010 (7). Nevertheless, data on the epidemiology of anthroponotic VL are sparse. To plan effective strategies for VL control and elimination, we must understand patterns of disease occurrence both at the community level and at broader geographic and ecologic levels. To elucidate the determinants at the community level, we studied spatial patterns and risk factors for kala-azar in a highly affected community in Bangladesh.

Methods

The study design was based on cross-sectional household surveys from January to April in 2002, 2003, and 2004. The surveys included leishmaniasis serologic studies and active kala-azar case detection. The study physician (M.A.) was present during the surveys and at regular intervals between surveys and offered free diagnosis for residents with suspected kala-azar; thus, additional ascertainment occurred between surveys. The protocol was approved by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) Research and Ethical Review Committees and the Institutional Review Board of the Centers for Disease Control and Prevention (CDC). Informed consent was obtained from all adult participants and a parent or guardian of all participating children. Assent was also obtained from children ≥7 years of age.

The study community is located in Fulbaria Thana, Mymensingh District, the “thana” (subdistrict) that has...
consistently reported the highest kala-azar incidence in Bangladesh since 2000. Community members identified the seasons as winter (October 15–March 15), summer (March 15–June 15), and rainy season (June 15–October 15). The community houses ≈12,000 people and is divided into 9 “paras” (sections) of ≈100 to 500 households. The paras are separated by 1 to 2 km and physically are much like separate villages, but politically they are considered parts of the same community. The study area comprised the 3 paras with the highest kala-azar rates during the previous several years according to villagers’ reports; these were designated paras 1–3 (8). The study included all members living in the study area for >6 months in the 3 years before the 2002 survey. Household questionnaires were used to collect data on births, deaths, inward and outward migration, socioeconomic factors, animal ownership, and house construction. Individual questionnaires focused on sleeping location, bed net use, and dietary practices.

Kala-Azar Case Ascertainment
During the 2002 survey, we attempted to retrospectively identify all kala-azar cases that had occurred in the study population. From January 2002 onward, ascertainment was prospective. Suspected kala-azar cases were identified through structured interviews by trained fieldworkers and referred to the study physician for complete medical history and physical examination. All seropositive persons were also examined. We defined a past case of kala-azar as an illness with >2 weeks of fever that resolved after 20 days of intramuscular injections and included a history of 1 or more of the following symptoms: weight loss, abdominal fullness, abdominal pain, or skin darkening. Patients who died with a disease consistent with kala-azar were also included; several patients died during treatment. While most patients could not identify the specific drug used, the 20-day course of injections corresponds to the sodium stibogluconate (SSG) regimen prescribed as first-line treatment by the national kala-azar management guidelines. We defined current kala-azar cases based on history and physical examination (symptoms as for past cases, plus splenomegaly or hepatomegaly, with or without measured fever or jaundice) and positive serologic test results. To confirm suspected kala-azar, we used the rK39 enzyme-linked immunosorbent assay (ELISA) during serosurveys and rK39 dipstick between serosurveys. The rK39 dipstick (Inbios International, Seattle, WA, USA) is a rapid test for kala-azar with very high sensitivity and moderately high specificity (9,10).

All persons with active kala-azar were referred to the Thana Health Complex located ≈1 km away. Patients with an atypical presentation or suspected relapse were referred to the district hospital for bone marrow aspiration and parasitologic confirmation. The study provided generic SSG (GlaxoWellcome-Bangladesh); each batch was tested by the International Dispensary Association (Amsterdam, the Netherlands) to ensure pharmacologic quality. Because of the ongoing SSG shortage in Bangladesh (8), the study provided SSG for all new kala-azar patients in the community, whether or not the patient lived in a study para.

Serologic Methods
Capillary blood specimens were collected from consenting participants ≥3 years of age. The serologic assay used recombinant K39 antigen (Corixa Corporation, Seattle WA, USA). In 2002, we used rK39 ELISA methods based on published protocols in which human antibodies reacting with plate-bound Leishmania donovani antigens were detected with horseradish-conjugated protein A (11). The positive cutoff was initially based on the mean optical density (OD) value of 4 wells of pooled negative control sera plus 3 standard deviations as described previously (11). Substantial plate-to-plate variation was seen in the negative control mean, and standard deviations were small; positive cutoff OD values were often close to the negative control mean. Therefore, to improve specificity for active kala-azar case confirmation, we used an alternative cutoff of 10 standard deviations (strong seropositive) for 2002 serosurvey data. We subsequently refined our methods to address these issues. For 2003 and 2004 surveys, we included a standard curve of dilutions of a pool of known positive sera and based our cutoff on concentration units from the standard curve for each plate (manuscript in preparation). We also used horseradish peroxidase–conjugated goat anti-human immunoglobulin (Ig) G, IgA, and IgM (Biosource International, Camarillo, CA, USA) because this reagent yielded better specificity than protein A conjugate. The negative cutoff was established by using serum specimens from persons from areas of Bangladesh not endemic for VL. Based on this assessment, we defined an ELISA reading of >60 concentration units as strongly seropositive.

Analytic Methods
All study households were mapped by Global Positioning System, and data were uploaded into ArcView Geographic Information System (GIS), v.3.3 (ESRI, Redlands, CA, USA). By using GIS data, distance was calculated from each household to the closest kala-azar case in the preceding year, and for multivariable modeling, to the closest case in any of the preceding years. To evaluate the effect of cattle (cows, oxen, or calves) on kala-azar risk for nearby residents, kernel density estimation was used to estimate cattle per 1,000 m². This index provides a smoothed measure of both proximity and number of cattle. Corresponding to where cattle were kept at night, the cattle shed was considered the center of gravity. If the house-
hold had no shed, house location was used based on the common practice of keeping cattle close to the house to prevent theft. A 50-m cell size was used to weight the analysis toward cattle in close proximity to a household, regardless of ownership.

Data were analyzed by using SAS 8.02 (SAS Institute Inc, Cary, NC, USA). Univariate and multivariable models were adjusted for within-household correlation by using Generalized Estimating Equations. Multivariable models were constructed by stepwise addition of variables significant at the p = 0.05 level in univariate analyses.

Results

A total of 2,439 persons in 506 households met the inclusion criteria. The surveyed population was 48% male and 52% female; median age was 18 years (range 0–80). Among those 20–29 years of age, 40% were male and 60% female; some adult men worked in Dhaka or Mymensingh and did not meet the inclusion criteria. For other age groups, the sex distribution was similar. The median household size was 5 persons (range 1–11).

During the 2002 serosurvey, 148 study participants had a history of kala-azar treatment, and active kala-azar was diagnosed in 16 patients. From the end of the 2002 survey through April 2004, active kala-azar was diagnosed in 49 additional participants, for a total of 65 prospectively ascertained kala-azar cases. In addition, probable relapses were diagnosed in 3 previously treated kala-azar patients; 6 previously treated patients were diagnosed with post–kala-azar dermal leishmaniasis. Altogether, we were able to reliably assign status with respect to kala-azar for 2,356 (97%) of 2,439 persons, of whom 213 (9%) had kala-azar or a history of kala-azar, 58 with onset before 2000 and 155 with onset in 2000 or later. Subsequent analyses focused on the 155 kala-azar patients with onset in 2000 or later because these data were considered the most reliable and complete.

The ELISA results were strongly positive for 33 (97%) of 34 prospectively ascertained kala-azar patients tested; the remaining patient had an ELISA reading of 57, just below the cutoff of 60 concentration units. The other 31 prospectively ascertained kala-azar cases were confirmed by rK39 dipstick (27 patients) or bone marrow aspirate (4 patients). Treated kala-azar patients also had strongly positive serologic test results that persisted for years after clinical recovery; 13 (24%) of 54 patients treated in 2001 and 13 (50%) of 26 patients treated in 2002 remained seropositive in 2004. Because so few patients had definitive parasitologic diagnosis, a formal analysis of serology performance characteristics was not performed.

Para 1 had a higher cumulative incidence (77 [14%] of 540) than para 2 (39 [3%] of 1,221) or para 3 (39 [7%] of 537, p<0.01 for all three 2-way comparisons). Kala-azar incidence peaked in 2001: 47 residents had illness onset in 2000, 57 in 2001, 23 in 2002, and 28 in 2003. No marked seasonal pattern was apparent by onset month (Figure 1A). Because ascertainment of cases with onset in 2003 was incomplete at the time of analysis, we combined quarterly data from January 2000 to December 2002 (Figure 1B). The quarterly analysis showed a trend for more cases to have onset from July to September and fewer cases from January to March (goodness-of-fit $\chi^2 = 5.63$, p = 0.13). The incidence was slightly higher in men than women (p = 0.27) and was higher among children and young adults than in the youngest and oldest age groups (p=0.01) (Table 1). The median symptom duration before treatment was 4.0 months. Among patients treated before the study began, the duration was longer for female patients (5.0 months) than male patients (3.0 months, p = 0.09). After we began active case finding, this trend disappeared (3.5 and 4.0 months for female and male patients, respectively, p = 0.82). The case-fatality rate was 9% (14/155), 14% among female patients and 5% among male patients (relative risk 2.7, p = 0.07).

From 2000 to 2003, kala-azar cases spread from a highly clustered pattern to one in which substantial sections of
the study village were saturated (Figure 2). Kala-azar risk was significantly higher among those living in the same household as or within 50 m of a kala-azar patient in the previous year (p = 0.0003 for closest patient in 1999 as predictor of kala-azar in 2000, p<0.0001 for closest patient in 2000 as predictor in 2001). By 2002, the pattern had disseminated so that the difference in incidence based on proximity was no longer significant in the overall study population (p = 0.12). In 2000, 21% of the study population lived within 50 m of a patient in the previous year; this figure rose to 37% in 2001 and 53% in 2002. When cumulative incidence during the study period was considered, 72% of the population lived within 50 m of a kala-azar case by 2003; in para 1, the proportion was 84% (Figure 2).

A number of other factors were associated with altered kala-azar risk (Table 2). The risk of kala-azar was highest for people in the 3- to 14-year and 15- to 45-year age groups. Consistent use of a bed net, especially in summer, was strongly protective (Table 2). The nets in use were not treated with insecticide and were locally produced. Other factors related to bed nets, such as net use in winter, household net ownership, and having ≥1 net per 3 household members, were associated with weaker levels of protection (Tables 2 and 3). Overall, 91% of households owned at least 1 net, and 87% of participants reported sleeping under a net at least some of the time.

We examined the effect of both cattle ownership and cattle density on kala-azar risk. Household cattle ownership was associated with lower risk, but this finding did not reach significance (p = 0.18, Table 3). However, the kernel density analysis demonstrated a significant protective effect for increasing cattle density: the mean density around the houses of persons without kala-azar was 1.4 cows/1,000 m² compared to 1.1 cows/1,000 m² for kala-azar patients (odds ratio 0.75, 95% confidence interval 0.62–0.92, p = 0.005). A dose-response relationship was seen; with 0 cows/1,000 m² as the referent, <1 cow/1,000 m² was associated with a 30% decrease, 1.1–2 cows/1,000 m² with 40% decrease, and >2 cows/1,000 m² with 43% decrease in kala-azar risk.

No difference was seen in kala-azar risk by income, education, or occupation; assets such as land, livestock, farm implements, radio, or cart; housing materials and condition; dietary intake of selected foods; keeping live-

### Table 1. Cumulative incidence of kala-azar (KA) from January 2000 to December 2003 in a Bangladeshi community*

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Male</th>
<th>Female</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>KA patients (%)</td>
<td>n</td>
</tr>
<tr>
<td>&lt;3</td>
<td>113</td>
<td>3 (2.7)</td>
<td>103</td>
</tr>
<tr>
<td>3–14</td>
<td>396</td>
<td>40 (10.1)</td>
<td>425</td>
</tr>
<tr>
<td>15–45</td>
<td>462</td>
<td>33 (7.1)</td>
<td>540</td>
</tr>
<tr>
<td>&gt;45</td>
<td>127</td>
<td>5 (3.9)</td>
<td>123</td>
</tr>
<tr>
<td>All</td>
<td>1,098</td>
<td>81 (7.4)</td>
<td>1,191</td>
</tr>
</tbody>
</table>

*Patients with onset before 2000 (n = 58) were excluded.
insecticide spraying in the last 5 years (Tables 2 and 3).

In the final multivariable model, 4 factors remained significant. Age from 3 to 45 years and proximity to a previous patient increased kala-azar risk, while higher numbers of cattle per 1,000 m² and consistent use of a bed net in summer were associated with protection (Table 4).

Discussion

This study is the first to examine spatial patterns and risk factors for anthroponotic VL in Bangladesh. Although we were not surprised that proximity to previous cases was a determinant of subsequent kala-azar risk, the strength of the association was remarkable. The 26-fold increase in risk for those living with a patient reflects the role of active kala-azar patients as the predominant infection reservoir. In this study, the mean duration of illness before treatment was 4 months; this delay provides ample opportunity for sand flies that feed inside the house to become infected and transmit disease. The risk associated with kala-azar patients within 50 m but not farther may reflect the relatively limited flight range of sand flies.

To present data applicable to regional control efforts, we chose to focus our analysis on risk factors for kala-azar, the form of VL reported in surveillance data and the predominant target of public health programs. In this analysis, we did not distinguish between uninfected participants and those with possible subclinical leishmanial infection. For this reason, we may have underestimated the strength of association for factors that alter risk of both leishmanial

Table 2. Individual-level factors associated with kala-azar (KA) in univariate analyses adjusted for household clustering, Bangladesh, 2000–2003

<table>
<thead>
<tr>
<th>Factor</th>
<th>N*</th>
<th>Cumulative KA incidence, n (%)</th>
<th>OR (95% CI)†</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>216</td>
<td>4 (1.9)</td>
<td>0.78 (0.22–2.78)</td>
<td>0.71</td>
</tr>
<tr>
<td>3–14</td>
<td>821</td>
<td>71 (8.7)</td>
<td>3.99 (1.77–8.99)</td>
<td>0.0008</td>
</tr>
<tr>
<td>15–45</td>
<td>1,002</td>
<td>74 (7.4)</td>
<td>3.28 (1.48–7.27)</td>
<td>0.0034</td>
</tr>
<tr>
<td>&gt;45</td>
<td>250</td>
<td>6 (3.9)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1,196</td>
<td>74 (6.2)</td>
<td>0.84 (0.62–1.15)</td>
<td>0.29</td>
</tr>
<tr>
<td>Male</td>
<td>1,102</td>
<td>81 (7.4)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Sleeps on ground</td>
<td>150</td>
<td>9 (6.0)</td>
<td>0.99 (0.51–1.90)</td>
<td>0.97</td>
</tr>
<tr>
<td>Sleeps on bed or cot</td>
<td>1,849</td>
<td>123 (6.7)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Always uses net in summer</td>
<td>1,769</td>
<td>96 (5.4)</td>
<td>0.43 (0.29–0.64)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Uses net sometimes or never in summer</td>
<td>370</td>
<td>46 (12.4)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Always uses net in winter</td>
<td>754</td>
<td>30 (4.0)</td>
<td>0.49 (0.33–0.73)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Uses net sometimes or never in winter</td>
<td>1,385</td>
<td>112 (8.1)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Always uses net in rainy season</td>
<td>1,277</td>
<td>76 (6.0)</td>
<td>0.79 (0.54–1.17)</td>
<td>0.24</td>
</tr>
<tr>
<td>Uses net sometimes or never in rainy season</td>
<td>862</td>
<td>66 (7.7)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Uses bed net ever</td>
<td>1,849</td>
<td>109 (5.9)</td>
<td>0.51 (0.32–0.80)</td>
<td>0.0033</td>
</tr>
<tr>
<td>Never uses bed net</td>
<td>290</td>
<td>33 (11.4)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Distance from previous KA patient</td>
<td>468</td>
<td>72 (15.4)</td>
<td>35.7 (21.1–60.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;50 m</td>
<td>1,178</td>
<td>67 (5.7)</td>
<td>3.51 (1.89–6.51)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;50 m</td>
<td>652</td>
<td>16 (2.5)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Eats beef at least twice a month</td>
<td>711</td>
<td>49 (6.9)</td>
<td>1.1 (0.76–1.59)</td>
<td>0.62</td>
</tr>
<tr>
<td>Eats beef less than once a month</td>
<td>1,299</td>
<td>83 (6.4)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Eats goat at least twice a month</td>
<td>206</td>
<td>9 (4.4)</td>
<td>0.59 (0.24, 1.43)</td>
<td>0.24</td>
</tr>
<tr>
<td>Eats goat less than once a month</td>
<td>1,804</td>
<td>123 (6.8)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Eats fish daily</td>
<td>1,402</td>
<td>96 (6.9)</td>
<td>1.25 (0.60–2.14)</td>
<td>0.33</td>
</tr>
<tr>
<td>Eats fish less than daily</td>
<td>610</td>
<td>36 (5.9)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Eats chicken at least twice a month</td>
<td>437</td>
<td>34 (7.8)</td>
<td>1.34 (0.86–2.11)</td>
<td>0.2</td>
</tr>
<tr>
<td>Eats chicken less than once a month</td>
<td>1,567</td>
<td>98 (6.3)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Goats kept in sleeping room</td>
<td>248</td>
<td>14 (5.7)</td>
<td>0.87 (0.48–1.57)</td>
<td>0.64</td>
</tr>
<tr>
<td>No goats in sleeping room</td>
<td>1,705</td>
<td>108 (6.3)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Chickens kept in sleeping room</td>
<td>1,269</td>
<td>80 (6.3)</td>
<td>1.04 (0.68–1.60)</td>
<td>0.85</td>
</tr>
<tr>
<td>No chickens in sleeping room</td>
<td>681</td>
<td>42 (6.2)</td>
<td>Referent</td>
<td></td>
</tr>
</tbody>
</table>

*Existing values for each variable.
†OR, odds ratio; CI, confidence interval.
infection and disease by including infected persons in our control group. At the same time, factors that may alter the risk of progression from infection to disease were not examined. For example, immunogenetic factors play a role in determining whether people infected with *Leishmania infantum* (*chagasi*) progress to clinical disease (12). Similar genetic factors are likely to affect progression of L. donovani and may contribute to the household clustering in our data. Poor nutritional status may also alter risk of progression (13). Although our analysis showed no significant risk variation with socioeconomic or dietary indicators, poor nutritional status may vary by household resources and practices, contributing to the high risk for members of kala-azar households.

Previous studies in South Asia demonstrated associations between kala-azar and poverty (14,15). However, the facility-based survey in India and case-control study in Nepal compared kala-azar patients to the general population, whereas in the current analysis we studied a relatively homogeneous, high-risk population. Our data showed no differences in risk by occupation, income, housing type, or assets aside from bed nets. A community-based study of kala-azar in India demonstrated an association with agricultural occupation, but like our study, no association between kala-azar and household income levels (16). The primary occupation in the Indian village was weaving; <10% of villagers were farmers. In contrast, 66% of our population lived in agricultural households. The lack of significance of socioeconomic factors may reflect the relative homogeneity of our study population and the fact that at the community level, more proximate factors determine kala-azar risk.

Our findings with respect to cattle answer a question raised by the Nepal case-control study (15). In that study, owning cattle or buffaloes conferred a strong protective effect. However, because of the study design, it was impossible to distinguish among 3 potential explanations for this effect: socioeconomic confounding, better nutritional status leading to decreased progression to kala-azar, and the role of bovines as a preferred sand fly bloodmeal source. The finding that cattle ownership was not as important as cattle density strongly suggests that cows decrease *leishmaniasis* transmission by sand flies in their immediate vicinity. Sand fly bloodmeal analysis in India confirms that *Phlebotomus argentipes* feed predominantly on bovines, with humans as their second choice (17). The proximity of cattle may diminish disease transmission by enabling sand flies to feed preferentially on animals not susceptible to

### Table 3. Household-level factors associated with kala-azar (KA) in univariate analyses adjusted for household clustering, Bangladesh, 2000–2003

<table>
<thead>
<tr>
<th>Household characteristic</th>
<th>N*</th>
<th>Cumulative KA incidence, n (%)</th>
<th>OR (95% CI)†</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farmer or farm laborer</td>
<td>1,459</td>
<td>99 (6.6)</td>
<td>0.99 (0.78–1.27)</td>
<td>0.97</td>
</tr>
<tr>
<td>Other occupation</td>
<td>753</td>
<td>49 (6.5)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Monthly income &gt;$10 per person</td>
<td>1,096</td>
<td>67 (6.1)</td>
<td>0.82 (0.55–1.22)</td>
<td>0.33</td>
</tr>
<tr>
<td>Monthly income &lt;$10 per person</td>
<td>1,112</td>
<td>81 (7.3)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Owns &gt;0.2 acres of land</td>
<td>1,186</td>
<td>80 (6.8)</td>
<td>1.06 (0.72–1.58)</td>
<td>0.76</td>
</tr>
<tr>
<td>Owns &lt;0.2 acres of land</td>
<td>1,026</td>
<td>68 (6.6)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Owns a bed net</td>
<td>2,012</td>
<td>125 (6.2)</td>
<td>0.58 (0.35–0.96)</td>
<td>0.033</td>
</tr>
<tr>
<td>Does not own a bed net</td>
<td>198</td>
<td>21 (10.6)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>≤3 household members per net</td>
<td>924</td>
<td>41 (4.4)</td>
<td>0.53 (0.35–0.79)</td>
<td>0.0023</td>
</tr>
<tr>
<td>&gt;3 household members per net</td>
<td>1,374</td>
<td>114 (8.3)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Owns bicycle</td>
<td>186</td>
<td>10 (5.4)</td>
<td>0.71 (0.36–1.42)</td>
<td>0.33</td>
</tr>
<tr>
<td>Does not own a bicycle</td>
<td>2,026</td>
<td>138 (6.8)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Owns radio</td>
<td>497</td>
<td>24 (4.8)</td>
<td>0.65 (0.39–1.08)</td>
<td>0.1</td>
</tr>
<tr>
<td>Does not own a radio</td>
<td>1,715</td>
<td>124 (7.2)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Head of household can read</td>
<td>652</td>
<td>48 (7.4)</td>
<td>1.2 (0.78–1.83)</td>
<td>0.41</td>
</tr>
<tr>
<td>Head of household cannot read</td>
<td>1,560</td>
<td>100 (6.4)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Keeps cattle</td>
<td>1,050</td>
<td>67 (6.4)</td>
<td>0.89 (0.75–1.06)</td>
<td>0.18</td>
</tr>
<tr>
<td>Keeps no cattle</td>
<td>912</td>
<td>63 (6.9)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Roof made of tin</td>
<td>1,441</td>
<td>93 (6.5)</td>
<td>1.12 (0.70–1.79)</td>
<td>0.64</td>
</tr>
<tr>
<td>Roof made of thatch or mixed</td>
<td>511</td>
<td>32 (6.3)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Earthen walls</td>
<td>1,689</td>
<td>113 (6.7)</td>
<td>1.81 (0.77–4.21)</td>
<td>0.17</td>
</tr>
<tr>
<td>Bamboo, tin, or concrete walls</td>
<td>269</td>
<td>12 (4.5)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Damp floor</td>
<td>1,607</td>
<td>103 (6.4)</td>
<td>1.17 (0.59–2.31)</td>
<td>0.65</td>
</tr>
<tr>
<td>Dry floor</td>
<td>346</td>
<td>19 (5.5)</td>
<td>Referent</td>
<td></td>
</tr>
</tbody>
</table>

*existing values for each variable.

†OR, odds ratio; CI, confidence interval.
leishmaniasis, thereby decreasing sand fly parasite acquisition, feeding on humans, or both.

Remarkably, untreated, locally available bed nets were associated with a 30% decrease in kala-azar risk in our multivariable model. Because the usual incubation period for kala-azar is 2–6 months (18), the strong protective effect of net use in March–June and the higher kala-azar incidence in the third quarter of the year are consistent with high transmission in the Bangladeshi summer. Nevertheless, some transmission probably occurs year-round, except for December–January when almost no sand flies are active. In addition, the extremes of the incubation period are highly variable, with a reported range from 10 days to >2 years (18,19), making strict seasonal correlation difficult.

The protective effect of untreated nets in this analysis is consistent with findings of the Nepal kala-azar case-control study (15). Intervention trials of insecticide-treated materials for anthroponotic cutaneous leishmaniasis in Afghanistan demonstrate strong protective efficacy (20), and the high rate of use in our data suggests that bed nets are already highly acceptable in VL-endemic communities. Indeed, at the community’s request, insecticide-treated nets were distributed to the population when the study ended. These findings highlight the promise of insecticide-treated nets as a VL control measure that could be implemented and sustained through community action (8). Our findings suggest that in VL-endemic areas where treated nets are not yet available, untreated nets should be used whenever possible.

The strong spatial clustering we found suggests that targeted vector-control efforts, such as spraying to the most affected foci, could be effective if they are instituted before the transmission pattern generalizes. Furthermore, our data suggest that generalization may occur within 2 to 3 years when transmission is intense. Thus, rapidity of response may be key to the success of a targeted intervention. The several-year delays that commonly occur before spraying in affected communities may help explain the low efficacy of current targeted spraying programs in South Asia. The incompleteness of kala-azar surveillance data may be another factor. Nonetheless, better disease control might be achieved through improved kala-azar surveillance systems that integrate a rapid, targeted, vector-control response mechanism. In addition, combining government-run spraying programs with community-level efforts to increase insecticide-treated net use could enhance vector control.

Maintenance of adequate kala-azar diagnostic and treatment facilities at the peripheral level will also be essential (8). Rapid diagnostic tests such as the rK39 dipstick and the direct agglutination test now make diagnosing most kala-azar cases possible without invasive procedures (21). Two new antileishmanial drugs, miltefosine and paromomycin, are or soon will be available in India, where antimonial drug resistance presents a major challenge to control (22). Both have advantages over currently used antileishmanial drugs, miltefosine because of its oral administration and paromomycin because of its excellent safety profile. The low rate of relapse in our study suggests that most VL in Bangladesh is still responsive to SSG. Nevertheless, if miltefosine and paromomycin can be made available in Bangladesh and Nepal at affordable prices, treatment could be made simpler and more effective. The resurgence of kala-azar in South Asia since 1990 has raised policymakers’ awareness of this historically neglected disease, which suggests that the political will may finally exist to address VL in a concerted fashion (23). The time has come to mount an aggressive, integrated effort to control anthroponotic visceral leishmaniasis.

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References


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Enzootic strains of Venezuelan equine encephalitis virus (VEEV) circulate in forested habitats of Mexico, Central, and South America, and spiny rats (*Proechimys* spp.) are believed to be the principal reservoir hosts in several foci. To better understand the host-pathogen interactions and resistance to disease characteristic of many reservoir hosts, we performed experimental infections of F1 progeny from *Proechimys chrysaeolus* collected at a Colombian enzootic VEEV focus using sympatric and allopatric virus strains. All animals became viremic with a mean peak titer of 3.3 log10 PFU/mL, and all seroconverted with antibody titers from 1:20 to 1:640, which persisted up to 15 months. No signs of disease were observed, including after intracerebral injections. The lack of detectable disease and limited histopathologic lesions in these animals contrast dramatically with the severe disease and histopathologic findings observed in other laboratory rodents and humans, and support their role as reservoir hosts with a long-term coevolutionary relationship to VEEV.

Venezuelan equine encephalitis (VEE) is an emerging disease that affected humans and equines in many parts of the Americas throughout the 20th century (1–6). The etiologic agent is VEE virus (VEEV), a positive-sense RNA virus in the family *Togaviridae* and genus *Alphavirus*. The first strain of VEEV was isolated and characterized serologically in 1938 (7,8). Numerous VEEV strains and closely related alphaviruses have since been classified into 2 epidemiologic groups: enzootic and epizootic strains. Enzootic strains (subtypes I, varieties D-F, and subtypes II-VI) are regularly isolated in lowland tropical forests in Florida, Mexico, and Central and South America, where they circulate between *Culex* (*Melanoconion*) spp. mosquito vectors and small rodents; these strains are generally avirulent for and incapable of amplification in equines (4, 9, 10). In contrast, epizootic VEEV strains (subtype I, varieties A-B and C), which are responsible for all major outbreaks in humans and equines, use several mosquito vectors and equines, which are exploited as highly efficient amplification hosts (11, 12). Epizootic viruses cause debilitating disease with high fatality rates in equines. Humans are tangential, spillover hosts in both epidemic and enzootic VEEV cycles and are affected by most strains. A severe febrile illness that can occasionally be life-threatening develops; although human death occurs in <1% of infections with enzootic and epizootic VEEV strains, neurologic sequelae occur in survivors, particularly children (13).

Reservoir hosts play an important role in the replication, maintenance, and dissemination of arthropodborne viruses (arboviruses). These hosts generally show little or no disease after infection, presumably reflecting long-term selection for host resistance and possibly for virus attenuation (14, 15). Changes in the habitats and ecology of reservoirs due to anthropogenic or natural causes can affect pathogen transmission to humans and domestic animals (16–18). Therefore, understanding how pathogens affect reservoir fitness, as well as how the reservoir affects pathogen replication and transmission, could facilitate prediction of emergence, reemergence, or extinction of sylvatic pathogens in response to environmental changes including deforestation. A better understanding of pathogen-reservoir interactions, particularly mechanisms of disease resistance, may also enhance the development of treatments for humans and domestic animals.

Field studies in Panama have identified antibodies to VEEV in many different species of mammals, including *Proechimys* spp. (spiny rats), *Sigmodon* spp. (cotton rats), *Marmosa* spp. (mouse opossums), *Didelphis marsupialis* (opossums), and *Chiroptera* (bats) (1, 19–22). However, *Proechimys* spp. (family *Echimyidae*) and *Sigmodon* spp. (family *Muridae*) are thought to be principal reservoir hosts.
hosts for enzootic strains because they are infected in nature, have high rates of immunity, and viremia develops in them after laboratory infection (20,23,24). These 2 rodents have different, but overlapping, geographic distributions; Proechimys spp. are found in Panama, northern Peru, Bolivia, Paraguay, and southern Brazil, whereas Sigmodon spp. are found from southern North America to northern parts of Venezuela and Peru. Proechimys spp. can be abundant in their forested habitats (25). They have a gestation period of 60 to 70 days and give birth to 2 to 3 pups per litter. Their natural life expectancy is ≈20 months and can exceed 2 years.

The relationships between rodent reservoir hosts and VEEV have received little study. Spiny rats (Proechimys semispinosus) captured in a VEEV-enzootic region of Panama exhibited ≈67% seropositivity (1). When spiny rats were infected with a local subtype ID VEEV strain, high-titer viremia developed, suggesting their role as reservoir hosts. Antibody was detectable by day 3 and persisted for up to 9 months (20). The role of spiny rats as VEEV reservoirs was reinforced by a study in Colombia and Venezuela in which a correlation was established between the abundance of these rodents and levels of enzootic circulation (10). Experimental infections of Sigmodon spp. also support their role as reservoir hosts, and horizontal transmission has been demonstrated among cage mates (20,23,24). However, none of these studies of spiny and cotton rats has investigated the clinical or histopathologic manifestations of VEEV infection in these reservoir rodents.

We examined interactions between VEEV isolates from an enzootic focus in the Middle Magdalena Valley of Colombia (10) and sympatric P. chrysaeolus. The lack of detectable disease and limited histopathologic effects on these animals contrast dramatically with the severe disease and histopathologic changes observed in other laboratory rodents and humans, and support their role as reservoir hosts with a long-term coevolutionary relationship to VEEV.

Materials and Methods

Animals

P. chrysaeolus (spiny rats) were obtained from a colony established at the Instituto Nacional de Salud, Bogotá, Colombia, from adults captured in the Monte San Miguel Forest in the Middle Magdalena Valley (10). The rats were identified using mitochondrial DNA sequence analysis and karotyping (J. Patton, University of California, Berkeley, CA, pers. comm.) (26). Male and female F1 offspring 3–36 months of age were used for experimental infections. The animals were housed in conventional rat cages and fed laboratory rat chow. All animals were tested for neutralizing antibodies against VEEV, and seronegative animals were infected in accordance with animal care and use guidelines of the Instituto Nacional de Salud. Organs were fixed for 48 h in 4% buffered formalin, embedded in paraffin, sectioned (5 µm), and stained with hematoxylin and eosin.

Viruses

Enzootic subtype ID VEEV strain Co97-0054 was isolated in 1997 from a sentinel hamster in the same Colombian forest where the spiny rats originated (10). This virus was passaged once in baby hamster kidney 21 cells before animal inoculations. Enzootic strain 66637 was isolated in 1981 from a sentinel hamster in Zulia State, Venezuela (27), and had 1 passage in suckling mouse cells and 1 passage in African green monkey kidney (Vero) cells.

Infections

Before infection, the animals were weighed and their body temperature was measured rectally. Animals were injected by the subcutaneous (SC) route into the left footpad with 3 log10 PFU/mL of virus in a 50-µL volume, a dose consistent with alphavirus saliva titers in mosquitoes (28).

Virologic and Histologic Tests

Infected animals were bled and weighed daily following ether anesthesia, and their body temperatures were recorded on days 1 to 4 and day 7. Blood samples were also collected from some animals at 1 month, 15 months, or both, postinfection. Blood was diluted 1:10 in Eagle minimal essential medium supplemented with 20% fetal bovine serum, gentamicin, and L-glutamine, and stored at –80°C. Viremia and levels of neutralizing antibodies were determined by plaque assay and 80% plaque reduction neutralization tests using Vero cells. For histologic analyses, 4 animals from days 1 to 4 postinfection and 2 from day 7 were killed and organs were collected. Samples containing virus and viral RNA from the heart, brain, liver, and kidneys of 2 animals killed on each of days 1–4 were homogenized and centrifuged for 10 min at 5,760 × g, and the supernatant fluids were stored at –80°C before virus titration or RNA extraction.

Neurovirulence Studies

Four animals were injected by the intracranial (IC) route with 3 log10 PFU of virus strain Co97-0054 following anesthesia with ketamine/xylazine (50/5 mg/kg). Animals were monitored for signs of illness, including loss of activity, ruffled coat, dehydration, anorexia, and neurologic disorder (erratic movements of legs or head), and bled daily for 4 days for viremia titration and on day 15 to determine seroconversion.
Isolation and Amplification of RNA

RNA was extracted from triturated tissues with Trizol LS (GIBCO, Grand Island, NY, USA) following the manufacturer’s protocol. DNA primers (5’-CGACA-GAAAACAGCAGACCTTG-3’, reverse primer: 5’-TCTAAGATGACGGCCTGTC-3’) were designed to amplify the VEEV genome at positions 8431–8677. cDNA was obtained by combining 1 µg of RNA in 10 mmol/L of dithiothreitol, 20 nmol/L of deoxynucleotide triphosphates (dNTPs), 4 µL of 5× first-strand buffer, 40 U of RNAse inhibitor, 100 ng/µL of minus sense primer, and water to give a final volume of 20 µL, and incubated for 2 min at 40°C. Two hundred units of reverse transcriptase (Superscript, GIBCO) were added, and the sample was incubated overnight at 42°C. A polymerase chain reaction (PCR) included 1 µg of cDNA, 300 ng of each primer, 10 µL of 10× Taq buffer (GIBCO), 1.25 mmol/L of Mg2+, 20 nmol/L of dNTPs, 5 U of Taq enzyme (GIBCO), and water to a final volume of 100 µL. Thirty cycles were performed, including denaturation at 95°C for 1 min, annealing at 59°C for 30 s, and extension at 72°C for 2 min, followed by a 10-min extension time at 72°C. PCR products were analyzed by electrophoresis on a 1% agarose gel, and DNA products were purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). PCR products were sequenced by using the sense primer and the ABI PRISM Big Dye Terminator v3.0 kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s recommendations.

Statistical Analyses

Linear regression was used to analyze the influence of age on peak level of viremia. One-way analysis of variance was used to analyze tissue and viremia data. Body temperature differences were analyzed using the SAS/STAT procedure MIXED for repeated measures (SAS/STAT Users Guide, Volumes 1, 2, and 3, Version 8, SAS Institute, Cary, NC, USA).

Results

Clinical Manifestations

Twenty rats were infected with the enzootic ID strain Co97-0059, and 2 rats were infected with the enzootic strain 66637. All animals were monitored twice a day for clinical signs of VEEV infection, and none showed any signs of disease or discomfort, abnormal activity, disturbed social behavior, or loss of appetite, as compared with the uninfected control. No significant changes in body temperature were observed (p>0.25; data not shown). The 3 animals injected IC with strain Co97-0054 survived and showed no signs of illness. Of all animals infected either SC or IC (n = 27), only 1 showed clinical signs (hyperthermia) and died on day 3 postinfection. Whether death was due to VEEV or another cause is not clear. Histopathologic results from this animal are presented below. Viral titers in this animal were similar to those of others infected with the same virus.

Viral Replication and Tissue Tropism

In animals infected SC with the sympatric enzootic strain Co97-0054 peak viremia levels of up to 3.3 log$_{10}$PFU/mL developed 24 h postinfection, but the virus was undetectable by day 4 (Figure 1). Similar viremia levels, and no detectable disease occurred in 3 spiny rats infected by the bite of an infectious mosquito (data not shown). No correlation between peak viremia titer and age could be established (n = 28, p = 0.08, R$^2$ = 0.11, slope = −0.1). Animals infected with the Venezuelan enzootic ID strain 66637 exhibited a viremia level 1 log$_{10}$PFU/mL higher viremia, with a delayed mean peak of 4.8 log$_{10}$PFU/mL on day 2 postinfection; this difference in titer between virus strains was significant (p = 0.001). Clearance of the virus occurred at the same time, by 4 days postinfection (Figure 1).

Virus titrations were performed on the spleen, heart, brain, liver, and kidneys from 2 animals on each of days 1 to 4 postinfection. Only the spleen had detectable levels of virus on days 1 (mean 2.6 log$_{10}$PFU/mg) to 4 (mean 3.9 log$_{10}$PFU/mg), with a peak on day 3 (4.1 log$_{10}$PFU/mg) (Figure 2). Peak virus titers in the spleen were reached 2 days later than with peak viremia level, and viremia was undetectable by day 4 when virus could still be isolated from the spleen. This finding suggests that the spleen is a major site of viral replication or clearance from the circulation after replication elsewhere. None of the other organs had detectable infectious virus during the peak viremia phase.

Figure 1. Viremia in spiny rats after subcutaneous infection with 3 log$_{10}$ PFU of the enzootic Venezuelan equine encephalitis virus strains Co97-0054 and 66637. Vertical bars represent standard errors of the means. **p = 0.001.
Because no VEEV was detected in organs other than the spleen, RNA extractions and reverse transcription–PCR, a more sensitive assay, were performed. The detection limit of the PCR was estimated at 0.3–1.3 PFU when virus stocks of known titer were used (29), while the detection limit of the plaque assay with the volumes available was 1.6 log₁₀ PFU/mL. Viral RNA was detected in all organs tested, and sequencing of the PCR amplicons confirmed the presence of VEEV strain Co97-0054. However, detection of viral RNA does not necessarily indicate the presence of replicating virus in the organs because the animals were not perfused, and residual virus could have been present in blood.

Animals injected IC with the subtype ID strain had a peak viremia level 48 h postinfection, with titers from 2.6 to 3.2 log₁₀ PFU/mL. Unlike SC infection, IC infection resulted in viremia levels for only 24 to 48 h (Table). The lack of detectable disease in these animals indicates that VEEV is not neurovirulent for these reservoir hosts.

Antibody Development

Neutralizing antibodies developed in all animals by day 7 postinfection, with a mean titer of 1:160. One year postinfection, antibody titers for surviving animals were 1:20–1:640. Detectable viremia did not develop in animals rechallenged with VEEV 1 year postinfection, but the animals did exhibit an 8-fold increase in antibody titers. All animals infected IC had detectable neutralizing antibodies 15 days postinfection (1:320–1:1,280).

Histopathologic Analysis

Histopathologic analysis was performed on small numbers of SC-infected animals on days 1 to 4 and day 7 postinoculation. Overall, pathologic changes were mild. All 4 animals examined at day 1 showed an acute lymphadenitis in the draining, left popliteal lymph node. This lesion was characterized by infiltration of the subcapsular sinus and cortical follicles with neutrophils, with minimal necrosis (Figure 3A and B). This lesion was not seen in the contralateral popliteal lymph node or in inguinal lymph nodes and disappeared by day 2. No splenic lesions attributable to VEEV were seen at any time. A striking degree of hemosiderosis of the red pulp, along with mineralization of the capsule and trabeculae, occurred in many animals, including uninfected controls, and appeared to be a normal, age-related phenomenon in this species. No brain or meningeal inflammation was seen at any time. Lesions of other organs were sporadic, including interstitial or periductal chronic inflammation in the salivary glands (Figure 3C) and multifocal chronic inflammation in the pancreas. Inflammatory foci were rarely seen in the heart on days 1, 4, and 7 but were not seen in controls. The kidney and thymus showed no lesions. Pathologic changes of the viscera appeared to peak on day 3, when 2 of 3 rats killed showed salivary gland pathology, 2 showed lung pathology, 1 showed pancreatic pathology (Figure 3D), and 2 showed liver pathology.

One spiny rat had hypothermia and weight loss 24 h after infection and died on day 3 postinfection. Necropsy showed extensive liver pathology, characterized by steatosis, and confluent coagulative necrosis with a mixed, mononuclear cell–predominant inflammatory infiltrate. This animal also showed pulmonary edema and striking alveolar hemosiderosis. Another animal exhibited alveolar edema and patchy acute inflammation in the lung 3 days after infection, while a different animal in this group displayed periportal acute inflammation in the liver.

| Table. Viremia and neutralizing antibody titers of spiny rats infected intracerebrally with the Co97-0054 strain of Venezuelan equine encephalitis virus |
|---|---|---|---|---|---|
| Rat no. | Viremia* | Neutralizing antibodies |
| 105 | Day 1† | 0.85 | Day 2† | 2.94 | Day 3† | 0.85 | Day 4† | 0.85 | Day 15† | 1:320 |
| 107 | 0.85 | <0.85 | 2.20 | <0.85 | 0.85 | 1:320 |
| 126 | 3.20 | 0.85 | 3.2 | 0.85 | 1:640 |
| 74 | 0.85 | 2.6 | <0.85 | 0.85 | 1:1,280 |

*Viremia levels are expressed in log₁₀ PFU/mL; 0.85 log₁₀ PFU/mL was the detection limit.
†Days postinfection
lesions were not seen in other animals. The lung histopathologic pattern seen in 2 animals on day 3 postinfection suggests that it might be VEEV-related; however, we cannot rule out other processes in these colony-reared animals.

**Discussion**

Subcutaneously VEEV infection induced little or no disease in *P. chrysaeolus*, the principal reservoir host in central Colombia. No signs of disease were seen after intracranial injection and at least 96% of animals showed no clinical signs after SC infection. Viremia was of low magnitude and short duration after SC inoculation, and was almost completely lacking after IC infection. Seroconversion occurred by day 7 and was persistent for at least 1 year. Histopathologic changes suggestive of viral cytopathic effects were visible in the draining lymph nodes 24 h postinfection and by day 3, mild pancreatic pathology was visible. The inability to detect infectious virus in the brain indicated that VEEV in these rats is probably not neuroinvasive; even after IC inoculation, detectable disease did not develop in any rat, indicating that VEEV is not neurovirulent in these animals. The only organ tested that showed evidence of viral production was the spleen; although viral RNA was detected in all other organs, blood contamination could not be ruled out.

In SC-infected spiny rats detectable levels of viremia developed for 3 days. The difference in viremia level between the 2 enzootic strains has been reported for another VEEV reservoir host, the cotton rat, using 2 strains of Everglades virus (30). Strain variation could explain the higher viremia titers reported for Panamanian spiny rats infected with Panamanian VEEV (20) as compared to our results with Colombian strains and animals. Other factors that could explain this difference include 1) the Panamanian virus used previously had a higher passage history than our strain Co97-0054 (3 suckling mice versus 1 baby hamster kidney cell) that could have led to adaptation for rodent replication (31); and 2) different species of *Proechimys* from a different locality were used in the previous studies.

Although the viremia titers we measured in spiny rats were lower than those generated in laboratory mice (3–4 log10 versus 6–7 log10 PFU/mL), they are sufficient to infect enzootic mosquito vectors that have been shown to be highly susceptible to infection by enzootic strains of VEEV (12,32,33). Furthermore, *Culex (Melanoconion) pedroi*, *Cx. (Mel.) spissipes*, *Cx. (Mel.) vomerifer*, *Cx. (Mel.) crybda*, and *Psorophora albipes* mosquitoes, some of which are natural VEEV vectors (12), captured in the Monte San Miguel forest became infected after feeding on viremic spiny rats (Carrara AS and Ferro C, unpub. data).

No virus was found in the feces (data not shown). Although saliva was not sampled, a previous study reported virus in throat swabs of both spiny rats and cotton rats (20). The pathologic changes we observed in the salivary glands, although nonspecific, suggest the possibility of VEEV infection in this site. Other studies have shown that VEEV is horizontally transmitted among rodents (23); in spiny rats, this transmission might occur orally during social contact and probably not through urinary and feces contamination. Further experiment designed specifically to address this issue are needed.

Virus titers in the spleen suggest it is a principal site of viral replication in spiny rats. The draining lymph node, as suggested by the presence of viral antigen and the high
level of neutrophil infiltration 24 h postinfection but absent after 48 h, may be a site of initial viral replication as in mice (34). Viral RNA detected in other organs indicates either a very small amount of replication (below the plaque assay detection limit) or the presence of RNA in viremic blood. The involvement of the pancreas as a target for VEEV replication during the later stages of infection is reminiscent of similar findings for TC-83 (attenuated VEEV vaccine strain) infection of mice and hamsters (3). In mice, VEEV also disseminates to the spleen after initial replication in the draining lymph node (35). However, an important difference is that enzootic VEEV appears to be nonneuroinvasive and nonneurovirulent in spiny rats, in contrast to its uniformly neuroinvasive and neurovirulent phenotype in laboratory mice.

Neutralizing antibodies were detected in spiny rat serum from 7 days to 1 year after VEEV infection. These antibodies apparently prevent reinfection by homologous (subtype ID) or heterologous (subtype IC, data not shown) VEEV subtypes 1 year after infection. Therefore, spiny rats appear unlikely to be susceptible to reinfection. Based on human data after vaccination with the TC83 strain, neutralizing antibodies are even longer lasting (36).

Seroconversion of spiny rats in nature may present a limiting factor to VEEV circulation, where the virus depends on the constant generation of naive rodents. Their long gestation and small litter sizes suggest that other reservoir animals may be required to maintain enzootic VEEV transmission when spiny rat populations are low (10). Further studies are needed to investigate other possible reservoir hosts, such as bats and opossums, which have also been shown to be seropositive in nature (12).

The high survival rates found in these rats after either enzootic or epizootic VEEV infection (A.S. Carrara and C. Ferro, unpub. data) support previous conclusions of their role as reservoir hosts (37). By comparison, laboratory mice (Mus musculus) exhibit a 100% mortality rate with subtype ID strains of VEEV, including those we tested (6). Horses have low mortality rates with enzootic strains, but deaths from epizootic strains can exceed 50% (11). Human mortality rates are generally <1% with both enzootic and epizootic VEEV strains, but severe neurologic disease develops in 4% to 14% of VEEV-infected patients <15 years of age; the mortality rate in these patients can reach 20% (38).

Not only did spiny rats survive VEEV infection (96%), but they also showed little sign of illness or discomfort, and their social behavior and fertility were not appreciably altered by infection. No significant change in the fecundity of infected females was observed compared to uninfected colony animals (p = 0.86, Student t test). In contrast, laboratory mice infected even with the attenuated VEEV vaccine strain TC-83 exhibit aggressive behavior with cage mates (39). The high survival rate of spiny rats in spite of abundant replication suggests selection for resistance to disease. The spiny rat is part of a very old (~25 million years) family (Echimyidae) (40), and long-term exposure to VEEV may have selected for resistance to disease in these animals.

Another possible explanation for the lack of disease in spiny rats is that VEEV has been selected for attenuation in these reservoir hosts. Other viruses, such as myxoma, have been shown to adapt to their hosts through attenuation. After its introduction into Australia for controlling the imported European rabbit population, myxoma virus underwent attenuation with concurrent selection for resistance in the rabbit population (14).

Spiny rats may be a useful tool for studying VEE pathogenesis and mechanisms of natural resistance. Additional studies of the immunologic responses in these rodents, particularly innate immunity, may provide valuable information that could be used to develop improved therapeutics for human and equine VEE.

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References


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Understanding people’s risk perceptions and motivations to adopt preventive behavior is important in preventing the spread of recreational water illnesses (RWI) and other emerging infectious diseases. We developed a comprehensive scale measuring parents’ perceived risk of their children contracting RWI. Parents (N = 263) completed a self-administered questionnaire with scale items based on 4 constructs of the Protection Motivation Theory: perceived vulnerability, perceived severity, response efficacy, and self-efficacy. Exploratory factor analysis identified 7 underlying factors, indicating 7 subscales of perceived risk for RWI. Cronbach α ranged from 0.60 to 0.81. The Precaution Adoption Process Model supported scale construct validity. This study provides the first perceived risk scale for exploring psychosocial factors that may predict or mediate the adoption of behaviors that prevent the spread of infectious diseases contracted by children while swimming. Findings from this study also provide implications for encouraging preventive behavior against other emerging infectious diseases.

Recreational water illnesses (RWI), or illnesses resulting from infectious agents acquired while swimming in pools, hot tubs, lakes, oceans, and other similar water venues, have been steadily increasing since the early 1990s, perhaps as a result of increasing numbers of bathers and the emergence of new infectious pathogens (1). Although outbreaks of RWI include a variety of illnesses, including skin, ear, eye, and respiratory infections, gastroenteritis is the most commonly reported illness (1,2). Common disinfectant agents for recreational water do not immediately destroy all pathogens, such as Cryptosporidium parvum (3), which pose a threat of prolonged outbreaks associated with contamination of chlorinated swimming pools (4–7).

Exposure to treated recreational water and infectious agents is high, with ≥350 million swimming visits in the United States annually (8). Children are particularly vulnerable to RWI because of their developing immune systems and high exposure to recreational water. However, many parents remain largely unaware of RWI, and most may underestimate their children’s risk of getting sick from swimming (9). Preventing the spread of RWI requires a 2-fold approach with steps to prevent self-exposure and contamination of others. Swimmers must refrain from contaminating the water (e.g., avoiding swimming while having a diarrheal illness), and swimmers must also avoid exposing themselves to contaminated water, especially by swallowing it. Because parents may not perceive their children to be at risk for RWI, they have little motivation to adopt behavior modifications that can reduce the risk of their children contracting RWI and contaminating recreational water.

A person’s perceived risk for an adverse outcome is considered an important factor in the adoption process of preventive behavior (10). Research on emerging infectious diseases and other health problems has found that perceived risk is an important predictor for persons taking protective actions (10–14). Perceived risk also is likely to have an important role in adopting preventive behavior against RWI (9). Although no known instrument currently exists to measure parents’ perceived risk for RWI transmission to their children, having a means of gauging perceived risk is valuable for exploring how and why persons are motivated to adopt RWI preventive behavior, identifying the educational needs of a target population, and evaluating efforts designed to promote the adoption of preventive behavior.
Our perceived risk scale was informed by the Protection Motivation Theory (PMT) (15), a theory on how persons make decisions to adopt health-related behavior (16) and its 4 constructs: perceived severity and perceived vulnerability to a health threat, and response efficacy and self-efficacy to respond to the threat. Risk is generally defined as the probability of a loss or an adverse outcome and usually consists of 2 elements: the likelihood that an adverse outcome will occur and the severity of that adverse outcome (17). However, the lay public often has a more intuitive definition of risk that is based on their perceptions of the likelihood, controllability, and information available about the hazard (18). Factor-analytic research (18,19) has shown that risk perception incorporates 2 prevailing factors: 1) dread risk, which involves evaluations of control, catastrophic potential, fatal consequences, and cost-benefit ratio, and 2) unknown risk, or whether the outcome of concern is new and observable, and if its effects are immediate. The constructs of the PMT are largely consistent with the primary factors found in previous research. For example, the characteristics of dread risk are equivalent to the perceived severity and self-efficacy of the PMT, while unknown risk is similar to perceived vulnerability. PMT provides a framework to explicitly measure additional dimensions of perceived risk that are likely to predict and explain behavior related to preventing RWI. Including PMT constructs in a perceived risk scale is supported by previous research (14,20,21).

The Precaution Adoption Process Model (PAPM) (22), which describes stages of behavior adoption from being unaware of a preventive behavior (stage 1) to maintaining the health behavior (stage 7), was used to validate our scales. Because people are often motivated to adopt preventive behavior when they feel vulnerable to, threatened by, and capable of mediating a health threat (23,24), we hypothesized that respondents in stage 7 would exhibit higher mean scale scores than respondents in stage 1.

Methods

Sample

A convenience sample of 263 parents of children ≤12 years of age were recruited from 1 elementary school and 5 nonprofit recreation-focused community organizations in Atlanta, Georgia. Questionnaires were retained for analyses if the respondent indicated having at least 1 child ≤12 years of age and if 80% of the survey scale items were complete. Seven surveys were excluded for failing to meet these criteria, yielding 256 analyzable surveys.

Of these respondents, 213 were recruited from the community organizations and 43 from the elementary school. Respondents ranged in age from 21 to 60 years (mean 38.3, standard deviation [SD] 6.6). Most (65.6%) were female, 28.9% were male, and the sex of the rest was unknown. The ages of the respondents’ children were 6 weeks to 25 years (mean 6.4, SD 4.3). Seventy-seven parents (30.1%) had children who wore diapers. Respondents’ children swam frequently in chlorinated venues; more than half (61.7%) swam in chlorinated venues at least once a week.

Procedures

After institutional review board approval was obtained from the sponsoring institution, the self-administered, anonymous, paper questionnaire was administered to parents in the fall of 2002. Parents were given verbal instructions from an oral script and asked to refer to their youngest child who swims when responding to the survey. Upon completion, respondents received an information packet on swimming safety and RWI and a $5 gift certificate.

Instrument Development

The items included for scale development were informed from focus group findings on parents’ perspectives of waterborne disease transmission in recreational water (9) and constructs from the PMT (15). Eighty-eight statements with 5-point Likert-type responses ranging from strongly disagree to strongly agree were created to reflect and capture the 4 PMT constructs. To reduce response bias, items about swimming safety were intermixed as foils. In addition, similar items were grouped by underlying construct, and items were keyed in both the positive and negative direction (25). The high internal consistency, as shown by the Cronbach α scores, indicated that keying responses in both the positive and negative direction did not compromise respondents’ interpretation of questions. The questionnaire also contained a PAPM scale to stage parents on the extent to which they actively protect their children from RWI. Five statements to which respondents could either agree or disagree were used to determine respondents’ stage. Before the study began, the PAPM scale was revised by 2 content experts for face validity and pilot tested among 7 parents for relevance, clarity, and readability of items.

Data Analysis

Exploratory factor analysis was conducted by using principal axis factoring and varimax rotation. The 4 PMT constructs were factor analyzed separately. Principal axis factoring, a common factor solution that is less biased than component factor solutions because unique and error variance is eliminated from the analysis, is recommended when the factor analysis includes <12 variables (26). Varimax rotation was used to facilitate interpretability of factors by maximizing the variance of loadings on each factor (27).
Questionnaire data were included for factor analysis for a particular construct if at least 80% of all items within that construct were answered, and median replacement was used for the <1% of missing items. Variables that fell outside of the skewness range of ±2 or the kurtosis range <7 were excluded from the factor analysis. Any item within each construct that was not correlated by at least ±0.30 with at least 1 other item was eliminated from analysis. The number of analyzable cases exceeded the minimum recommended number of 5 cases per item (28) with at least 100 cases (29).

Factorability of items was confirmed by using the Bartlett test of sphericity and the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy. The number of factors extracted was determined by scree plot by using the recommended criteria (27) (i.e., eigenvalue >1 and at least 2 items loading on a theoretically interpretable factor) to yield a solution that was parsimonious yet reliable. Items with a factor loading ≥0.4 and no secondary factor loading ≥0.30 were retained (30). All emerging factors were combined to form a perceived risk of RWI scale; perceived vulnerability and perceived severity factors were combined to form a threat appraisal of RWI scale, and factors from each construct were retained as subscales. Once each scale and subscale was finalized, Cronbach α was calculated to determine scale reliability.

Results

Scale Development

Two hundred fifty-five cases were included in the factor analysis for perceived vulnerability items (Table 1), 1 of the 4 primary constructs informed by PMT. The KMO measure of sampling adequacy was 0.830 with a significant Bartlett test of sphericity (p<0.001). Of the 16 items entered into the factor analysis, 2 items were dropped; the 14 remaining items were in a 2-factor solution. The first factor, disease vector acknowledgment, accounted for 21.5% of the variance with an α of 0.76. This factor pertained to recognizing the swimming pool as a source of transmission of infectious agents. The second factor, knowledge of transmission of infectious agents, accounted for 7.6% of the variance with an α of 0.73. This factor referred to modes and types of diseases spread through swimming pools. These 2 factors were moderately correlated (r = 0.346, p<0.01), and combining them yielded an α of 0.79 and explained 29.1% of the variance.

Two hundred forty-five cases were included in the perceived severity factor analysis (Table 1). The KMO measure of sampling adequacy was 0.762 with a significant Bartlett test of sphericity (p<0.001). A 2-factor solution emerged, retaining 7 of the 8 items. The first factor, severity of diarrheal illness, accounted for 28.1% of the variance with an α of 0.65. The second factor, severity of nongastrointestinal illness, accounted for 8.7% of the variance with an α of 0.63. These factors assessed perceptions of child illness severity for the most common illness (diarrhea) and other illness from RWI. These factors were moderately correlated (r = 0.316, p<0.001), and the combined α was 0.69, with 36.8% of the variance explained.

The response efficacy factor analysis included 247 cases (Table 1). The KMO measure of sampling adequacy was 0.680 with a significant Bartlett test of sphericity (p<0.001). Seven items were retained on a 2-factor solution, yielding 2 response efficacy subscales. The first factor, efficacy of behavioral modifications, accounted for 27.7% of the variance with an α of 0.71, and related to steps parents can take to reduce infectious agents in a pool. The second factor, efficacy of swim diapers, an important means of keeping fecal matter out of recreational water, accounted for 17.9% of the variance with an α of 0.78. Both subscales combined yielded an α of 0.63 with 45.6% of variance explained.

The factor analysis for self-efficacy items used 213 cases because items marked as not applicable were excluded from analysis (Table 1). The KMO measure of sampling adequacy was 0.668 with a significant Bartlett test of sphericity (p<0.001). Four items were retained in a 1-factor solution, self-efficacy for gastrointestinal RWI prevention, that explained 29.1% of the variance with an α of 0.60.

The 4 perceived vulnerability and perceived severity subscales were combined to form a threat appraisal of RWI scale with an α of 0.81. In addition, all 7 subscales from the 4 PMT constructs were combined to form a comprehensive perceived risk of RWI scale with an overall α of 0.74.

Construct Validity

The mean scores on the 7-risk perception subscales were compared for respondents in stage 1 and stage 7 of the PAPM (Table 2). As hypothesized, respondents in stage 7 had significantly higher mean scores on the 2 perceived vulnerability subscales (p<0.001) and the 2 perceived severity subscales (p<0.001) than respondents in stage 1. The scale on the efficacy of swim diapers produced significant results (p = 0.049) in the opposite direction. The other efficacy scales produced nonsignificant differences, although some were in the hypothesized direction. Stage 1 and stage 7 respondents were also compared on the threat appraisal of RWI scale and the comprehensive perceived risk for RWI scale, and for both scales, parents in stage 7 scored significantly higher than parents in stage 1 (p<0.001), as hypothesized.

Discussion

A comprehensive perceived risk scale of RWI is an important tool for examining the psychosocial factors that
predict or mediate the adoption of recommended behavior for preventing the spread of infectious diseases while swimming. This study describes the first known effort to develop a scale that offers a detailed and comprehensive assessment of parents’ perceived risk for RWI for their children. The 4 components of PMT (perceived vulnerability, perceived severity, response efficacy, and self-efficacy) served as the theoretical framework for scale development, and a 7-factor solution emerged.

Factor analysis showed 2 moderately correlated factors among the perceived vulnerability items, which accounted for nearly 30% of the variance. That perceived vulnerability subscales capture hazard-related knowledge is an important attribute in forming perceived risk (31,32) because one must know how one is exposed to a hazard and the nature of the hazard to perceive being at risk. Previous research on public perceptions of food-related risks similarly found salient factors related to awareness or knowledge of food hazards (33,34).

Two moderately correlated perceived severity factors were identified that explained more than one third of the variance. A threat appraisal scale can be created by

<table>
<thead>
<tr>
<th>Item</th>
<th>Factor 1</th>
<th>Factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Disease vector acknowledgment (perceived vulnerability) (α = 0.76)†</td>
<td>0.610</td>
<td>0.121</td>
</tr>
<tr>
<td>A well-maintained pool is germ-free.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorinated pool water is just as clean as drinking water.</td>
<td>0.607</td>
<td>0.005</td>
</tr>
<tr>
<td>Chlorine kills all germs instantly.</td>
<td>0.541</td>
<td>0.141</td>
</tr>
<tr>
<td>A swimming pool contains fewer germs than oceans or lakes that can make my child sick.</td>
<td>0.516</td>
<td>0.178</td>
</tr>
<tr>
<td>My child is more likely to get sick from germs in a restaurant than from a swimming pool.</td>
<td>0.501</td>
<td>0.173</td>
</tr>
<tr>
<td>Pool management makes sure that the pool my child swims in is germ-free.</td>
<td>0.483</td>
<td>0.007</td>
</tr>
<tr>
<td>My child is more likely to get sick from germs from a public restroom than a swimming pool.</td>
<td>0.459</td>
<td>0.009</td>
</tr>
<tr>
<td>Swimming in chlorinated water with other swimmers can spread germs.</td>
<td>0.404</td>
<td>0.271</td>
</tr>
<tr>
<td>2. Knowledge of germ transmission (perceived vulnerability) (α = 0.73)†</td>
<td>0.140</td>
<td>0.698</td>
</tr>
<tr>
<td>It is possible that there are germs in a pool that can cause eye infections.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>It is possible that there are germs in a pool that cause skin infections.</td>
<td>0.211</td>
<td>0.653</td>
</tr>
<tr>
<td>Swallowing water while swimming in a pool increases the risk of getting sick from germs.</td>
<td>0.165</td>
<td>0.586</td>
</tr>
<tr>
<td>My child can get sick if she or he swims in a pool when another swimmer has diarrhea.</td>
<td>0.009</td>
<td>0.492</td>
</tr>
<tr>
<td>It is possible that there are germs in a pool that cause ear infections.</td>
<td>0.120</td>
<td>0.485</td>
</tr>
<tr>
<td>If one child in my family were to get sick with diarrhea from swimming in a chlorinated pool, she or he could infect the rest of the family.</td>
<td>0.004</td>
<td>0.423</td>
</tr>
<tr>
<td>3. Perceived severity of diarrheal illness (α = 0.65)‡</td>
<td>0.725</td>
<td>0.190</td>
</tr>
<tr>
<td>Diarrhea is dangerous to my child’s health.</td>
<td>0.611</td>
<td>0.150</td>
</tr>
<tr>
<td>Diarrhea threatens a child’s health.</td>
<td>0.422</td>
<td>0.185</td>
</tr>
<tr>
<td>It is difficult for children to get well from diarrhea.</td>
<td>0.416</td>
<td>0.003</td>
</tr>
<tr>
<td>Compared to other children, diarrhea is more dangerous to my child’s health.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Perceived severity of nongastrointestinal illness (α = 0.63)‡</td>
<td>0.161</td>
<td>0.739</td>
</tr>
<tr>
<td>An eye infection from a germ in the pool is easily treated.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children recover easily from earaches caused by germs in a chlorinated pool.</td>
<td>0.007</td>
<td>0.604</td>
</tr>
<tr>
<td>I am not worried about skin rashes that are caused by germs in the pool.</td>
<td>0.249</td>
<td>0.441</td>
</tr>
<tr>
<td>5. Response efficacy of behavioral modifications (α = 0.70)§</td>
<td>0.759</td>
<td>0.101</td>
</tr>
<tr>
<td>Taking children on frequent bathroom breaks will reduce the feces in the pool.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taking children on frequent bathroom breaks will reduce the amount of urine that will get into the pool.</td>
<td>0.690</td>
<td>−0.008</td>
</tr>
<tr>
<td>If parents keep their children who are sick with diarrhea out of the pool, illness to other children will be reduced.</td>
<td>0.623</td>
<td>0.002</td>
</tr>
<tr>
<td>Maintaining chlorine levels will reduce the number of germs in the pool.</td>
<td>0.488</td>
<td>0.008</td>
</tr>
<tr>
<td>Parents who avoid changing diapers near the pool help keep germs out of the pool.</td>
<td>0.409</td>
<td>0.103</td>
</tr>
<tr>
<td>6. Response efficacy of swim diapers (α = 0.78)§</td>
<td>0.003</td>
<td>0.812</td>
</tr>
<tr>
<td>Swim diapers are effective in preventing feces from getting into the pool.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swim diapers prevent germs from spreading in a pool.</td>
<td>0.129</td>
<td>0.796</td>
</tr>
<tr>
<td>7. Self-efficacy for gastrointestional RWI prevention (α = 0.60)¶</td>
<td>0.632</td>
<td></td>
</tr>
<tr>
<td>It is difficult to interrupt my child for bathroom breaks while she or he is playing in the pool.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>It would be difficult to stop my child from swimming for 2 weeks after his or her diarrhea stops.</td>
<td>0.524</td>
<td></td>
</tr>
<tr>
<td>It is difficult to tell my child that she or he cannot swim when she or he has diarrhea.</td>
<td>0.523</td>
<td></td>
</tr>
<tr>
<td>It is difficult to constantly supervise my children while they are playing in the pool.</td>
<td>0.465</td>
<td></td>
</tr>
</tbody>
</table>

*Bold numbers indicate the factors on which the items load. RWI, recreational water illness.
†Scales 1 and 2 combined: total variance 29.1%, α 0.79.
‡Scales 3 and 4 combined: total variance 36.8%, α 0.66.
§Scales 5 and 6 combined: total variance 45.6%, α 0.63.
¶Scale 7: total variance 38.2.
combining the perceived vulnerability and perceived severity subscales, and this scale can be useful for evaluating the impact of RWI awareness campaigns. Threat appraisal scales can assess changes in beliefs and can be effective in predicting different phases of behavior change (35).

The response efficacy subscales and the prevention self-efficacy subscale explained a great deal of variance (45.6% and 38.2%, respectively) but produced a slightly lower $\alpha$ when combined than when considered separately. We found that the perceived vulnerability and response efficacy subscales had sufficient ($\alpha$>0.70) internal consistency (26), as did the combined threat appraisal and comprehensive perceived risk scales, but the internal consistency of the perceived severity and self-efficacy subscales were slightly lower (from 0.60 to 0.65).

Construct validation of the scales using the PAPM showed that differences in the perceived risk for RWI scale, as well as the perceived vulnerability and perceived severity subscales, were significant in the hypothesized directions, with respondents in stage 7 exhibiting lower mean scores that respondents in lower stages. However, opposite of the hypothesized direction, a significant difference in the efficacy of the swim diapers subscale was found. One explanation for this finding is that parents who are most actively engaged in preventing RWI (stage 7) may already recognize that swim diapers are not efficacious at preventing leakage of fecal matter that can contain infectious pathogens (3). Although the other response efficacy and self-efficacy subscales were not significantly different for respondents in stages 1 and 7, the difference in the self-efficacy for gastrointestinal RWI prevention was in the hypothesized direction. The lack of significant differences on these efficacy scales may be due to low levels of awareness about RWI prevention among parents, which leads to more variability on the threat scales and less on the efficacy scales.

Because a person’s individual perception of risk can be influenced by a number of biases, such as personal experience and information from the media, public health practitioners need instruments to accurately assess risk perception of pediatric RWI. The scale we developed quantifies the multiple dimensions that can contribute to risk perception. This scale can be used to understand the public’s perceived risk of pediatric RWI by obtaining a baseline measurement of risk perception and its contributing factors, which can inform the extent and type of educational efforts. Furthermore, scale scores can identify groups for intervention, such as those who underestimate and those who overestimate the risk for RWI. Intervention is important for these groups because an underestimation of risk will result in persons being unprepared to handle a health threat, and an overestimation of risk can result in public panic, distrust of authority, and the adoption of counterproductive behavior (36).

Factors to be emphasized in a program that aims to reduce pediatric RWI will depend on the awareness level of the targeted audience. For example, if an audience is relatively unaware of RWI, perceived vulnerability and perceived severity factors are likely to be most influential in raising risk perception. Once the risk for RWI has been acknowledged, the response efficacy and self-efficacy constructs may be more important in promoting the adoption of recommended preventive behavior modifications.

Our study had several limitations. We used a convenience sample, as is common for developing scales. Our sample was largely well-educated and consisted primarily of above-average income earners. In addition, recruitment occurred at locations where RWI awareness may be higher than average. Additional testing should involve larger and more diverse populations. In addition, the questionnaire length may have contributed to response bias, particularly toward the end of the questionnaire. Third, some respondents’ self-reported RWI answers may have been influenced by social desirability bias; however, the inclusion of pool-based injury items was intended to reduce the focus on RWI.

Table 2. Differences in perceived risk scales and subscales between stage 1 and stage 7*  

<table>
<thead>
<tr>
<th>Scale</th>
<th>Stage 1</th>
<th>Stage 7</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease vector acknowledgment</td>
<td>23.08 (2.47)</td>
<td>27.44 (4.59)</td>
<td>21.04†</td>
</tr>
<tr>
<td>Knowledge of germ transmission</td>
<td>21.60 (2.96)</td>
<td>24.12 (3.18)</td>
<td>13.05†</td>
</tr>
<tr>
<td>Perceived severity of diarrheal illness</td>
<td>11.74 (2.56)</td>
<td>13.03 (2.51)</td>
<td>5.004‡</td>
</tr>
<tr>
<td>Perceived severity of other illnesses</td>
<td>8.70 (2.20)</td>
<td>10.52 (1.96)</td>
<td>15.90†</td>
</tr>
<tr>
<td>Efficacy of behavioral modifications</td>
<td>21.50 (2.15)</td>
<td>21.26 (2.23)</td>
<td>0.24</td>
</tr>
<tr>
<td>Efficacy of swim diapers</td>
<td>6.17 (2.22)</td>
<td>5.22 (2.09)</td>
<td>3.95‡</td>
</tr>
<tr>
<td>Self-efficacy for gastrointestinal RWI prevention</td>
<td>14.70 (3.85)</td>
<td>15.53 (2.77)</td>
<td>1.25</td>
</tr>
<tr>
<td>Threat appraisal of RWI prevention</td>
<td>64.78 (7.12)</td>
<td>75.29 (8.60)</td>
<td>29.76†</td>
</tr>
<tr>
<td>Perceived risk for RWI</td>
<td>107.11 (9.44)</td>
<td>117.52 (9.04)</td>
<td>19.62†</td>
</tr>
</tbody>
</table>

*RWI, water recreational disease.
†p<0.001.
‡p<0.05.
In addition to expanding our understanding of RWI risk perceptions, the scale developed in this study may provide insights for studying how people understand and adopt preventive behavior for other emerging infectious diseases. For example, risk perception has been shown to be an important factor in obtaining vaccine for influenza (37), which can lead to serious illness in vulnerable populations, including young children (38). While vaccination is considered to be the best protection against influenza, information presented by the media might exaggerate the risks of vaccination, and the benefits of vaccine, i.e., disease prevention, are either undervalued or ignored, leading some parents to perceive vaccines to be risky (39,40). With future research, the perceived risk of the RWI scale developed in this study can be adapted to other populations, disease vectors, and pathogens, and may be useful in preventing and controlling future outbreaks.

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References


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A telephone survey of 986 Hong Kong households determined exposure and risk perception of avian influenza from live chicken sales. Householders bought 38,370,000 live chickens; 11% touched them when buying, generating 4,220,000 exposures annually; 36% (95% confidence interval [CI] 33%–39%) perceived this as risky, 9% (7%–11%) estimated >50% likelihood of resultant sickness, whereas 46% (43%–49%) said friends worried about such sickness. Recent China travel (adjusted odds ratio 0.35; CI 0.13–0.91), traditional beliefs (1.20, 1.06–1.13), willingness to change (0.29, 0.11–0.81) and believing cooking protects against avian influenza (8.66, 1.61–46.68) predicted buying. Birth in China (2.79, 1.43–5.44) or overseas (4.23, 1.43–12.53) and unemployment (3.87, 1.24–12.07) predicted touching. Age, avian influenza contagion worries, husbandry threat, avian influenza threat, and avian influenza anxiety predicted perceived sickness risk. High population exposures to live chickens and low perceived risk are potentially important health threats in avian influenza.

The risk of a pandemic human influenza strain emerging from coinfection of a human influenza carrier by avian influenza H5N1 virus is small (1); however, the potential global public health impact could be catastrophic. The circumstances that would facilitate events of low probability may be highly prevalent, which increases the public health importance of such potential events. Modern travel and transportation links would distribute a new human-transmissible influenza strain worldwide within days and overwhelm most healthcare systems within weeks. Preventing such an event is a vital public health enterprise.

Domestic waterfowl, chickens, and pigs act as aberrant hosts (2), both for avian influenza from migratory waterfowl and shorebirds (3,4) and human influenza viruses moving in the opposite species direction (2). Genetic reassortment of influenza viruses is likely more rapid in aberrant hosts because of adaptive selection pressures (5). Domestic animal and human avian influenza infection may therefore increase reassortment opportunities and the chance of a potentially pandemic strain emerging.

Most human-animal contact is domestic (pets and husbandry), or commercial, (farming, wholesale and retail marketing). Most human avian influenza infections occur among persons working or living with domesticated birds (6). Traditional Asian wet markets provide major contact points for people and live animal mixing (because of lack of refrigeration, animals are usually alive when sold), making them important potential sources of viral amplification and infection (7). Severe acute respiratory syndrome–associated coronavirus probably emerged from the selling in wet markets of Himalayan palm civets and other wild species. Traditional local demand for live animals from wet markets maintains this practice. In the wet markets of Hong Kong Special Administrative Region, ≤10 chickens are enclosed in small (approximately 25 cm x 60 cm x 60 cm) plastic cages in stacks of 5. Distressed chickens defecate, which contaminates feathers with feces. Frequent cleaning of cages and transportation and storage areas does not prevent this. Although direct hand-to-face contact is the most likely path for infection, the flapping by distressed chickens inverted during inspection by shoppers raises fecal-dust aerosols and exposes sellers, shoppers, and passers-by to any virus particles on an infected bird. Highly dense urban populations maximize opportunities for infection and transmission in any outbreak.

Minimizing unnecessary mixing between people and domestic poultry by replacing live animal sales in wet markets with hygienic central slaughtering and chilling is epidemiologically compelling. Since the 1997 Hong Kong avian influenza outbreak, which killed 6 people, all ducks and quail have been centrally slaughtered, but live chicken sales at wet markets continue, supported by chicken vaccination and intensified immunologic surveillance. The current avian influenza epidemic occurred in Asia in January

The Hong Kong government suspended imports and sales of live chickens in early 2004. Local poultry farms remain free from H5N1 infection. In May 2004, limited importation of screened live birds from China was resumed. Public consultation on central slaughtering evoked commercial and some public support for live chicken sales. To determine population knowledge of risk and self-protection practices and estimate degree of population exposure from live chicken sales, we carried out a survey in Hong Kong during February and March 2004.

Methods

Nearly 100% of the Hong Kong population have telephones. After receiving institutional review board approval, we conducted a telephone survey of the general population from 10 a.m. to 10 p.m. from mid-February to mid-March 2004, at the peak of the avian influenza epidemic in Asia. Households were selected by using random digit dialing and, within households, respondents were selected by using random number tables based on varying household sizes. Inclusion criteria were Cantonese-Chinese speakers, age of 16 to 95 years, and residence in Hong Kong >12 months.

Instrumentation

The draft questionnaire was examined by a panel of experts, including epidemiologists and psychologists, to determine face validity, then pilot tested with respondents, who were questioned on item comprehensibility and relevance. Interrater performance was examined by comparing response rates for recruitment and item completion, and additional training was given to improve these rates. Several households completed the questionnaire twice, with no significant differences in reported buying frequencies. Interviewers gave additional information on respondents’ reactions to certain questions, which were reworked, and the instrument was retested as necessary to obtain satisfactory responses. Rater response rates were monitored throughout the study.

Of the 6-section questionnaire, 3 sections are addressed here. Section 1 consisted of Likert scale items assessing self-rated health (excellent to very poor) and influenzalike symptoms (ILI): fever, chills, cough, headache, myalgia, breathing difficulties, coryza, sore throat, diarrhea and low back pain (“yes,” “no,” “don’t know”) (8). Section 2 consisted of 13 questions on household practices of buying live birds and 3 that assessed risk perceptions: worries about catching avian influenza from buying live chickens, likelihood of self/family members getting sick from buying live chickens (all using 5- or 7-point categorical ordinal response formats) and a decile anchored 0%-100% probability assessment for likelihood of getting sick (9) from buying live chickens.

To help identify attitudinal and knowledge predictors of risk perceptions and behavior change, respondents expressed agreement or disagreement using 5-point Likert scales (strongly agree to strongly disagree) with 32 statements addressing attitudes, avian influenza protection practices, and perceptions of live chicken sales. Section 3 consisted of 9 items concerning demographic information.

Data Analysis

Categorical data were analyzed with the χ² test and continuous data with t tests. Average annual live chicken purchase rates were calculated by using a conservatively estimated number of live chicken purchases per response category. To households reporting ≤1 live chicken purchase per year, 1 live chicken purchase was attributed; to households reporting “a few times a year,” 4 were attributed; to households reporting “monthly,” 12 were attributed; to households reporting “a few times per month,” 24 were attributed; to households reporting “weekly,” 52 were attributed; and to those reporting “a few times a week,” 100 purchases annually were attributed.

Perceived risk moderates behavior (10–12). To identify predictors of greater risk perception and behavior, purchase (yes/no) (model 1) and touching during purchase (yes/no) (model 2) of live chickens, and perceived likelihood of getting sick from buying live chickens (dependent variable 50th percentile dichotomized 0%-100% probability assessment responses to the question, “How likely is it that you will get sick from buying live chickens?”) (model 3) were regressed in forward-stepped multivariate logistic equations on 5 attitudinal factors, adjusted for demographics. Attitudinal factors were derived by reducing the 32 attitudinal statements with varimax-rotated principal components factor analysis by using scree-plot and Eigen vector-driven factor extraction. Dichotomization and logistic regression were required for binary dependent variables in models 1 and 2 and to overcome multimodal distribution difficulties (13,14) on the response scale used in model 3. All proportions are rounded to the nearest whole number. Analyses were performed using SPSS 11.0. (SPSS Inc., Cary, NC, USA).

Results

Seven interviewers called 6,603 telephone numbers in 4 weeks. Of these, 2,596 were invalid (fax or answering machines), and those reached at 1,765 numbers were ineligible (either non-Cantonese speakers, resident <12 months, or businesses). Of 2,240 eligible respondents, 1,256 declined to participate or complete the survey (556 were “too busy,” 688 refused for other reasons), leaving
986 eligible respondents who completed the survey, a response rate of 44% (986/2,240).

The sample comprised 589 women and 397 men closely matching the most recent population census data. Men had a wider age distribution than did women ($\chi^2 = 16.3$, degrees of freedom [df] 5, $p = 0.006$), more likely to be single ($\chi^2 = 23.84$, df 3, $p<0.001$), born in Hong Kong ($\chi^2 = 21.67$, df 4, $p<0.001$), and better educated ($\chi^2 = 10.52$, df 3, $p = 0.015$) (Table 1).

### Purchase of Live Chickens

One female respondent in 5 (116/589, 20%, 95% confidence interval [CI], 17%–23%) reported that her household never bought live chickens, compared to 1 in 4 (96/396, 24%, 20%–28%) male respondents who reported this. In households (78%) that reported buying live chickens, 76% (72%–78%) of female and 31% (26%–36%) of male respondents personally bought live chickens; the remainder were bought by other family members or domestic helpers. The remainder of this section only considers households that reported buying live chickens.

Of male respondents, 18% (14%–22%) reported that all family members bought live chickens, 14% (10%–18%) were the sole purchasers, while 69% (64%–74%) reported that live chickens were bought by other household members but not the respondent. The corresponding rates among females were 11% (8%–14%), 65% (61%–69%), and 24% (20%–28%). Detailed purchase patterns and rates are given in Table 2.

Because 65% of women but only 14% of men personally bought live chickens, we adjusted for sex differences in purchasing rates by applying the female rate to the remaining proportion of purchases in male-respondent households (86%), and all but 14% in female respondent households, the remainder attributed to the male rate. The sex-adjusted household purchase rate for all 2,051,890 households in Hong Kong is given in Table 2.

### Contact with Live Chickens during Purchase

Of the 78% of respondents who reported their household bought live chickens, 13% (10%–16%) of female and 19% (14%–23%) of male purchasers touched chickens when

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### Table 1. Sample characteristics and thematic household survey, Hong Kong, 2002

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. female (%)</th>
<th>No. male (%)</th>
<th>Total (%)</th>
<th>Thematic household survey proportion</th>
<th>Effect size*</th>
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<td>Male</td>
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<td>397 (40)</td>
<td>986 (100)</td>
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<td>(40)</td>
<td>(60)</td>
<td>(120)</td>
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<td></td>
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<td>Age (y)†</td>
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<td></td>
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</tr>
<tr>
<td>18–34</td>
<td>136 (23)</td>
<td>111 (28)</td>
<td>247 (25)</td>
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<tr>
<td>35–44</td>
<td>176 (30)</td>
<td>93 (23)</td>
<td>269 (27)</td>
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<td>45–64</td>
<td>215 (36)</td>
<td>134 (34)</td>
<td>349 (35)</td>
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<td>≥65</td>
<td>62 (10)</td>
<td>58 (15)</td>
<td>120 (12)</td>
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<td>Marital status‡</td>
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<td>117 (29)</td>
<td>225 (23)</td>
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<td>Married</td>
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<td>262 (66)</td>
<td>690 (70)</td>
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<td>Divorced/separated</td>
<td>12 (2)</td>
<td>8 (2)</td>
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<tr>
<td>Widowed</td>
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<td>9 (2)</td>
<td>48 (5)</td>
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<td>3 (0)</td>
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<tr>
<td>Place of birth§</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Hong Kong</td>
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<td>279 (70)</td>
<td>615 (62)</td>
<td>59.7§</td>
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<tr>
<td>China province</td>
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<td>108 (27)</td>
<td>333 (34)</td>
<td>33.7</td>
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<tr>
<td>Elsewhere</td>
<td>28 (5)</td>
<td>10 (2)</td>
<td>38 (4)</td>
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<td></td>
<td></td>
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<tr>
<td>None/primary 1–6</td>
<td>140 (24)</td>
<td>74 (18)</td>
<td>214 (22)</td>
<td>30.5</td>
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<td>Secondary 7–11</td>
<td>311 (53)</td>
<td>188 (48)</td>
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<tr>
<td>Matriculated 12–13</td>
<td>32 (5)</td>
<td>33 (8)</td>
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<tr>
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<td>102 (26)</td>
<td>208 (21)</td>
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<td></td>
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<tr>
<td>Employed</td>
<td>236 (41)</td>
<td>260 (65)</td>
<td>496 (51)</td>
<td>61.2</td>
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<tr>
<td>Unemployed</td>
<td>27 (5)</td>
<td>45 (11)</td>
<td>72 (7)</td>
<td>5.5</td>
<td></td>
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<tr>
<td>Student</td>
<td>28 (5)</td>
<td>23 (6)</td>
<td>51 (5)</td>
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<tr>
<td>Homemaker</td>
<td>254 (43)</td>
<td>1 (0)</td>
<td>255 (26)</td>
<td>16.3</td>
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<td>Retired</td>
<td>44 (7)</td>
<td>67 (17)</td>
<td>111 (11)</td>
<td>13.0</td>
<td></td>
</tr>
</tbody>
</table>

*Three levels of effect sizes: 0.1, small; 0.3, medium; 0.5, large.
†Differences between male and female participants: $\chi^2 = 16.30$, degrees of freedom (df) 5, $p = 0.006$.
§$\chi^2 = 21.67$, df 4, $p<0.001$.
¶$\chi^2 = 23.84$, df 3, $p<0.001$.
# $\chi^2 = 10.54$, df 3, $p = 0.015$.
Note: Totals may not be summed to 1 due to rounding.
Overall, 14% (9%–13%) of purchases involved physical contact with a live chicken. Extrapolating these exposures (14% of 78% = 11%) by the average number of chickens purchased annually (18.7), multiplied by the number of Hong Kong households (2,051,890), gives 4,220,738 person-chicken exposures annually. Of those reporting that they touched live chickens when buying, only ≈30% said they "always" or "usually" wash hands afterwards. Anxiety scores did not differ between those who bought live chickens and those who did not.

### Risk Perception

This section addressed all respondents, not just those buying live chickens. Four separate items tapped perception of risk from buying live chickens. The first assessed perceived “objective” risk. Overall, 36% (33%–39%) of respondents agreed with the statement “Buying live chickens is risky to health.” The next 2 items considered perceived consequences of risk (odds of getting sick). Statement-based probability estimates for “getting sick from buying live chickens” indicated that 34% (31%–37%) of respondents considered that they would “never” or were “very unlikely” to get sick from buying live chickens, while 27% (24%–30%) thought it was “unlikely,” 24% (21%–27%) “chances are even” and 15% (13%–17%) “likely” or “very likely.” The third item (0%–100% probability estimates of sickness risk) produced lower risk estimates than the second item, with 53% (50%–56%) perceiving the likelihood of getting sick at below 26%, 38% (35%–41%) in the range 26%–50%, and 9% (7%–11%), exceeding a 51% likelihood. Item 4 assessed the risk expressed by others. Overall, 46% (95% CI 43%–49%) of respondents reported that their friends had expressed worries about catching avian influenza. Risk perceptions did not differ by age, sex, education, income, or occupation.

### Factor Analysis

The 32 attitude statements produced a 5-factor best-fit solution, which accounted for 38.5% of the score variance (see online Appendix Table, available at http://www.cdc.gov/ncidod/EID/vol11no05/04-1125_app.htm). These 5 factors were labeled according to their item content. Factor 1, “animal husbandry risk” (10% of variance), included items attributing avian influenza to market practices, live animal sales, and poor home and market hygiene. Factor 2, “traditional market practices” (9% of variance), items supported traditional markets, their low health risks, live chicken sales, and trivialized health “scares.” Factor 3, “protective practice” (8%), items reflected unwillingness to continue live chicken purchases despite risks, unwillingness to take risks for enjoyment, risks from zoonotic infections, and responsibility for own health. Factor 4, “avian influenza anxieties” (6%), items reflected avian influenza worries, effect of media reports, and sense of vulnerability. Factor 5, “feel protected” (6%), items reflected reassurance from media reports, trust in government, and confidence in existing avian influenza control measures.
of birth, years of residence in Hong Kong, and recent China travel (see online Appendix Table). All models also included factors 1–5 plus attitudinal items not included in the factor scores.

Model 1 produced 6 independent predictors of buying live chickens: 1) travel; respondents reporting recent Chinese mainland travel were less likely to buy (adjusted odds ratio [AOR] 0.35, 95% CI 0.1–0.9); 2) employment status; unemployed people were less likely to buy (AOR 0.18, 0.05–0.6); 3) traditional market practices (Factor 2 score); persons supporting traditional markets were more likely to buy (AOR 1.2, 1.06–1.1); 4) protective practice (Factor 3 score); persons reporting high protective practices were more likely to buy (AOR 1.2, 1.6–1.5); 5) willingness to change buying habits if other persons do the same (AOR 0.3, 0.1–0.8); and 6) belief that cooking food thoroughly is the best protection against bird flu (AOR 8.7, 1.6–46.7).

Model 2 estimated independent predictors of touching chickens when buying, using only respondents who reported buying live chickens themselves (n = 451). Two variables independently predicted higher risk of touching: place of birth; persons born outside of Hong Kong (AOR 8.7, 1.6–46.7). These estimates, though highly uncertain, quantify the potential risk magnitudes involved.

Discussion

Women are usually responsible for food shopping; shopping practices differ by sex, and reporting differences by sex are found elsewhere (15). The observed purchase (and therefore exposure) rate of 18.7 live chickens/household/year (38,370,343 purchases annually) matches government figures of ≈38,325,000 live chickens purchased annually in Hong Kong, (Government of Hong Kong, 2004). This provides important independent validation of our data accuracy.

How much risk this exposure represents is difficult to accurately quantify. A highly conservative estimation assumes that genetic reassortment of human and avian influenza viruses can occur only on day 1 of a 5-day infectious period (16) in a person with human influenza. During the two 10-week human influenza seasons that occur annually in Hong Kong (17,18), sentinel data for ILI from live chicken sales, risk magnitude seldom exceeded 60%, and peaks at 25% and 50% are partially artifactual (13,14). Almost 50% indicated that their friends had expressed anxieties about avian influenza. Attributing greater concerns to others than to oneself reflects optimistic attribution bias, a protective response enabling expression of concern while preserving “face” (22). Sickness anxieties reflected the fact that the markets and live chicken sales were perceived as health threats. Older persons, possibly due to past experience of buying live chickens, or past “chicken plagues,” viewed the present avian influenza outbreak as low risk. Hazard familiarity and experience reduce associated risk perceptions (23). Yet respondents who reported higher anxiety and greater risk were no less likely to buy live chickens.

Raising population anxiety levels by warnings about disease produces only transient, inconsistent, and therefore often ineffective results as a means of reducing long-term high-risk behavior for 3 main reasons. First, persons perceiving control over dubious “hazards” may underestimate the associated risk, which reduces the likelihood of behav-
ior change (24). Second, persons who perceive little or no control over a threat adopt fatalistic responses, continue with established behavior, and direct coping efforts towards controlling emotions rather than risks (25,26). Third, hazard exposure causes familiarity, thus reducing perceptions of risk (10–12,27). Therefore, persons may dismiss the warnings as exaggerated or unrealistic.

However, some persons are willing to change buying habits if others do. Consequently, health warnings can produce short-term effects that rapidly attenuate in the absence of increased perceived threat, particularly where established behavior is involved. This suggests that large group changes may be more probable than individual level changes, consistent with evidence from health “ scares.” Once confidence in food safety is lost, recovery time may be protracted (28).

In conclusion, perceptions of risk from buying live chickens were moderate, but sickness anxieties did not predict buying or touching habits. Buying was, importantly, strongly predicted by the erroneous belief that cooking is the best way to protect from avian influenza. This is an important message for health education groups seeking to increase preventive practices to control possible avian influenza outbreaks.

Dr. Fielding is associate professor of medical psychology, head of the Behavioral Sciences Unit, and director of the Behavioral Medicine and Wellness Group, at the Department of Community Medicine and School of Public Health, University of Hong Kong.

References

Alkhurma hemorrhagic fever virus (genus *Flavivirus*, AHFV) was recently identified as the agent of a viral hemorrhagic fever in Saudi Arabia and characterized serologically and genetically as a variant genotype of Kyasanur Forest disease virus (KFDV). Since viral diagnosis and vaccine development may be hindered by genetic diversity, this study was intended to address AHFV genetic heterogeneity. Eleven strains isolated from hospitalized patients from 1994 to 1999 in Saudi Arabia were sequenced in the envelope, NS3, and NS5 genes. Homologous sequences were compared and used to look for patterns reflecting specific evolution associated with spatiality, temporality, infection pathway, and disease prognosis. Genetic analyses showed low diversity, which suggests a slow microevolution. Evaluation of divergence times showed that AHFV and KFDV ancestral lineage diverged 66–177 years ago, and the diversity observed within the studied AHFV strains reflected a 4- to 72-year period of evolution.

Low Diversity of Alkhurma Hemorrhagic Fever Virus, Saudi Arabia, 1994–1999

Rémi N. Charrel,* Ali Mohamed Zaki,† Mazen Fakeeh,† Amany Ibrahim Yousef,† Reine de Chesse,* Houssam Attoui,* and Xavier de Lamballerie*

Alkhurma hemorrhagic fever virus (AHFV) was first isolated in Jeddah, Saudi Arabia, in the 1990s from the blood of a butcher admitted to the hospital with a severe infectious syndrome. To date, 24 cases have been recorded in a 10-year period. Clinical manifestations include fever, headache, retroorbital pain, joint pain, generalized muscle pain, anorexia and vomiting associated with leukopenia, thrombocytopenia, and elevated levels of liver enzymes. In addition, some patients had clinical symptoms of hemorrhagic fever or encephalitis; overall, 5 patients, of 24 infected, died, for a 25% fatality rate (1–4).

AHFV was identified as a flavivirus on the basis of immunofluorescence assay performed with the flavivirus-specific monoclonal antibody 4G2 and polymerase chain reaction (PCR) amplification of a 220-bp genome fragment that exhibited 89% nucleotide (nt) sequence homology with the Kyasanur Forest disease virus (KFDV) NS5 gene. Recently, the complete coding sequence of AHFV was determined; comparative analysis with other tickborne flaviviruses confirmed that AHFV was most closely related to KFDV, and genetic distances suggested that AHFV was a subtype of KFDV (5). In this study, 11 human isolates of AHFV, obtained in a 5-year period, were studied. Partial envelope and NS3 and NS5 genes were sequenced for each isolate and used to conduct detailed genetic analyses. The results of these analyses are presented and discussed.

Materials and Methods

Samples

Virus isolation was performed from 1994 to 1999 at the virology laboratory of Dr. Suliman Fakeeh Hospital in Jeddah from blood samples from 11 patients. After centrifugation, serum was injected into suckling mice both intracerebrally or intraperitoneally, and mice were observed twice daily to detect death or signs of illness. Mice showing symptoms were killed, and their brains were harvested, suspended in 10% Hanks balanced salt solution with 20% fetal bovine serum, and centrifuged at 3,000 rpm for 30 min. The supernatant was used to inject tissue culture and for passage in another litter of mice. All isolates included in this study have been passaged twice in mice, except isolate 1176, which was passaged twice in mice, 3 times in Vero cells, once in sheep, and finally once in mice. One hundred microliters of mouse brain suspension was mixed with 900 mL of RNA NOWTM TC-Kit (Biogentex, Inc., Seabrook, TX, USA) and shipped to the Unité des Virus Emergents laboratory in Marseille, France.
RNA Purification, Amplification, and Sequencing

RNA was purified in a BSL-3 laboratory according to the manufacturer’s instructions. Reverse transcription (RT) of virus-specific RNA was carried out at 42°C in a 20-μL reaction that included 11 μL of RNA extract, 200 U of Superscript IITM RNase H-Reverse Transcriptase (Gibco BRL, Life Technologies, Inc., Grand Island, NY, USA), and 1 pmol of primer ALK-NS5R. Truncated noninfectious cDNA molecules were produced. PCR products were generated independently from the envelope, NS3 region, and NS5 region by using ALK-ES/ALK-ER, ALK-NS3S/ALK-NS3R, and ALK-NS5S/ALK-NS5R pairs of primers, respectively. PCR reactions were carried out in a volume of 100 μL that included 10 mmol/L Tris-HCl (pH 9.0), 1.5 mmol/L MgCl2, 50 mmol/L KCl, 0.1% Triton X-100, 200 mmol/L each deoxynucleoside triphosphate, 0.2 mmol/L of each primer, 3 μL of cDNA and 1.5 U of Taq DNA polymerase (Promega Corp., Madison, WI, USA). The thermocycler profile was 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 55°C, and 2 min at 72°C, and terminated by a final extension for 7 min at 72°C. PCR products of the expected size were purified from agarose gel slices with the Wizard PCR Preps DNA Purification System (Promega). Both strands of each PCR product were sequenced directly, with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 on an Applied Biosystems 3730x1 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the company Genome Express (Meylan, France).

Sequence Data and Phylogenetic Analysis

Sequences from the 11 AHFV isolates were compared with homologous sequences representing other tickborne flaviviruses retrieved from the GenBank database: Langat virus (LGTV) (M73835), Powassan virus (POVV) (L06436), deer tick virus (DTV) (AF311056), Omsk hemorrhagic fever virus (OHFV) (AY323489), Kysanur Forest disease virus (KFDV) (AY323490) for envelope sequence, and personal data for NS3 and NS5 sequence), tick-borne encephalitis virus (TBEV) Neudoerfl strain (U27495), Hypr strain (U39392), Vasilchenko strain (L40361), Sofjin strain (AB062064), and Louping ill virus (LIV) (Y07863). Nonvectored flaviviruses (Rio Bravo virus [NC_003675] and Apoi virus [NC_003676]) were used to root the phylogenetic tree. The nucleotide sequence alignments were generated with Clustal W 1.7 (6). Nucleotide sequence identities were calculated by the pairwise distance algorithm with the MEGA software program (7). Colinearized sequences derived from envelope, NS3, and NS5 regions were used for studying evolutionary mechanisms. Phylogenetic relationships were determined by using the Jukes-Cantor algorithm combined with either neighbor-joining (NJ) or Minimum Evolution methods implemented in MEGA. Maximum parsimony analyses were also performed in MEGA. The robustness of the resulting branching patterns was tested by bootstrap analysis with 500 replications.

Macroevolution and Microevolution

Previous data estimated the times of divergence between LIV and other member viruses of the tickborne flavivirus complex on the basis of an analysis of the rates of nonsynonymous substitutions within complete E gene sequences (8). A comparable analysis was performed here with the dataset of colinearized partial envelope, NS3, and NS5 sequences obtained as described above. In a first step, it was shown that, within the group of viruses encompassing LIV, TBEVNEU, TBEVSOF, OHFV, LGTV, KFDV, and POWV, rates of nonsynonymous substitutions in colinearized E-NS3-NS5 sequences are a linear function of rates of nonsynonymous substitutions in complete E gene sequences ($R^2 = 0.995$). Distances observed by using nonsynonymous sites were calculated according to the Nei and Gojobori algorithm implemented in MEGA (9). This method allowed plotting genetic distances (nonsynonymous substitutions) between colinearized sequences against divergence times calculated by Zanotto et al. (8), which permitted the evaluation of divergence times among AHFV isolates and between AHFV and KFDV. Using the same method, we estimated divergence times between LIV and other tickborne flaviviruses selected in this study.

Results

Epidemiologic Findings

Available epidemiologic data are presented in Table 1. The 11 isolates were recovered during a 5-year period; the first case was recorded in May 1994 and the last in June 1999. Analysis of the seasonal distribution of cases showed that they occurred in 2 peaks, lasting from March to June and from September to October, respectively (Figure 1). All cases occurred in patients originating from Mecca or Jeddah, 2 cities in Saudi Arabia 75 km apart, representing a zone of 5,000 km².

Sequence Analysis

RT-PCR amplification using primers ALK-ES/ALK-ER, ALK-NS3S/ALK-NS3R, and ALK-NS5S/ALK-NS5R produced 742-bp, 757-bp, and 723-bp products, respectively (Tables 2). Once primer sequence was excluded, the respective lengths of the sequences included in the study were 699 nt, 713 nt, and 685 nt. These sequences were deposited in GenBank under accession numbersAY727543–AY727575. All AHFV sequences were of the same length, and an optimal alignment was obtained without incorporating any gap. The genetic diversity observed
among the 11 strains of AHFV included in this study was up to 0.4%, 0.6%, and 0.9% in E, NS3, and NS5 regions, respectively, and p distance of nonsynonymous substitutions per nonsynonymous sites reached 0% in the envelope, 0.19% in the NS3 gene, and 0.77% in NS5. A total of 21 nt positions were variable, 6 of them associated with a modification of the encoded amino acid (Table 3). Fourteen of 28 observed mutations were nonsynonymous and therefore affected the protein sequence. Thirteen were located in the NS5 at nucleotide positions NS5-1679, NS5-1693, NS5-1720, NS5-1930, and NS5-2096, and 1 was located in NS3 at nucleotide position NS3-1177. The multipassaged 1176 strain did not develop more mutations than the low-passage isolates. We sequenced the homologous regions of KFDV strain P9605 (corresponding to the first human isolate, isolated from blood in 1957 by Dandavate at the Virus Research Center at Vellore field station) for comparative analysis (R.N. Charrel and X. de Lamballerie, unpub. data). In the 3 regions, the genetic heterogeneity observed between the sequences of the 11 AHFV isolates and KFDV strain was 7.3%–7.6%, 6.6%–7.2%, and 8.2%–8.8% at the nucleotide level for E, NS3, and NS5 regions, respectively.

**Phylogenetic Analysis**

Phylogenetic analyses performed independently with envelope, NS3, and NS5 gene sequences did not provide an accurate picture of the recently published topology deduced from complete coding sequences (5,10). Accordingly, a dataset of colinearized sequences was used to increase the discrimination of analysis and provide branching patterns concordant with complete sequence-based analysis. Such procedures were recently reported to reflect adequately complete genome analysis of West Nile virus strains (11). Figure 2 represents the phylogenetic reconstruction based on the analysis of E-NS3-NS5 colinearized sequences. All phylogeny algorithms used provided very similar results in terms of branching patterns. All AHFV isolates clustered together. Their closest relative was KFDV. The existence of a more divergent lineage common to AHFV and KFDV was supported by a 100% bootstrap value.

**Discussion**

Little information exists on seasonal patterns and host preferences of the tick species circulating in Saudi Arabia. However, extensive investigations of the most closely related virus, KFDV, have established that 2 species of ticks (Ixodes petauristae, I. ceylonensis) are involved in viral transmission and that they exhibit different seasonal peaks; similarly, each developmental stage has a peak of activity that corresponds to different seasons. Whether AHFV has similar features is unknown, but if so this finding could account for 2 peaks of cases during the year (8 cases from March to June, 3 cases from September to October). Two additional cases reported through ProMED in 2002 and 2004 (2,4) also occurred in March and April, thus reinforcing the evidence of a peak in frequency in the spring. Camels and sheep are believed to be the hosts that replicate AHFV, but other mammals may be involved in the natural cycle. Recent reports posted on the ProMED Web site suggest that since 1999, additional cases have occurred (3). Accordingly, AHFV is maintained through a

Table 1. Epidemiologic data available for the 11 male patients infected with Alkhurma hemorrhagic fever virus (AHFV)

<table>
<thead>
<tr>
<th>AHFV isolate</th>
<th>Date of isolation</th>
<th>Nationality</th>
<th>Occupation</th>
<th>Source of infection</th>
<th>Origin</th>
<th>Outcome</th>
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<td>87</td>
<td>May 1994</td>
<td>Egyptian</td>
<td>Butcher</td>
<td>Wound</td>
<td>Mecca</td>
<td>Recovery*</td>
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<td>228</td>
<td>May 1994</td>
<td>Egyptian</td>
<td>Butcher</td>
<td>Wound</td>
<td>Mecca</td>
<td>Death</td>
</tr>
<tr>
<td>1176</td>
<td>September 1995</td>
<td>Egyptian</td>
<td>Butcher</td>
<td>Wound</td>
<td>Mecca</td>
<td>Death</td>
</tr>
<tr>
<td>MOS</td>
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<td>Egyptian</td>
<td>Butcher</td>
<td>Wound</td>
<td>Jeddah</td>
<td>Recovery*</td>
</tr>
<tr>
<td>1209</td>
<td>October 1995</td>
<td>Saudi</td>
<td>Soldier</td>
<td>Camel raw milk</td>
<td>Jeddah</td>
<td>Recovery*</td>
</tr>
<tr>
<td>5975</td>
<td>June 1997</td>
<td>Saudi</td>
<td>Driver</td>
<td>Camel raw milk</td>
<td>Jeddah</td>
<td>Death</td>
</tr>
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<td>7344</td>
<td>March 1998</td>
<td>Saudi</td>
<td>Engineer</td>
<td>Tick bite</td>
<td>Jeddah</td>
<td>Recovery*</td>
</tr>
<tr>
<td>7466</td>
<td>March 1998</td>
<td>Egyptian</td>
<td>Butcher</td>
<td>Wound</td>
<td>Jeddah</td>
<td>Recovery*</td>
</tr>
<tr>
<td>7471</td>
<td>March 1998</td>
<td>Egyptian</td>
<td>Butcher</td>
<td>Wound</td>
<td>Jeddah</td>
<td>Recovery*</td>
</tr>
<tr>
<td>7586</td>
<td>April 1998</td>
<td>Saudi</td>
<td>Student</td>
<td>Tick bite</td>
<td>Jeddah</td>
<td>Death</td>
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<tr>
<td>9518</td>
<td>June 1999</td>
<td>Eritrean</td>
<td>Poultry worker</td>
<td>Camel raw milk</td>
<td>Jeddah</td>
<td>Recovery*</td>
</tr>
</tbody>
</table>

*Recovery without sequelae.

Figure 1. Annual distribution of the 11 cases of Alkhurma hemorrhagic fever virus infections in Saudi Arabia, 1994–1999.
cycle that needs to be clarified through veterinary and entomologic surveillance programs. As discussed, analysis of genetic data provided no evidence for multiple introductions of the virus in Saudi Arabia during the period studied; however, this must be confirmed with sequence data covering a larger time period.

Although the number of reported cases is low, case histories suggest that AHFV may have infected humans through various routes (oral, direct contact, tick bite), as previously demonstrated for other flaviviruses vectored by ticks (12). Based on a questionnaire administered by the physician when a patient was admitted, 3 different modes of infection were considered probable, i.e., skin wound (n = 6), tick bite (n = 2), and consumption of unpasteurized raw camel milk (n = 3). Working as a butcher in a slaughterhouse was the occupation with the most exposure; the 6 butchers most probably acquired AHFV infection through skin abrasions or wounds and contact with sheep-infected blood. Although, the 5 other patients did not have clear occupational exposures, 2 of them (or their relatives) reported tick bites shortly before the episode, and the 3 others (or their relatives) reported raw milk consumption. Therefore, these 5 patients may have acquired AHFV infection through tick bite or raw milk consumption, as previously documented for tick-borne encephalitis virus (12,13).

Although the human cases were acquired from different sources and through different routes, the clinical and biological features were similar; whether asymptomatic infection may occur is not known and would require seroepidemiologic studies. From 1999 to 2004, the ProMED Web site reported on 3 occasions a total of 9

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Position, per AHFV prototype sequence</th>
<th>PCR product size (bp)</th>
<th>Sequence used for analysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK-ES</td>
<td>GGATATGTTGATGATGCTCAATAA</td>
<td>1342–1364</td>
<td>742</td>
<td>699</td>
</tr>
<tr>
<td>ALK-ER</td>
<td>GCTGCAATTCACAGAAACCT</td>
<td>2083–2064</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALK-NS3S</td>
<td>CAATGAGGCTATGAGGAAGC</td>
<td>5064–5085</td>
<td>757</td>
<td>713</td>
</tr>
<tr>
<td>ALK-NS3R</td>
<td>CACAAAATCTGGCTTCTCTTC</td>
<td>5820–5799</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALK-NS5S</td>
<td>AGCAAAATCTGGCAGTTATCTGA</td>
<td>9308–9328</td>
<td>723</td>
<td>685</td>
</tr>
<tr>
<td>ALK-NS5R</td>
<td>GTCCCTGCCGGGACCAGC</td>
<td>10030–10014</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*AHFV, Alkhurma hemorrhagic fever virus; PCR, polymerase chain reaction.
†Primers excluded.

Table 2. Positions of primers used for PCR amplification and sequencing of AHFV genome and resulting sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Position, per AHFV prototype sequence</th>
<th>PCR product size (bp)</th>
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</tr>
<tr>
<td>ALK-NS5R</td>
<td>GTCCCTGCCGGGACCAGC</td>
<td>10030–10014</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*AHFV, Alkhurma hemorrhagic fever virus; PCR, polymerase chain reaction.
†Primers excluded.

Table 3. Mutated positions in the sequences of the 11 AHFV isolates included in the study

<table>
<thead>
<tr>
<th>Position/ genotype</th>
<th>Coding sequence</th>
<th>Strain</th>
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</thead>
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<td>E</td>
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<td>1062</td>
<td>1905</td>
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<td>NS3</td>
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<td>600</td>
<td>1905</td>
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<tr>
<td></td>
<td>687</td>
<td>1323</td>
</tr>
<tr>
<td></td>
<td>714</td>
<td>1356</td>
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<td></td>
<td>600</td>
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<td></td>
<td>2096</td>
<td>1905</td>
</tr>
<tr>
<td></td>
<td>2244</td>
<td>9783</td>
</tr>
</tbody>
</table>

*AHFV, Alkhurma hemorrhagic fever virus.
†By reference to the nucleotide sequence of AHFV prototype strain deposited in GenBank under accession number AF331718; shaded cell indicates mutated position; underlined nucleotides indicate nonsynonymous mutations.
cases of infection; at least 2 were fatal. The overall rate of death observed with AHFV (6/24, 25%) appears to be much higher than that currently reported for KFDV (3.0%–8.9%) (14,15) and stable during the period 1994–2004.

The genetic variability between the different isolates was low regardless of 1) time of the year during which the strain was recovered, 2) disease symptoms in the infected patients, 3) presumed route of infection, and 4) environment in which the patient lived. This pattern was observed for KFDV isolates in India, although these conclusions were established from antigenic methods rather than sequence analyses (14). This evolutionary pattern is consistent with the existence of a recent common ancestral lineage for all AHFV isolates characterized in this study. This lineage went through a period of in situ evolution in this region. The fact that all strains were recovered from humans, after suckling mouse inoculation, could bias the results by acting as a genetic filter. Whether AHFV genetic diversity is as low as implied in this study remains to be seen. This point will only be resolved when strains obtained from cattle, camels, and ticks from Saudi Arabia and neighboring countries are studied. However, because these strains were transmitted to humans through distinct pathways, they do not likely represent a specific genetic cluster associated with a specific pathogenicity.

Evolution of AHFV and closely related tickborne flaviviruses was addressed on the basis of previous estimation of the times of divergence of flavivirus species (8) (Figure 3). By using the same method, we estimated that KFDV and AHFV diverged 66–177 years ago. By comparison, the divergence between POWV and DTV, according to the same algorithm, was estimated to have occurred 275–393 years ago, and divergence between TBENEU and LIV occurred 291–411 years ago (estimated 364–498 years ago [8]). Accordingly, the divergence between KFDV and AHFV appears to be a recent event in the evolution of tickborne flaviviruses, comparable with the divergence observed between isolates of LIV (8). Regarding the common ancestor to the AHFV isolates included in this study, and when one takes the life cycle of ticks into account, the algorithm indicated that strains diverged 4–72 years ago, which implies a limited number of generations between this ancestor and the strains currently circulating in Saudi Arabia.

The high genetic similarity between all AHFV strains makes designing and developing specific and sensitive RT-PCR diagnostic assays possible. Close antigenic properties are also important for vaccine development. Among the many issues that merit future investigations, cross-protection conferred by KFDV and commercially available tickborne encephalitis virus vaccines should be tested since slaughterhouse workers appear to rank high on the risk scale and therefore may be the first population to benefit from this information.

Seroepidemiologic studies are needed in various population groups to determine the extent of AHFV infection and its geographic distribution in the Middle East.
peninsula (Yemen, Oman). Studies are needed to achieve a better understanding of the natural history of this virus but its pathogenicity in humans, specifically the prevalence of asymptomatic and symptomatic cases. The most important issues to resolve are the origin of the virus, how it is dispersed, and how it came to be in Saudi Arabia so that disease control strategies can be devised.

Acknowledgments

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References


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The spread of infectious agents through the mail has concerned public health officials for 5 centuries. The dissemination of anthrax spores in the US mail in 2001 was a recent example. In 1901, two medical journals reported outbreaks of smallpox presumably introduced by letters contaminated with variola viruses. The stability and infectivity of the smallpox virus are reviewed from both a historical (anecdotal) perspective and modern virologic studies. Bubonic plague was the contagious disease that led to quarantines as early as the 14th century in port cities in southern Europe. Later, smallpox, cholera, typhus, and yellow fever were recognized as also warranting quarantine measures. Initially, attempts were made to decontaminate all goods imported from pestilential areas, particularly mail. Disinfection of mail was largely abandoned in the early 20th century with newer knowledge about the spread and stability of these 5 infectious agents.

In January 1876, William Osler, a young Canadian physician, was recovering from a mild case of smallpox contracted while attending patients at Montreal General Hospital (Figure 1 [2]). In a letter written that same month to an old schoolmate (Arthus Jarvis), Osler described his illness and noted in closing, “You need not be afraid of this letter. I will disinfect it before sending” (2). Concern about disseminating smallpox through this letter was well founded. In his medical textbook of 1892, Osler would later write that smallpox can be conveyed by fomites: “the dried scales [of variola scabs] … as a dust-like powder … become attached to clothing and various articles …” (3).

Stability of the Smallpox Virus

Long before Osler’s time, the stability and infectivity of variola virus was well known, as illustrated by an example of germ warfare during the French and Indian War. In 1763, the British general Sir Jeffrey Amherst ordered that scab-laden “Sundries” be delivered to the Ottawa Indians of Pennsylvania. Amherst hoped thereby to induce a debilitating smallpox epidemic among the Indians and conquer them (4).

Much later in the 1860s, a professional grave robber for the Medical College of Ohio in Cincinnati became incensed at tricks played on him by medical students. He delivered the corpse of a smallpox victim recently buried to the dissecting laboratory and intentionally infected many anatomy students (5).

The stability of the smallpox virus was often noted by 18th-century physicians in debates over the comparative merits of variolation and vaccination. Razzell cited a 1792 article describing how an English amateur inoculator dried smallpox scabs in peat smoke, stored them underground covered with camphor, and used them as long as 8 years later (6).
Razzell also reported the longest supposed survival of the variola virus, which caused an outbreak of smallpox in a town in Somerset in 1759. The coffin of a villager who had died of smallpox 30 years before was exhumed for transfer to a new grave site. The sexton accidentally put his spade through the oak coffin, which released “a most nauseous stench.” The deceased was of such eminence that most of the villagers had attended the exhumation and reburial. “In a few days afterwards [sic], fourteen persons were seized with smallpox in one day” (6).

Today we would be skeptical of this story from 18th-century Somerset, but outbreaks of smallpox appeared occasionally in other isolated communities in England without any recognized living human source. For example, from 1908 to 1952, sporadic cases of variola appeared in Lancaster and Cheshire, textile mill counties. These outbreaks were likely introduced there by cotton imported from Egypt, where months before it had been contaminated with smallpox scabs (7).

Samples of the smallpox virus freeze-dried in a laboratory have been revived after storage for 20 years at Liverpool University (6). But long-term survival of this virus under natural conditions is another issue. In 1957, two London virologists using cell cultures examined smallpox scabs that had been stored in test tubes under ambient laboratory conditions. They found that the variola virus survived as long as 18 months (7). A comparable study from Leiden in 1968 used variola minor scabs collected and stored in 12 unsealed envelopes kept at room temperature. Each year the contents of a single envelope were cultured. The final envelope was examined 13 years later and, like the previous ones, showed virus particles still capable of replicating in culture (8).

The search for variola viruses surviving even longer was pursued in 1991 near Novosibirsk, Russia (9). “Bioweapons experts” searched for the variola virus in 19th-century smallpox victims mummified in the permafrost above the Arctic Circle. In the event of unusual thawing and flooding, the concern was that these corpses might become exposed and release infectious virus into the environment. In the 19th century, this region of Russia (Sakha Republic) was “ravaged by smallpox strains of extraordinary lethality” (9). Isolating and comparing them with preserved modern strains might identify genes contributing to virulence. To date, no live variola viruses have been isolated from Sakha. But the threat now is that “a sophisticated terrorist team might … go smallpox hunting on the permafrost” (9).

Smallpox Transmitted in Letters

In 1876, Osler’s concern was the danger his letter might pose to his friend Jarvis and not to cell cultures. Indeed, years later in 1901, articles in 2 respected medical journals incriminated letters as sources of 2 separate epidemics of smallpox (10,11).

The New York Medical Journal reported that smallpox had developed in a young lady in Saginaw, Michigan, after she received a letter from her sweetheart, a soldier in Alaska. He had written it while recovering from this disease. The infection subsequently spread to 33 other persons in Saginaw (10).

In addition, the April 1901 issue of the British Medical Journal reported an outbreak of 5 cases of smallpox at the Mormon headquarters in Nottingham, England, apparently after receipt of “letters or other fomites” from Salt Lake City, Utah, where smallpox was widespread (11). According to February 1901 issue of the Journal of the American Medical Association, 314 cases had been reported during the previous 3 months in Salt Lake City itself (12). The year before, the New York Times noted that Mormons opposed vaccination and had introduced a bill in the state legislature making it unlawful to compel vaccination (13).

Other Fearsome Epidemics

In the fall of 2001, anthrax spores were sent in letters through the US mail. This event resulted in 18 confirmed cases of the disease, 5 deaths, and cross-contamination of perhaps 5,000 letters (14). Not since the fifth plague of Egypt, which may have been anthrax (“a very grievous murrain,” Exodus 9), have people so panicked over the threat of this disease. However, in centuries past, many have fled from the sudden appearance of 5 other contagious diseases: smallpox, bubonic plague, yellow fever, typhus, and cholera. Malaria was such an expected seasonal affliction in many parts of the world that it was never perceived as an acute contagion.

When a smallpox epidemic struck Rome around 164 A.D., Galen is said to have hastily returned to his home in
When plague returned to London in 1665, Thomas Sydenham, a physician, prudently sought safety in the countryside. In 1779, yellow fever swept through Philadelphia, then our federal capital. Alexander Hamilton left town, and President George Washington remained at Mount Vernon until the fall frost had been reported up North. In 1813, typhus decimated the French army in Moscow, forcing Napoleon to retreat to Paris. And in 1832, when cholera came to Kentucky, US Senator Henry Clay established a tent city on his estate outside Lexington for the 2,000 citizens who fled the town.

Origin of Quarantines

In the 14th century, most citizens could not flee pestilences threatening their towns, but civil authorities sought to protect them by excluding suspected human carriers and merchandise from outside. Garrison gives a succinct history of early quarantines (15). When bubonic plague reached Europe in 1347, ports on the Mediterranean and Adriatic Sea were among the first to deny entry to ships coming from pestilential areas, notably from Turkey, the Middle East, or North Africa. Florence, on the Arno River, issued restrictions on travelers and goods as early as 1348. The Venetian Republic formally excluded “infected and suspected ships” in 1374 (15). The earliest such action in the Americans was by the Massachusetts Bay Colony in 1647 to 1648, when it barred ships coming from the West Indies thought to be carrying yellow fever (16).

The first official quarantine system is commonly ascribed to Ragusa (now called Dubrovnik), a port city located on the Dalmatian coast of the Adriatic Sea. There in 1377, and later when pestilences were abroad, incoming persons and ships were first isolated on a nearby island for 30 days (trentina) to await clinical signs of a contagion or evidence of continued good health. Detention of 40 days (quarantina) was instituted by the city of Marseille in 1383 and soon became the standard period of quarantine. Later, other cities established isolation stations on shore or on nearby islands. Ragusa’s use of an offshore island in 1377 was an early example of such a quarantine station. In spite of Ragusa’s seeming priority, various sources claim that the first such station was a pest house built on the island of Sardinia in 1453 or buildings erected at Pisa near the church of San Lazzaro in 1464 (15,17). In North America during the 1743 epidemics of smallpox and yellow fever, an early quarantine station was established in Philadelphia on Providence Island in the Schuylkill River (18). Other major U.S. cities soon thereafter organized quarantine stations to cope with later epidemics of smallpox, yellow fever, typhus, and cholera.

Lazarettos

Quarantine stations in southern Europe were originally called lazarettos. The origin of the term is uncertain. One 19th-century historian suggested that it is a corruption of the name of the church of Santa Maria di Nazaret, used as pest house in 15th-century Rome (18). But the Crusaders, who captured Jerusalem in 1099, had isolated and treated people with contagious diseases outside the city in the Hospital of St. Lazarus, the patron saint of lepers (17). In Venice (1403) quarantined ships were anchored at Lazzaretto Vecchio, an island in the lagoon. When the island acquired this particular name is not known. As noted above, in Pisa in 1464, persons were quarantined in a special building near the Church of San Lazzaro (15). An exhaustive, illustrated survey of lazarettos is given in John Howard’s 1789 treatise on the subject (Figure 2) (19).

Early Decontamination Measures

During the early Renaissance, clothing and other possessions of plague victims were often burned. In Italy and France during this period, the threat of plague compelled the destruction of great quantities of cloth prepared from cotton, wool, and silk recently imported from suspect countries. This precaution resulted in enormous economic loss and often in an immediate devastating local poverty (20).

The earliest attempts to decontaminate merchandise on ships coming from pestilential shores were made in Venice in the mid-1400s. Cargo was unloaded and fumigated with smoke from burning straw, pitch, tobacco, or even gunpowder. Cargo was also “perfumed.” This term likely derived from burning fragrant herbs, juniper berries, aromatic gums (e.g., myrrh), and resinous wood in attempts to sterilize items. Early on, smoking sulfur was frequently employed, while in 18th-century Germany a mixture of sulfur, potassium nitrate (salt peter), and wheaten bran (Raucher Pulver) was used (18).

Figure 2. Lazaretto at Genoa, founded in 1467 (19).
Disinfection of Mail

Karl F. Meyer, a physician-pathologist from Louisville, Kentucky, spent a lifetime researching the disinfection of mail (18). He determined that disinfection was first attempted in Venice around 1493 by dipping letters in vinegar. Later, other methods were used. By the early 1600s, decontamination of mail was practiced in much of Europe. In the United States in 1712, when yellow fever threatened Boston, mail from docking ships was first exposed to burning sulfur (17).

Very few letters from the early centuries of decontaminating mail are available today. But 1 rare specimen from 1485 does show evidence of having been dipped in vinegar (18). Since such treatment often rendered parts of a letter illegible, other less-damaging methods were employed, such as exposure to smoke and various fumes. The eventual widespread use of burning sulfur yielding sulfur dioxide (with its “sharp, irritating odor”) may have been based on an ancient idea that the more foul a medicine, the more effective it might be.

In the late 19th century, sulfur gave way to chlorine or formaldehyde gas. In November 2001, chlorine dioxide gas was sprayed into the partly contaminated Hart Senate Office Building, while the Postal Service used a 10% solution of bleach to “sterilize” its mail sorting centers (21). Ion beam sterilization (high-energy electrons) and x-ray radiation have been considered for use on individual letters.

Decontaminating Letters “Inside and Outside”

Sterilizing the outside of sealed envelopes did not ensure that the letter inside was safe. To allow penetration of sterilizing fumes or gases, initially envelopes were breached by cutting a small tip off one or more corners without exposing the content of the letter inside. In later years, multiple small holes were made in the envelope and its letter by means of a rastel, a hairbrush-size instrument consisting of 2 hinged metal plates (jaws), one of which held several rows of nails or small metal spikes (Figure 3 [18]). Clamping each letter between the jaws of the rastel produced several rows of small holes through the envelope and its contents, enabling gas to penetrate the interior (18).

When thousands of letters required fumigation, perforating each individually was not practical. Instead, they were laid out on screens, placed in an air-tight box (or in a boxcar; see figure on Table of Contents), and exposed to burning sulfur for \( \approx 6 \) hours (18). Sterilizing the outside of envelopes protected the mail handlers, but the reader still remained at risk from the interior.

Certification of Decontamination

Once letters had been decontaminated, some sort of certification had to be noted on them. As late as 1837, a paste or wax seal was affixed to fumigated letters. But this certification was impractical with large numbers of letters, so soon each letter was simply stamped, much like a modern-day postal cancellation. The following descriptions of these cancellation marks are taken from illustrations of some early 19th-century cachets (Figure 4 [18]).

A letter that had passed through Genoa in 1813 during the Napoleonic occupation bore the French stamp “Purifié à Gênes.” Letters stamped at Leghorn in 1829 showed “LAZZERETTO SAN ROCCO DI LIVORNO.” San Rocco was 1 of the 2 plague saints. During the early period when only the outside of envelopes were disinfected, an Italian cachet read “NETTA FUORAI E SPORCA DENTRO,” or “clean outside and dirty inside.” An 1830 cachet with the Papal insignia noted “NETTA DENTRO E FUORI” (clean inside and out). An 1831 stamp from Vienna read “Rein von innen und aussen” (clean within and out). A first-class letter from Jacksonville, Florida, in 1888 read simply “Fumigated” and thus did not define the extent of the procedure.
Decline in Mail Disinfection

By the early 20th century, plague, typhus, and yellow fever were known to be transmitted by arthropod vectors, and cholera was known to be waterborne. Since letters seemed an unlikely means of spreading pestilences, disinfection of mail declined, but some authorities continued to see a potential risk in mail from patients with tuberculosis and leprosy. Meyer noted that as late as 1953, letters leaving a German tuberculosis sanatorium were first fumigated with formaldehyde fumes. Likewise in the United States, as late as 1968 mail leaving the leprosarium at Carville, Louisiana, was first sterilized by baking in electric ovens (18).

Conclusions about Osler’s Letter

Osler did not say how he would disinfect his 1876 letter to Jarvis. The letter shows no vinegar stains. The sterilizing value of dry heat (oven) and moist heat (autoclave) was not established until 1881 by Koch and others (22). The causative agent of smallpox was not visualized microscopically until 1887, when Buist first observed small clumps of virus particles now called Guarnieri bodies in infected tissues. The variola virus was first cultivated in 1935 by Torres and Teixeria on the chorioallantoic membrane of embryonated eggs (23).

Being a pathologist, Osler may have used formaldehyde vapors to sterilize his letter. In any case, we do not hear that Jarvis ever contracted smallpox from it. Indeed, correspondence between Osler and Jarvis continued at least through 1910 (24). Osler died in 1919 and Jarvis in 1936 (L. Russell, pers. comm.).

Dr. Ambrose is professor of microbiology at the University of Kentucky School of Medicine. He has published studies on antibody production in cell cultures and has taught widely in pathogenic microbiology and the history of medicine and microbiology.

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Salmonella Derby Clonal Spread from Pork

Sylvia Valdezate,* Ana Vidal,† Silvia Herrera-León,* Javier Pozo,† Pedro Rubio,† Miguel A. Usera,* Ana Carvajal,† and M. Aurora Echeita*

The genetic diversity of the Derby serotype of Salmonella enterica in Spain was examined by pulsed-field gel electrophoresis (PFGE). Out of 24 identified PFGE profiles, a major clone was detected in 19% of strains from humans, 52% from food, and 62% from swine. This clone (clone 1) was isolated from pork products, suggesting swine as its source.

Salmonellosis, a major public health concern worldwide, is one of the most common causes of human gastroenteritis. It is caused by the ingestion of contaminated food because of a failure to control Salmonella infection in animal husbandry. Its carriers are swine, poultry, and cattle, along with eggs, milk, and fresh seafood (1). Salmonella in pork carcasses is a result of fecal contamination during slaughtering and processing. In this case, the carrier swine is the main initial source of contamination (2,3), and bacteria are usually located in the pharynx, lymph nodes, stomach, and feces (4).

During 2002, Salmonella enterica subsp. enterica serotype Derby was the second and sixth most common Salmonella serotype in clinical and nonclinical nonhuman sources, representing 24.0% and 3.7% of isolates reported to the Centers for Disease Control and Prevention and the National Veterinary Service, respectively (5). The frequency of S. Derby among isolates from animals received at the Spanish National Reference Laboratory for Salmonella and Shigella (LNRSSE) was higher than in previous years because of isolates received from swine farms. The incidence of S. Derby in Spain has remained stable in isolates of human and food origin, accounting for 0.55% and 2.5%, respectively, of Salmonella recovered from these sources from 1997 to 2003 (M.A. Echeita, unpub. data).

The persistence and clonal survival of S. Derby in swine populations and in closed environments and the role of pork and pork products as sources of outbreaks in humans have been described previously (6–8). S. Derby is one of the most common serotypes of Salmonella in swine. It accounted for 24.2% of the serotypes at 3 nurseries and 9 finishing farms of 2 commercial swine production companies in North Carolina (9), and for 37.1% at 5 swine-finishing units in Quebec, Canada (10).

To obtain epidemiologic insights into human acquisition of the S. Derby serotype compared with swine-related strains, antimicrobial susceptibility profiles were obtained and genetic characterization by pulsed-field gel electrophoresis (PFGE) was conducted in strains isolated from humans, food, and the environment. Unlike more common serotypes such as Enteritidis or Typhimurium, this serotype has not been subjected to intensive molecular epidemiologic studies.

The Study

A total of 110 S. Derby isolates collected during a 3-year period (2000–2002) were studied. Forty-seven of these isolates (LNRSSE group) were submitted by 34 Spanish laboratories and had the following origins: humans (n = 16), food (n = 23), ill animals (n = 6), and environmental sources (n = 2), as shown in Table 1. The remaining 63 isolates (swine group) were obtained from the stools and mesenteric lymph nodes of 48 swine slaughtered at 4 swine operations (A, B, C, and D) and 6 finishing units (units 1–6) (Table 2) in Spain. The swine reared on these farms shared the same feed supply and had similar genetic backgrounds.

The isolates were screened for antimicrobial resistance by the disk diffusion method (Clinical and Laboratory Standards Institute, Wayne, PA, USA). All isolates were susceptible to amoxicillin-clavulanate, cephalothin, cefuroxime, cefotaxime, ceftriaxone, cefoxitin, imipenem, ciprofloxacin, and enrofloxacin. All strains but 1 were resistant to spectinomycin and had variable resistances to the remaining antimicrobial agents tested (Tables 1 and 2).

A total of 70% and 85.7% of the isolates in the LNRSSE and swine groups were resistant to tetracycline, 36.6% and 85.7% to streptomycin, 33.3% and 71.4% to sulfonamides, 23.3% and 50% to ampicillin and ticarcillin, 16.6% and 42.8% to cotrimoxazole, and 13.3% and 7.1% to nalidixic acid, respectively (Tables 1 and 2). Phenotypes in both groups were most commonly resistant to tetracycline and sulfamethoxazole/tetracycline. Multiple drug resistance (resistance to ≥4 drugs), was observed in 23.3% of LNRSSE isolates and 64.3% of swine isolates.

In addition, the MICs for nalidixic acid and ciprofloxacin were determined in 7 nalidixic acid–resistant strains by the E test (AB Biodisk Dalvagen, Solna, Sweden). The results of these tests are shown in Tables 1 and 2. GyRA, GyRB, ParC, and ParE point mutations in these isolates were genetically characterized (11). The results showed amino acid substitutions only in GyRA: Ser83 → Phe or Tyr and Asp87 → Asn or His (GenBank accession no. AY858891) (Tables 1 and 2).

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Table 1. Characteristics of *Salmonella enterica* Derby serotype isolates (N = 47) from the LNRSSE Group (Spanish National Reference Laboratory for *Salmonella* and *Shigella*)

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Isolation date</th>
<th>Geographic origin</th>
<th>PFGE profile</th>
<th>Resistance profile</th>
<th>GyrA mutation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human origin (16 strains)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9/4/01</td>
<td>Barcelona</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3/5/02</td>
<td>Lugo</td>
<td>4</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4/25/02</td>
<td>Madrid</td>
<td>3</td>
<td>S,Su</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4/25/02</td>
<td>Unknown</td>
<td>NT</td>
<td>A,S,Su,T,C,Nx</td>
<td>Tyr 83 (&gt;256/0.12)</td>
</tr>
<tr>
<td>5</td>
<td>5/23/02</td>
<td>Orense</td>
<td>10</td>
<td>A,S,Su,T,C</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7/27/02</td>
<td>Castellón</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7/27/02</td>
<td>Barcelona</td>
<td>11</td>
<td>A,S,G,To,Ap,Su,T,SxT</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>7/8/02</td>
<td>Zaragoza</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>8/8/02</td>
<td>Barcelona</td>
<td>1</td>
<td>Su,T,SxT</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8/20/02</td>
<td>Tarragona</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>8/22/02</td>
<td>Alicante</td>
<td>16</td>
<td>S,T,Nx</td>
<td>Asn 87 (64/0.09)</td>
</tr>
<tr>
<td>12</td>
<td>7/8/02</td>
<td>Oviedo</td>
<td>1</td>
<td>S,Su,T</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>9/1/02</td>
<td>Zamora</td>
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<td></td>
</tr>
<tr>
<td>14</td>
<td>9/30/02</td>
<td>Huesca</td>
<td>16</td>
<td>T</td>
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</tr>
<tr>
<td>15</td>
<td>11/28/02</td>
<td>Ciudad Real</td>
<td>23</td>
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<tr>
<td>16</td>
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<td>Caceres</td>
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<td>Food origin (23 strains)</td>
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<td>18</td>
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</tr>
<tr>
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<td>Cádiz</td>
<td>7</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>Pork</td>
<td>6/18/00</td>
<td>Granada</td>
<td>23</td>
<td>Nx</td>
<td>Phe 83 (&gt;256/0.09)</td>
</tr>
<tr>
<td>Pork</td>
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</tr>
<tr>
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<tr>
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<td>NT</td>
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<td>Hamburger</td>
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<td>Nx</td>
<td>Phe 83 (&gt;256/0.1)</td>
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<td>T</td>
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<td>Pork</td>
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<td>Environmental origin (2 strains)</td>
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<td>Valencia</td>
<td>12</td>
<td>S,T</td>
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<td>Beach</td>
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<td>Bilbao</td>
<td>1</td>
<td>Su,T,SxT</td>
<td></td>
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<tr>
<td>Ill animal origin (6 strains)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>Soria</td>
<td>14</td>
<td>A,T</td>
<td></td>
</tr>
<tr>
<td>Swine</td>
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<td>Toledo</td>
<td>1</td>
<td>S,Su,T</td>
<td></td>
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<tr>
<td>Swine</td>
<td>3/7/02</td>
<td>León</td>
<td>6</td>
<td>S,T</td>
<td></td>
</tr>
<tr>
<td>Swine</td>
<td>3/7/02</td>
<td>León</td>
<td>6</td>
<td>S,T</td>
<td></td>
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<tr>
<td>Swine</td>
<td>3/6/02</td>
<td>León</td>
<td>6</td>
<td>S,T</td>
<td></td>
</tr>
<tr>
<td>Swine</td>
<td>1/2/02</td>
<td>León</td>
<td>9</td>
<td>Su,T,SxT</td>
<td></td>
</tr>
</tbody>
</table>

*PFGE, pulsed-field gel electrophoresis; Nx, nalidixic acid; Cp, ciprofloxacin; T, tetracycline; S, streptomycin; Su, sulfamethoxazole; NT, not typeable; A, ampicillin; C, chloramphenicol; G, gentamicin; To, tobramycin; Ap, apramycin; SxT, cotrimoxazole.

†MIC for Nx/Cp, μg/mL.
To define clusters, PFGE was carried out according to the Salm-gene protocol (12) by using the CHEF-DR II System (Bio-Rad Laboratories, Hercules, CA, USA). Fingerprints were compared with GelCompar (Applied Maths, Kortrijk, Belgium). Pattern clustering was performed by using the unweighted pair group method with an arithmetic mean and the Dice coefficient (13) with a tolerance of 0.8%. Each PFGE profile or clone, which differed by at least 1 band from a previously recognized type, was considered a distinct profile. Twenty-four PFGE profiles were observed among the 110 strains (Figure 1). A total of 10, 9, 2, 4, and 6 clones were detected in strains from human, food, environmental, sick animal, and slaughtered swine sources, respectively (Tables 1 and 2). Twelve clones displayed closely related profiles, and paired PFGE profiles differed from each other by 1 band (1-2, 2-3, 4-5, 9-10, 12-13, and 12-14), 2 bands (1-3 and 13-14), or 3 bands (16-18) (Figure 2).

Eleven PFGE profiles (10.0% of the strains) were unique. Conversely, PFGE profile 1 accounted for 52.7% of all isolates studied: 3 from humans, 12 from food, 1 from the environment, 1 from an ill swine, and 42 from 30 slaughtered swine (Tables 1 and 2). PFGE profile 8 represented 10.0% of the characterized strains isolated from 11 swine slaughtered at operation B finishing unit B3. No other PFGE profile accounted for >3% of the strains. In the 63 strains obtained from 48 swine slaughtered, 6 PFGE profiles were observed: 1 (62.5%), 2 (4.2%), 8 (22.9%), 15 (4.2%), 21 (2.8%), and 22 (4.2%).

The corresponding values of genetic similarity ranged from 32% to 96% according to the dendrogram constructed with the different PFGE profiles (Figure 2). Typeability was ≈96.3% because autodigestion occurred in 4 strains. The discriminatory ability calculated by using the Simpson index was 0.9931 for the LNRSSE group, which was selected without a geographic link for any isolates.

### Table 2. *Salmonella enterica* Derby strains (N = 63) from the swine group

<table>
<thead>
<tr>
<th>Swine operations/ finishing units</th>
<th>No. of PFGE profiles/resistance patterns</th>
<th>No. of PFGE profiles (no. of swine)</th>
<th>Resistance phenotype (no. of swine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1 (6 strains)</td>
<td>1/2</td>
<td>1 (5)</td>
<td>S,G,T,Su,T,C (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A,S,G,T,No,Su,T,Nr,C (4)†</td>
<td></td>
</tr>
<tr>
<td>A-2 (3 strains)</td>
<td>1/1</td>
<td>1 (3)</td>
<td>A,S,G,Su,T,C (3)</td>
</tr>
<tr>
<td>B-3 (16 strains)</td>
<td>2/7</td>
<td>2 (4)</td>
<td>S,Su,T (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 (11)</td>
<td>S,Su,T,C (1)</td>
</tr>
<tr>
<td>C-4 (1 strain)</td>
<td>1/1</td>
<td>1 (1)</td>
<td>S,Su,T (1)</td>
</tr>
<tr>
<td>D-5 (32 strains)</td>
<td>3/3</td>
<td>1 (17)</td>
<td>S,Su,T (17)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (2)</td>
<td>S,Su,T (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21 (1)</td>
<td>A,S,Su,T,SxT (1)</td>
</tr>
<tr>
<td>D-6 (5 strains)</td>
<td>2/4</td>
<td>22 (2)</td>
<td>A,S,G,T,Ap,Su,SxT,C (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 (2)</td>
<td>A,S,G,T,No,Su,T,SxT,C (1)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>T (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A,S,G,T,No,T (1)</td>
</tr>
</tbody>
</table>

*Identified in 48 swine slaughtered at 4 swine operations (A–D) and 6 finishing units (1-6) in Spain. PFGE, pulsed-field gel electrophoresis; S, streptomycin; G, gentamicin; T, tobramycin; Su, sulfamethoxazole; T, tetracycline; C, chloramphenicol; A, ampicillin; Ni, netilmicin; SxT, cotrimoxazole.
†GyrA mutation (MIC nalidixic acid/ciprofloxacin): Hys 87 = 48.0.09 g/mL.
‡Susceptible to all antimicrobial agents tested except spectinomycin.

Figure 1. Twenty-one pulsed-field gel electrophoresis profiles identified in Derby strains of *Salmonella enterica*. A) profiles 3, 1, 2, 4, 5, and 6 (lanes 1–6); B) profiles 20, 21, 23, 14, 12, 14, 13, 14, 15, 9, 11, 10, 16, 17, 7, and 19 (lanes 1–15). The control strain (Braenderup serotype) used as a molecular marker in shown in lane M.
Conclusions

The study was conducted because of the *S.* Derby isolate, detected through *Salmonella* surveillance programs, emerged on several swine farms in Spain. We investigated the genetic diversity of *S.* Derby isolates in these herds by using PFGE to obtain information on their genetic backgrounds.

The predominant clone (clone 1) was identified in 42 strains isolated from 30 animals at 4 swine operations and 5 finishing units. It appeared as a single clone in 3 swine operation finishing units (A-1, A-2, and C-4), or in combination with other PFGE profiles (8 for B-3; 2 and 21 for D-5).

When the persistence of a clone was detected, we tracked its spread from food products to humans by testing *S.* Derby strains isolated from humans, food, the environment, and ill animals in different areas of Spain. Clone 1 was identified in 18.7% of human strains, 52.2% of food strains, as well as in a strain collected on a beach and in another from an ill animal. Moreover, a closely related clone (clone 2) was detected with clone 1 in the swine operation finishing unit D4. This finding might be the result of these clones sharing a common ancestor. The dissemination of clone 1 may be more widespread because LNRSSE group isolates were selected on the basis of no epidemiologic links and were not associated with national or local food outbreaks.

Because resistance of *Salmonella* to antimicrobial agents is a worldwide problem, drug susceptibility was also examined. Resistance to amoxicillin-clavulanate, cephalosporins, imipenem, and fluoroquinolones was not detected. However, resistance to tetracycline was high (>75%), which is similar to that recorded in a study of 304 *S.* Derby swine isolates (9), but differs from that obtained in human and food isolates (49%) (14). In addition, susceptible isolates from the swine group showed increased resistance to other drugs when compared with those in the LNRSSE group. No relationship was observed between the time of isolation of a specific clone and increased resistance.

A novel GyrA mutation (Hys 87) was identified in 4 swine strains of clone 1 that showed resistance to nalidixic acid and low-level resistance to ciprofloxacin. These strains, which also showed resistance to other antimicrobial agents (ampicillin, streptomycin, gentamicin, tobramycin, netilmicin, tetracycline, and sulfamethoxazole), may be attributed to multiple resistance factors present on transmissible genetic elements (15).

In summary, this study detected a clone of *S.* Derby in pork-derived products and subsequently in humans. Our results emphasize the need for safe food-handling practices to limit the intake of raw or undercooked pork, and procedures to avoid colonization of swine herds with *Salmonella*. They also support establishing guidelines to reduce foodborne pathogens on swine farms and in slaughterhouses. Moreover, because of the emergence of bacterial resistance to antimicrobial agents as a result of their extensive use in animal husbandry, measures such as modification of doses of antimicrobial agents, control of *Salmonella* infections in primary production facilities, and effective epidemiologic surveillance must be implemented to determine potential routes and spread of infection.

Acknowledgments

We thank the laboratories in Spain for sending strains to LNRSSE.

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Dr. Valdezate is a microbiologist in the bacteriology department at the Instituto de Salud Carlos III in Majadahonda, Spain. Her research focuses on the transmission and dynamics of nosocomial and foodborne infectious diseases by using molecular typing methods.

References


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Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
Probable Tiger-to-Tiger Transmission of Avian Influenza H5N1

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During the second outbreak of avian influenza H5N1 in Thailand, probable horizontal transmission among tigers was demonstrated in the tiger zoo. Sequencing and phylogenetic analysis of those viruses showed no differences from the first isolate obtained in January 2004. This finding has implications for influenza virus epidemiology and pathogenicity in mammals.

In mid-January 2004, an epizootic outbreak of highly pathogenic avian influenza (HPAI H5N1 strain) was reported in poultry and various other birds in Thailand (1). Two tigers (Panthera tigris) and 2 leopards (P. pardus) in a zoo in Suphanburi, Thailand, died after experiencing high fever and respiratory distress; H5N1 infection was later confirmed as the cause of the illness (2). The animals had been fed raw chicken carcasses that were possibly contaminated with the HPAI H5N1 virus. A tiger zoo in Sriracha, Chonburi, Thailand, was affected by HPAI beginning on October 11, 2004.

The Study

The site of the HPAI outbreak is the biggest tiger zoo in Thailand, housing 441 tigers in 3 zones: breeder, nursery, and grower. The outbreak initially involved 16 tigers from 6 to 24 months of age in the grower zone. This zone was open for display; the other 2 zones were restricted areas. Laboratory findings in specimens from these animals included severe leukopenia and thrombocytopenia and increased levels of the liver enzymes alanine aminotransferase and aspartate aminotransferase (data not shown). Three days later, 5 tigers had died and 14 displayed varying degrees of clinical symptoms, including high fever and respiratory distress. All specimens submitted to the National Institute of Animal Health laboratory were positive for HPAI H5N1 by real-time polymerase chain reaction (PCR) and virus isolation and later confirmed by reverse transcriptase-PCR (3). On October 16, 2004, the tigers were fed cooked chicken carcasses or pork. Samples of raw chicken carcasses from the local suppliers were tested by using egg inoculation; 1 sample was positive for H5N1 virus. All animals that died had serosanguinous nasal discharge, and some had neurologic signs of infection. Necropsy was performed on 3 tigers on October 18. The lungs were severely congested and had been hemorrhaging. Serosanguinous exudate was seen throughout the tracheal and bronchiolar lumen, and pleural effusion was also seen. Microscopic findings showed moderate congestion of the brain with mild nonsuppurative meningoencephalitis (Figure 1A), severe diffuse lung hemorrhage and edema, and moderate multifocal necrotizing hepatitis. Immunohistochemical procedures were performed on all tissue by using mouse monoclonal antibody to the nucleoprotein of influenza A H5N1 (B.V. European Veterinary Laboratory, Woerden, the Netherlands). Strongly positive, brown staining was prominently displayed in the nuclei of the hepatocytes and in the cerebral neurons. Positive staining of both nuclei and cytoplasm was also apparent in the neurons (Figure 1B) and bronchiolar epithelium.

Because the number of sick animals increased, the moribund animals were euthanized on October 20, 2004. Oseltamivir (Roche, Basel, Switzerland) (75mg/60 kg) was administered twice daily to the healthy tigers (4). On October 23, another sick tiger was found in the grower zone. The increasing numbers of sick tigers demonstrated the potential of tiger-to-tiger transmission (Figure 2). The incubation period was determined to be ≈3 days, since the infected tigers died 3 days after clinical signs were observed. Nasal swabs and blood samples were obtained from both infected and healthy tigers in the other 2 zones. Only 3 nasal swab samples from the infected zone were positive. Rectal swabs were then taken from the 3 sick animals; 1 was positive on egg inoculation. The remaining 42 tigers in the infected zone were euthanized to stop tiger-to-tiger transmission on October 28; a total of 147 tigers died or were euthanized. One week after the tigers in the grower zone were euthanized, 55 nasal swabs and serum
samples (18.7%) obtained from the tigers in the other 2 zones were tested every other week for a 2-week period by using real time-PCR and egg inoculation. None of the samples was positive. Four weeks after the last tiger was infected, 58 serum samples were obtained from all humans that had been in contact with either the animals or their tissue or fluid, including veterinarians; these samples were tested twice by microneutralization for the H5N1 virus. Seroconversion (1:80, ≥1:640) occurred 6 weeks after the incident in only 2 (3.5%) persons who had shown no clinical signs of illness. To mitigate the risk for human infection and the potential for genetic reassortment, all persons involved in the incident were advised to take the commercially available human influenza vaccine (H3N2) for the 2003–2004 season.

Epidemiologic surveillance tests for the H5N1 virus were performed on the zoo animals and 11 wild avian species near the tiger zoo. Neither sickness nor death from the H5N1 virus had been observed in any of these animals before. Before reopening the zoo 3 weeks after the last infected tigers had died or been euthanized, water and soil samples or swabs from the tigers’ cages were tested by using egg inoculation. None of the samples was positive.

Influenza A virus was isolated from the sick tigers’ nasal swabs; A/Tiger/Thailand/CU-T3/04 (HA and NA gene; AF842935-6; PB2 and NS gene; AY907672-3) was initiated from those pretreated with oseltamivir, and A/Tiger/Thailand/CU-T7/04 (hemagglutinin [HA] and neuraminidase [NA] gene; AY866475-6, PB2; AY907671 and NS; AY907674) was isolated from those posttreated. Sequencing and phylogenetic analysis of the HA and NA genes of the H5N1 isolates in this outbreak showed that they were similar to each other as well as to those of the virus obtained from the earlier cases identified in 2004 (data not shown). The HA gene contained a glutamine at position 222 (226 in H3) in HA1 and 4 polybasic amino acid insertions at the cleavage site, which had also been found in other recent H5N1 isolates from chicken and tigers (1,2). However, no mutation of histidine to tyrosine was seen at position 274 of the NA molecule after oseltamivir treatment. In both isolates, a single amino acid substitution, glutamine to lysine, was observed at the position 627 in the PB2 protein responsible for H5N1 pathogenicity in mammals (5), and a 5-codon deletion was found in the NS gene, similar to the H5N1 viruses isolated in the same epidemic.

Conclusions

The H5N1 virus is fatal to tigers, a conclusion also drawn in our previous report (2). The last case of the H5N1 virus infection was found in the tigers on October 28, 2004. The tigers had not been fed raw chicken carcasses in ≈12 days, and no other avian or mammal species kept in the zoo had been infected during this outbreak. Our results demonstrated that tigers kept in captivity are at risk for
infection with and dying of the H5N1 virus; moreover, they could be infected by horizontal transmission since raw chicken carcasses represent the main food item for them. Alternative food for the tigers should be considered to reduce the risk for infection. Epidemiologic data obtained from this study demonstrated that all tigers that became ill after October 23, 2004, were probably infected by horizontal transmission since the animals had not been fed raw chicken carcasses since October 16. Administration of oseltamivir therapy could suppress and prolong the incubation period of the H5N1 virus infection, but it is unlikely.

To date, illness in tigers due to H5N1 infection is of the same severity as that in the H5N1 virus in cats (6). The serosanguinous nasal discharge seen in the sick tigers before death is likely due to severe thrombocytopenia. Results of laboratory findings, except liver enzyme levels, for the sick tigers were similar to the findings reported earlier in the pediatric cases (7). Positive staining for the NP protein of influenza A in the nuclei of the hepatocytes might indicate that a heavy virus load had passed through the digestive tract after the infected chicken carcasses were eaten, affecting the liver, particularly the hepatocytes, and possibly causing hepatic failure. Unlike results derived from experiments with cynomolgus monkeys (8), we were able to demonstrate H5N1 viral antigen in several organs of the infected tigers. The evidence of nonsuppurative encephalitis shown in the previous study (2) confirmed the involvement of H5N1 virus, as was apparent by using immunohistochemical procedures. H5N1 infection in tigers can induce neurologic signs and encephalitis similar to that observed in other mammals (9). Neurotropism of the H5N1 virus in mice as part of the pathogenesis subsequent to infection by human influenza virus isolates has been reported (10). Further studies will be required to elucidate the pathogenesis of the H5N1 virus in felines.

In vitro studies have demonstrated the potent antiviral activity of oseltamivir against all strains of influenza A tested, including the avian H5N1 virus recently implicated in human influenza cases in Hong Kong (11). In this study, the failure of treatment might have been attributable to various factors such as dosage, pharmacokinetics, or host metabolism, since no changes were seen in the neuraminidase. Application of antiviral therapy to those sick tigers has not been sufficiently researched and, hence, requires additional data.

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This work was conducted at the Faculty of Veterinary Science and Faculty of Medicine, Chulalongkorn University.

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References


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The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.
Highly Pathogenic H5N1 Influenza Virus in Smuggled Thai Eagles, Belgium

Steven Van Borm,* Isabelle Thomas,† Germaine Hanquet,† Bénédicte Lambrecht,* Marc Boschmans,* Gérald Dupont,† Mireille Decaestecker,* René Snacken,† and Thierry van den Berg*

We report the isolation and characterization of a highly pathogenic avian influenza A/H5N1 virus from Crested Hawk-Eagles smuggled into Europe by air travel. A screening performed in human and avian contacts indicated no dissemination occurred. Illegal movements of birds are a major threat for the introduction of highly pathogenic avian influenza.

The 2003–2004 highly pathogenic avian influenza epidemic caused by an A/H5N1 virus has become established in 8 Southeast Asian countries (1). It affects not only birds but several mammals (2–4). As of February 2005, 55 laboratory-confirmed human cases, 42 fatal, have occurred after direct transmission of the virus (available from http://www.who.int/csr/disease/avian_influenza/country/en/).

The Study

On October 18, 2004, 2 Crested Hawk-Eagles (Spizaetus nipalensis) smuggled into Europe from Thailand were seized at Brussels International Airport (5). Clinical examination of the birds showed no symptoms. As import of birds and products from several Asian countries into the European Union (EU) is forbidden (DG Sanco Decision 2004/122/EC), the birds were humanely sacrificed and immediately sent to the Veterinary and Agrochemical Research Centre for routine diagnosis to exclude influenza and Newcastle disease viruses.

The eagles were transported in a hand luggage (Figure 1), with the zipper not totally closed to allow air to enter. The bird smuggler, a Thai resident, took connecting flights from Bangkok to Brussels, with a stopover in Vienna; he placed his hand luggage in an overhead compartment during both flights. When interviewed, the Thai man declared that he “bought the eagles on a major Bangkok market, a few days before departure, as a present for [his] brother living in Belgium.” The eagles reportedly had no contact with domestic animals before departure. A few days later, a Belgian falconer declared he had ordered the eagles and offered €7,500 for each bird.

Necropsy and further testing were carried out at the Veterinary and Agrochemical Research Centre (virologic procedures according to EU Council Directive 92/40/EEC of 19 May 1992). Both eagles had enteritis, and 1 had bilateral pneumonia. Samples were taken from lungs and injected into embryonating eggs, which died in <2 days. The isolated virus was denominated A/crested eagle/Belgium/01/2004. The antigenic subtyping as H5N1 was made by hemagglutination inhibition using 12 monospecific polyclastra. The diagnosis was confirmed by nucleoprotein gene (general for type A influenza) and H5-specific reverse transcriptase–polymerase chain reaction (RT-PCR) (primers summarized in Table). Sample quality was controlled by using 18S rRNA as housekeeping control gene

Figure 1. Crested Hawk-Eagles confiscated at Brussels International Airport in the hand luggage of a Thai passenger. The birds were wrapped in a cotton cloth, with the heads free, and each of them inserted in a wicker tube ~60 cm in length, with 1 end open. Pictures courtesy of Paul Meuleneire, custom investigations officer, antidrug group.
with bird-conserved primers (Table), validated against a variety of bird species (including chicken, duck, goose, and crow) and tissues (spleen, brain, lung, trachea, cecum, liver; unpub. data). The high pathogenicity of the virus was confirmed by measuring the intravenous pathogenicity index in chickens (IVPI = 2.94).

After tracing birds that had passed through the customs inspection center during the at-risk period, the Federal Agency for Food Chain Safety killed several batches of birds, notably 2 parrots at the customs inspection center, 200 parrots in a quarantine center, and 450 birds in another quarantine center. They were tested by RT-PCR and virus isolation on embryonating egg or cell culture. All were negative for the H5N1 strain. As soon as the results from the tested hawk-eagles were known, 25 persons who had been in direct or indirect contact with the infected eagles in Brussels airport were rapidly traced back, examined, and given oseltamivir prophylaxis. The Thai man and a close contact went to the police immediately after hearing about the diagnosis of avian influenza in the news. They were immediately brought to Antwerp University Hospital, put into an isolation unit for 4 days, monitored, and given oseltamivir prophylaxis. All exposed persons remained asymptomatic, except for the veterinarian who euthanized the birds; bilateral conjunctivitis developed 2 days after he examined the birds. His family was given prophylaxis as well.

The Scientific Institute of Public Health informed the health authorities of all EU member states, as well as the appropriate EU authorities. Passengers who had taken the same flights as the bird smuggler were informed by the media to seek medical advice if they had any influenzalike symptoms within 7 days after the flight. Swabs (2 nasal and 1 throat) from 23 persons (21 custom officers, the smuggler, and a close contact) as well as a tear swab collected from the veterinarian, who had conjunctivitis, were tested by using nested RT-PCR to achieve maximal sensitivity: one to detect influenza A and B and the second for subtyping of influenza A H5 (Table). Primers from the human β-actin gene were used as housekeeping genes for control and designed by using a published sequence (GenBank accession no. M10277) (Table). All samples (including the tear swab) were negative for influenza A or B and confirmed negative for H5. The conjunctivitis tear swab sample was confirmed to be negative by injection into embryonating eggs.

A 645-bp part of the hemagglutinin (HA) gene, spanning the HA cleavage site, was sequenced 3 times from each of 3 independent RT-PCR amplification products (primers specified in Table). The amplicons were cloned into a pCR2.1-TOPO vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA) and plasmid DNA from positive colonies was purified (Qiaprep miniprep kit, Qiagen, Valencia, CA, USA) before sequencing (BigDyeTerminator v.3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA, USA). The 645-bp partial HA sequence (GenBank accession no. AY861372) of A/crested eagle/Belgium/01/2004 contained 5 mutations relative to its nearest isolate A/Ck/Thailand/9.1/2004 (H5N1) (GenBank accession no. AY651328; identity score

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**Table. Primers used for human (h) and avian (a) diagnosis, subtyping, and sequencing**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Specificity</th>
<th>Host</th>
<th>Primer sequence (5′–3′)</th>
<th>PCR product (bp)</th>
<th>Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP1200F</td>
<td>Flu A</td>
<td>h, a</td>
<td>CAGRTACTGGCCHATAAGRAC</td>
<td>330</td>
<td>(6)</td>
</tr>
<tr>
<td>NP1529R</td>
<td>Flu A</td>
<td>h, a</td>
<td>GCATTGTCTCGGAAGAATAAG</td>
<td>900</td>
<td>(7)</td>
</tr>
<tr>
<td>NP 1222 F</td>
<td>Flu A</td>
<td>h</td>
<td>CAGRAYGGGAGGAGGACAAYAC</td>
<td>245</td>
<td>This study (IT)</td>
</tr>
<tr>
<td>NP 1467 R</td>
<td>Flu A</td>
<td>h</td>
<td>CGGGYTCGTGGCTTTCGTGC</td>
<td>767</td>
<td>(7)</td>
</tr>
<tr>
<td>BHA2</td>
<td>Flu B</td>
<td>h</td>
<td>GTGACTGTTGTATACACTC</td>
<td>545</td>
<td>(6)</td>
</tr>
<tr>
<td>H5-155F</td>
<td>Flu A/H5</td>
<td>h, a</td>
<td>ACACATGCYACGACATACT</td>
<td>401</td>
<td>Mod. from (8)</td>
</tr>
<tr>
<td>H5 2 R</td>
<td>Flu A/H5</td>
<td>h</td>
<td>TAATTTCTCTATCCTCCTTTCAA</td>
<td>645</td>
<td>This study (IT)</td>
</tr>
<tr>
<td>H5 IS 1F</td>
<td>Flu A/H5</td>
<td>h</td>
<td>CTTGCCAGTGCGCTCAGAAT</td>
<td>181</td>
<td>This study (IT)</td>
</tr>
<tr>
<td>β act 5</td>
<td>β lactine</td>
<td>h</td>
<td>AACACCCAGCCATGTAC</td>
<td>144</td>
<td>This study (IT)</td>
</tr>
<tr>
<td>β act 6</td>
<td>β lactine</td>
<td>h</td>
<td>GAGTCTAGTCAGTCCCG</td>
<td>181</td>
<td>This study (IT)</td>
</tr>
<tr>
<td>β act 7</td>
<td>β lactine</td>
<td>h</td>
<td>AACACCCAGCCATGTAC</td>
<td>144</td>
<td>This study (IT)</td>
</tr>
<tr>
<td>β act 8</td>
<td>β lactine</td>
<td>h</td>
<td>GCCAGCCAGGCAGGCA</td>
<td>401</td>
<td>This study (SVB)</td>
</tr>
<tr>
<td>18S-F908</td>
<td>18S</td>
<td>a</td>
<td>AGGGAAGCCTTTGCAAGA</td>
<td>645</td>
<td>This study (MB)</td>
</tr>
<tr>
<td>18S-R1309</td>
<td>18S</td>
<td>a</td>
<td>AGTCTCGTGGTTATCGAAAT</td>
<td>645</td>
<td>This study (MB)</td>
</tr>
<tr>
<td>H5-614F</td>
<td>HA sequencing</td>
<td>a</td>
<td>GARGAYCTTTRATHTRTGG</td>
<td>645</td>
<td>This study (MB)</td>
</tr>
<tr>
<td>H5-1259R</td>
<td>HA sequencing</td>
<td>a</td>
<td>CTYCRXNHTGRTXGTCTATT</td>
<td>645</td>
<td>This study (MB)</td>
</tr>
</tbody>
</table>

*IT, Isabelle Thomas; SVB, Steven Van Borm; MB, Marc Boschmans; Mod., modeled.*
0.992), including 1 change in the HA cleavage site, resulting in a unique arginine (R) > lysine (K) replacement, which contains 6 basic amino acid residues KRRKKR, whereas all Thai isolates from 2003 and 2004 have RRRKKR. As the occurrence of multiple basic amino acids in the cleavage site marks highly pathogenic H5 and H7 influenza A strains (9), this finding confirms the results of the IVPI experiment. We aligned the partial HA sequence to 35 representative GenBank entries (ClustalW multiple alignment) and calculated a neighbor-joining phylogenetic tree based on this alignment (Figure 2 [10]). High bootstrap values support the clustering of A/crested eagle/Belgium/01/2004 together with strains from the current Asian H5N1 epidemic. Moreover, all of these 2004 Thai-Viet strains clearly belong to the previously described highly pathogenic Z genotype cluster (10).

Conclusions

The simultaneous resurgence of the H5N1 epidemic across several countries during the summer of 2004 indicated that the virus has become endemic in Asian poultry. This situation represents an increasing threat for public health since constant multiplication and circulation of the virus might help selecting mutations that adapt the virus to new hosts. This continuous evolution (10) is supported by our study, which showed that, although the A/crested eagle/Belgium/01/2004 virus still belongs to the Z genotype, an amino acid change was observed in the HA cleavage site. The exchange of an arginine by a lysine conserved 6 basic amino acids in the cleavage site, a molecular structure demonstrated by reverse genetics studies as being the optimal sequence for highly pathogenic avian influenza virus cleavability and pathogenicity (11).

Although *Spizaetus nipalensis*, a CITES2-listed species (Convention on International Trade in Endangered Species), is well distributed in the H5N1 affected regions in Thailand (12), no details are currently available that might explain how the birds got infected. One possibility is that they were fed infected chicken carcasses before their departure to Europe. The birds may thus have been infected very shortly before their transportation. This scenario might explain why no clinical symptoms were found. Alternatively, avian wildlife may have a higher resistance to the disease before they exhibit clinical signs and then die suddenly. Preliminary quantitative real-time RT-PCR data collected in our laboratory (data not shown) support this explanation as they show a viral load in the eagles similar to poultry infected with highly pathogenic avian influenza (13). In a zoo in Cambodia, a variety of free-flying and captive birds, including raptors, were reported as being infected with the H5N1 virus (available from http://www.fao.org/docs/eims/upload/159535/AVIbull016.pdf). The disease appeared first in raptors, including hawk-eagles, in the first 2 to 3 days, indicating their high susceptibility. They were most probably fed infected chicken carcasses. The only other report of H5N1 in wild raptors consists of a single peregrine falcon found dead in Hong Kong (available from http://www.oie.int/eng/info/hebdo/AIS_60.htm). Unfortunately, no histopathologic confirmation of the cause of death or viral load assessment studies about this case have been communicated. There have also been 2 reports of avian influenza infections of falcons with H7 highly pathogenic avian influenza virus. Manvell et al. (14) reported the isolation of a highly pathogenic avian influenza virus of H7N3 subtype from a peregrine falcon dying in the United Arab Emirates. During the highly pathogenic avian influenza virus outbreaks in Italy in 2000, an H7N1 virus was isolated from a Saker Falcon that died 3 days after normal hunting activity (15). The raptor had a sudden onset of depression, weakness, and anorexia the day after normal hunting activity and died 2 days later without further clinical signs.

This study demonstrates that international air travel and smuggling represent major threats for introducing and disseminating H5N1 virus worldwide. Hunting with falcons is practiced in several countries around the world. Here,
the falconer who ordered the birds already owned 4 other eagles of the same species. The 2 birds detected by customs may reflect a much larger underlying problem of bird smuggling. Such birds easily remain undetected because customs officers are essentially focused on metal objects, although airport scanners can theoretically detect bones of animals. Specific methods for systematically detecting live animals (e.g., trained dogs) should be considered at airports and borders (5). This method is now under evaluation in Belgium.

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Human T-cell Leukemia Virus Type 1 Molecular Variants, Vanuatu, Melanesia

Olivier Cassar,*† Corinne Capuano,‡ Laurent Meertens,† Eliane Chungue,* and Antoine Gessain†

Four of 391 Ni-Vanuatu women were infected with variants of human T-cell leukemia virus type 1 (HTLV-1) Melanesian subtype C. These strains had env nucleotide sequences ≈99% similar to each other and diverging from the main molecular subtypes of HTLV-1 by 6% to 9%. These strains were likely introduced during ancient human population movements in Melanesia.

**Human T-cell leukemia virus type 1 (HTLV-1),** a human oncoretrovirus, is the etiologic agent of adult T-cell leukemia and of tropical spastic paraparesis/HTLV-1–associated myelopathy. Molecular epidemiologic studies have shown HTLV-1 proviruses to be remarkably stable genetically. The low levels of genetic drift in this virus have been used as a means for monitoring viral transmission and the movement of ancient human populations (1,2). The few nucleotide substitutions observed in HTLV-1 strains are specific to the geographic origin of the patient and are unrelated to viral pathology (1,2). Four major geographic HTLV-1 subtypes have been described: subtype A, cosmopolitan (1,2); subtype B, central African; subtype C, Melanesian (3–6); and subtype D, present in central Africa, mainly in pygmies.

Previous reports have indicated that HTLV-1 is endemic in some remote or ancient populations in Melanesia (3–14). These populations include a small number of tribes from Papua New Guinea (especially the Hagahai people) (5) and some inhabitants of the Solomon Islands (7). Evidence of HTLV-1 infection has also been found in some aboriginal groups from Australia (8). Rare cases of adult T-cell leukemia and tropical spastic paraparesis/HTLV-1–associated myelopathy have also been described in these populations (9).

Genetic characterization of the few available Melanesian HTLV-1 strains has indicated that these HTLV-1 strains are the most divergent, constituting molecular subtype C (also called Melanesian subtype [3,4,6,10]) in phylogenetic analyses. The discovery of such divergent variants has increased our understanding of the migration of HTLV-1–infected populations throughout the Pacific region. Furthermore, 1 of the calibration methods frequently used, in phylogenetic analyses, to estimate a time scale for the evolution of HTLV and simian T-cell leukemia virus (STLV) appears to coincide with the first human migrations to Melanesia and Australia 40,000–60,000 years ago (2).

We carried out a large serologic and molecular study to determine the prevalence of HTLV-1 and associated diseases in the Vanuatu Archipelago. Vanuatu, formerly known as the New Hebrides, is a Y-shaped archipelago made up of ≈80 islands. It is located in Melanesia, in the South Pacific region, northeast of Australia and south of the Solomon Islands. Vanuatu has a population of ≈200,000 inhabitants, most of whom (95%) are of Melanesian origin and are known as the Ni-Vanuatu.

Very few seroepidemiologic studies on HTLV-1 in Vanuatu have been carried out, and these studies examined mostly small populations more than a decade ago and were not based on stringent serologic criteria (11–14). No molecular characterization data are available for HTLV-1 from this area. The main goals of this study were to evaluate the situation concerning HTLV-1 infection in a remote Ni-Vanuatu population by using stringent serologic criteria for Western blotting and molecular characterization of the viruses.

**The Study**

In February 2002, we recruited 391 women during a clinical survey for sexually transmitted diseases in various remote rural communities of western Ambae Island in the Penama Province of the Vanuatu Archipelago. Ambae Island, also known as Aoba, has a population of ≈9,500. The women participating in this survey were offered a complete clinical examination, with Papanicolaou test analysis for all women >25 years of age. For each participant, we obtained plasma anduffy coats from 5 mL of blood obtained by venipuncture. The blood samples were rapidly transferred to Institut Pasteur de Nouvelle-Calédonie, where plasma anduffy coats were isolated, frozen, and stored (at –80°C) until HTLV screening. Informed consent was obtained from each woman participating in the field survey. This study was approved by the Ministry of Health of the Republic of Vanuatu and was supported by the Vanuatu Family Health Association, a local nongovernmental organization. Samples were taken from 391 women (mean age 36 years, range 16–82 years) with the following stratification by age: 11.2% from women 15–24 years of age, 28.4% from women 25–34...
years of age, 31.2% from women 35–44 years of age, 17.4% from women 45–54 years of age, and 11.8% from women ≥55 years of age.

Plasma HTLV-1 antibodies were detected by enzyme-linked immunosorbent assay (ELISA) (HTLV-I+II, Abbott-Murex, Kent, United Kingdom) with Western blot (HTLV-I+II Blot 2.4, Diagnostic Biotechnology, Singapore) used for confirmation. On Western blot, plasma samples were considered HTLV-1–positive if they reacted to the 2 Gag proteins (p19 and p24) and both env-encoded glycoproteins: the HTLV-1–specific recombinant gp46-I peptide (MTA-1) and the specific HTLV-1/HTLV-2 recombinant GD 21 protein. Plasma samples were considered negative when no band were shown and indeterminate when partially reactive (15,16).

Forty-nine of the 391 plasma samples studied tested positive or borderline by ELISA, and 4 of these samples displayed full reactivity on Western blot (Figure 1). One sample also displayed a typical HTLV gag-indeterminate profile (16), and 6 displayed weak reactivity (19 or GD 21 bands). The 4 plasma samples testing positive by Western blot had higher immunofluorescence assay titers on MT2 (HTLV-1) cells than on C19 (HTLV-2) cells and high particle agglutination titers (Table 1). We carried out a second serologic survey on 64 members of the families of the 4 women seropositive for HTLV-1. This survey identified 2 more infected women; 1 was the mother of an index patient, and the other was the sister-in-law of another index patient (Table 1). These results confirm the circulation of HTLV-1 in this population.

High molecular-weight DNA was extracted from buffy coats from the 4 HTLV-1–seropositive women, 5 HTLV-1–seronegative persons, and 6 others with indeterminate Western blot results, by using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The 15 DNA samples studied were subjected to polymerase chain reaction with primers specific for the human β-globin gene to check that cellular DNA was amplifiable for all samples (17). We then subjected DNA samples to 2 series of polymerase chain reaction to obtain the complete long terminal repeat (LTR) (755 bp) and a 522-bp region of the env gene as previously described (18). Fragments of the appropriate size were amplified for the 4 HTLV-1–seropositive women, whereas the other 11 samples yielded negative results. The amplified products were cloned and sequenced, and phylogenetic studies were performed as previously described (18). Both the complete LTR and the 522-bp env fragment were obtained for the 4 HTLV-1–seropositive women.

**Conclusions**

The gp21 gene sequences of the 4 HTLV-1 strains involved were almost identical (99.6%–99.8 % nucleotide similarity) and were very similar to those of Melanesian strains. These strains were closely related (99.4%) to...
certain strains from Solomon Islanders (Mel 4, 8) but were only 97.1%–98.3% similar to strains from Papua New Guinea residents (Mel 2, 7) and from Australian aborigines (MSHR-1), respectively. Finally, the sequences of these new strains diverged from those of HTLV-1 strains from the 3 other main molecular subtypes (A, B, D) by 6% to 9%.

The 4 new HTLV-1 LTR sequences were also very closely related (98%–100% nucleotide similarity). They displayed 2% nucleotide divergence from Mel 5 (from a Solomon Islander), the only available LTR from all the HTLV-1 subtype C strains. However, they also displayed up to 11% nucleotide divergence from HTLV-1 strains from other molecular subtypes.

Phylogenetic analyses were performed on all the available env and LTR HTLV-1 sequences from Melanesia, and on several representatives of HTLV-1 and STLV-1 strains from the various subtypes/subgroups as described (18), by the neighbor-joining (NJ) method. Similar tree topologies were obtained for both genomic regions (Figure 2 and Appendix Figure, which is available online at http://www.cdc.gov/ncidod/EID/vol11no05/04-1015_app.htm). Analyses of these trees confirmed that the 4 novel Vanuatu HTLV-1 strains were closely related to all available HTLV-1 subtype C strains (Table 2). Indeed, in the env analysis, which included 71 HTLV-1 strains (including 12 Melanesian strains and 1 from an Australian aborigine, Table 2) and 55 STLV-1 strains, the 4 new HTLV-1 strains clustered with subtype C (Figure 2). This subtype only includes strains from Australia, Papua New Guinea, the Solomon Islands, and Vanuatu. Within this clade are at least 2 subgroups, strongly supported phylogenetically: 1 comprises the Vanuatu strains and most of the strains from the Solomon Islands (bootstrap values of 88%), and the other comprises the 3 isolates from Papua New Guinea (the Hagahai population), with a bootstrap value of 100%. Two other unique and divergent strains, the only strain available from an Australian aborigine (MSHR-1) and the other from a Solomon Islander (Mel-12), may represent prototypes of 2 other clades within the Melanesian subtype C.
In conclusion, we report, for the first time, the presence of HTLV-1 infection in a Ni-Vanuatu population living in remote villages. We also demonstrate that the viruses infecting these Ni-Vanuatu persons are novel HTLV-1 molecular variants belonging to the Melanesian divergent C subtype. This finding suggests that these viruses were introduced into Vanuatu by ancient migrations of Melanesian populations. The first people to reach Santa Cruz, Banks, Vanuatu, and the Loyalties Islands 3,600 years ago seem to have been Austronesian speakers (19). Epidemiologic and clinical surveys are under way in this area to determine the extent of such retroviral infection and associated neurologic and hematologic diseases. In addition, studies of viral and mitochondrial/nuclear DNA are being conducted and should provide insight into the migrations of the first settlers and the origin, evolution, and modes of dissemination of such retroviruses.

Acknowledgments

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Mr. Cassar is a PhD student whose primary research interests are the clinical and molecular epidemiology and physiopathology of dengue viruses. He is currently working on the epidemiology of HTLV-1 in Melanesian populations.

References


Table 2. Epidemiologic data and GenBank accession numbers of the human T-cell leukemia virus type 1 (HTLV-1) strains of the Melanesian subtype C

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Birth</th>
<th>Residence</th>
<th>Clinical status</th>
<th>Virus name</th>
<th>env GenBank accession no.</th>
<th>LTR GenBank accession no.</th>
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<tr>
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<tr>
<td></td>
<td>36</td>
<td>F</td>
<td>Ambae</td>
<td>Ndai Ndai</td>
<td>AC</td>
<td>HTLV-1 VAN 136</td>
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<td>F</td>
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</tbody>
</table>

*LTR, long terminal repeat; F, female; M, male; AC, Asymptomatic carrier; TSP/HAM, tropical spastic paraparesis/HTLV-1-associated myelopathy; NA, not available.


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Clonal Comparison of Staphylococcus aureus Isolates from Healthy Pig Farmers, Human Controls, and Pigs

Laurence Armand-Lefevre,* Raymond Ruimy,* and Antoine Andremont*

Pig farming is a risk factor for increased nasal Staphylococcus aureus colonization. Using sequence typing and phylogenetic comparisons, we showed that over-colonization of farmers was caused by a few bacterial strains that were not present in nonfarmers but often caused swine infections. This finding suggests a high rate of strain exchange between pigs and farmers.

The Study

The S. aureus strains studied included 44 nasal isolates from healthy pig farmers and 21 from healthy nonfarmer controls (i.e., bank or insurance workers). These participants all had been part of the population included in a previously published epidemiologic study in which the resistance rates in commensal bacteria from healthy pig farmers were compared with the rates in controls matched for age, sex, and county of residence (4). This population was disseminated over 7 French departments, chosen because they were the leading areas of porcine production. A department is a French administrative territory roughly the size of a British or American county. Each pig farmer worked on a different pig farm. We also studied 14 S. aureus isolates from the following types of swine infections: cutaneous, for isolates CA-1, CA-2, CA-6, F-9, and F-10; urinary, for isolates CA-3, CA-5, F-8, F-9, IV-11, IV-13, and IV-14; blood, for IV-12; and bone for CA-4. Isolates were collected from 1996 to 2002 in 4 of the 7 departments in which the pig farmers were working and were kindly provided by state veterinary laboratories. All strains had been identified with conventional techniques, and their susceptibility to antimicrobial agents had been determined by the disk-diffusion technique (available from www.sfm.asso.fr).

S. aureus strains were lysed with 30 µg/mL lysostaphin, which was incubated for 10 min at 37°C, and DNA was extracted by using MagNA Pure LC automat (Roche, Mannheim, Germany), as recommended by the manufacturer. DNA concentrations were measured by optical density, and extracts were diluted to obtain concentrations of 50 ng/µL DNA for amplification.

The presence of mecA and nuc genes was determined by multiplex polymerase chain reaction (PCR) using mecA1, mecA2, nuc1, and nuc2 primers (6). Mixes contained 250 µmol/L of each primer, 400 nmol/L of each deoxynucleoside triphosphate (Boehringer GmbH, Mannheim, Germany), 1 x reaction buffer supplied by the manufacturer with 1.5 mmol MgCl2, 1 U of AmpliTaq DNA polymerase (Applied, Courtaboeuf, France), and 100 ng of DNA extract in a final volume of 50 µL. The PCR was carried out for 1 cycle of 5 min denaturation at 94°C and 20 cycles of 10 s at 94°C, 10 s at 60°C, and 30 s at 72°C. PCR products were visualized under UV irradiation after electrophoresis.

MLST analysis was carried out by sequencing fragments of 7 housekeeping genes (arcC, aroE, glpF, gmk, pta, tpi, and yqiL), as described (available form www.mlst.net), except that the primers used for tpi amplification were tpi1a 5′-GCATTAGCAGATTAGGGCTT- TA-3′ and tpi2d 5′-TGACACCTTCAAATGTAGCA-3′. All PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France) and sequenced using an ABI Prism sequence (Applied) with Big Dye reaction mixes, using the primers chosen for the
initial amplification, and analyzed on the BioEdit biological sequence editor 5.0.6 (7). Each allele of the 7 housekeeping genes was assigned to a number, and each isolate was characterized by a sequence type (ST), defined by the allelic profile of the housekeeping genes. These profiles were compared to those present in the *S. aureus* MLST database (available from www.mlst.net). The 2 new allele sequences of the *yqiL* gene and the 1 new sequence of the *aroE* gene were deposited in the MLST database under numbers 72, 73, and 91, respectively. The new STs have also been deposited in the MLST database, under numbers ST432 to ST438, ST440, and ST457.

For each strain, the sequences of all 7 housekeeping genes were concatenated to produce an in-frame sequence of 3,198 bp. A phylogenetic tree (Figure) was generated by using the neighbor-joining method, and the robustness of branches was estimated by the bootstrap method. Both are included in Mega version 2.1 software (available from www.megasoftware.net).

All 79 isolates studied were identified as *S. aureus* by conventional techniques and harbored the *nuc* gene. The *mecA* gene was present in the 5 methicillin-resistant isolates. The Figure shows an unrooted tree in which the aligned sequences of the 79 isolates are compared; it also indicate the ST number and antimicrobial resistance of each isolate.

Nineteen STs were identified among the 65 nasal isolates from pig farmers and nonfarmer controls. Nine (STs 432 to 438, ST440 and ST457) had not been previously described. Twelve of the 19 STs were each found in only 1 isolate, 1 (ST 437) in 2 isolates, and the remaining 6 (ST5, ST8, ST9, ST15, ST34, and ST398) in at least 4 isolates. Only 3 of these 6 STs (ST5, ST15, and ST34) were found in isolates from both pig farmers and nonfarmer controls. ST5 was present in 10 isolates (7 from farmers, 3 from controls), ST15 in 7 (5 from farmers, 2 from controls), and ST34 in 6 (3 from farmers, 3 from controls). Comparison with isolates from the entire MLST database showed that ST5 had previously been reported in 90 isolates from the United Kingdom, Japan, United States, and Poland; ST15 in 33 isolates from the United Kingdom, Australia, and Canada; and ST34 in 15 isolates from the United Kingdom only. The other 3 STs (STs 8, 9, and 398) were only found in isolates from pig farmers. ST8, retrieved from 4 isolates from pig farmers, had previously been reported in 86 isolates from the United Kingdom, Australia, United States, Canada, France, Germany, Netherlands, Denmark, and Greece. ST9 was found in as many as 18 of the 44 pig farmer isolates that we studied but had only been previously described in 5 isolates, all from the United Kingdom. ST398 was retrieved from 6 isolates from pig farmers; previously, it had only been reported in 1 isolate from the Netherlands.
Analysis of the geographic distribution of STs 8, 9, and 398, which were only found in pig farmers, showed that they were dispersed throughout the 7 departments studied. The 18 ST9 isolates were from pig farmers working in 6 of the 7 departments, the 4 ST8 isolates from pig farmers in 3 of 7, and the 6 ST398 isolates from pig farmers in 4 of 7 departments.

Thirteen of the 14 isolates from swine infections had STs that were only found elsewhere in strains from pig farmers. Two of these 13 swine isolates had ST433, which we found in a single pig farmer isolate, 7 had ST9, and 4 ST398 (Figure). STs 9, 398, and 433 in the swine isolates originated from 3, 2, and 2 different departments, respectively. The remaining swine isolate had ST97, which was not observed in another isolate. In all, 25 (57%) of the 44 pig farmers isolates had STs identical to those of swine strains. No control isolate was identical to those of the swine farmers isolates had STs identical to those of swine not observed in another isolate. In all, 25 (57%) of the 44 pig farmers isolates had STs identical to those of swine strains. No control isolate was identical to those of the swine.

Four of the 5 methicillin-resistant Staphylococcus aureus (MRSA) strains found in pig farmer isolates had STs (ST5 and ST8) previously reported in MRSA (available from 222.mlst.net) or new (ST438). The remaining strain had ST398, which was grouped together with pig farmer isolates that were susceptible to methicillin. Differences in susceptibility to antimicrobial agents other than methicillin were also observed between isolates with identical STs. Although 25 of 25 isolates with ST9 were resistant to penicillin, only 17 were resistant to lincomycin and erythromycin. Of the latter, 5 were coresistant to pristinamycin. One was resistant to kanamycin and pefloxacin. Similar variations in antimicrobial susceptibility were observed among strains with the other STs. Resistance to erythromycin was more frequent in pig farmers than controls (29/44 [66%] vs. 2/21 [10%], as previously reported (4). Resistance was intermediate in swine strains (5 [38%] of 14).

Conclusions

Our results strongly suggest that the high risk for nasal Staphylococcus colonization that we previously reported in pig farmers (4) was due to strains exchanged with swine: 25 (57%) of the 44 pig farmer isolates grouped together with the swine isolates and had 3 STs (9, 398, and 433) that were not found in control isolates. If these pig farmers had not been taken into account, the rate of nasal carriage in pig farmers would have been close to that found in controls (5). The number of pig strains tested was small because swine mastitis, unlike bovine mastitis, is rare and because no other collection of Staphylococcus aureus from swine was available for testing (we did not sample pigs when we performed the previous (4) study of pig farmers and controls).

The hypothesis that pig farmers exchanged strains bearing these specific STs with swine was not formally demonstrated in our study because we did not test strains isolated from swine and from farmers on the same farms at the same time. However, MLST is a powerful tool for comparing strains and determining their phylogenetic and epidemiologic relatedness (8). This tool, together with the similarity of the pig farmer and swine strains (which both had STs 9, 398, and 433) and the absence of these STs in strains from epidemiologically matched controls, argues strongly in support of an exchange of specific strains between pig farmers and pigs.

The widespread geographic distribution of strains with similar STs in strains in pig farmers and swine only was puzzling because pig farmers were working in different farms scattered among the 7 major French pig-raising departments and separated by tens or sometimes hundreds of kilometers. The potential sources of contamination of pigs and farmers by strains such as those with STs 9, 398, or 433, which were common to both, were not investigated. One possibility might be that the sows used for pig production are the vectors of transmission because young sows are transferred from 1 farm to another when production needs to be increased. A specific investigation is needed to explore this possibility. Contamination of 9 sheep farms and their dairy products by a single Staphylococcus aureus strain was previously demonstrated in France, but the routes of dissemination were not investigated (9). In California, the widespread distribution of a multidrug-resistant clone of Escherichia coli that caused community-acquired urinary tract infections was suspected to be of animal origin, but again, the routes of dissemination were not investigated (10).

We observed differences in susceptibility to antimicrobial agents between strains with the same ST, which suggests that the final phenotypes were selected locally, depending on the use of antimicrobial agents. This finding was particularly striking with susceptibility to macrolides and related drugs, a class of antimicrobial agent widely used in pig farming (11), although individual farms may use it differently. Unfortunately, data on the use of antimicrobial agents by each farm were not available.

Animal-to-human transmission during farming has been demonstrated for enterobacteria and enterococci in several instances (1,2) but only once before for Staphylococcus aureus (9). Although they are rare (12), animal MRSA have been suggested as a source of infection for humans (13). Our results suggest that such transmission may be frequent, particularly since virtually no barrier precautions were used by the pig farmers studied in our previous investigation (4).

In the few published studies on the molecular epidemiology of nasal strains from carriers, genetic backgrounds of the strains were very diverse (14), just as in our controls. This finding further underlines the particular way in which most pig farmer strains are grouped together. Whatever the
exact route of transmission, single *S. aureus* strains, probably acquired from pigs, colonized the nostrils of pig farmers throughout large geographic areas and that this colonization probably caused their overall increase in nasal *S. aureus* colonization. Since nasal carriage is a recognized source of *S. aureus* bacteremia with severe consequences (15), our findings suggest that pig farming could be a staphylococcal hazard for farmers, under the conditions in which it is practiced today. Several points could not be addressed in the study, including whether colonization of the farmers by *pig* *S. aureus* isolates was permanent or temporary, whether the pig isolates were also disseminated in the farmer’s families and to other persons living in the area, whether skin and soft tissues of pig farmers were infected and, if so, whether or not it was due to *S. aureus* isolates identical to those from pigs. These questions will be addressed in further, specifically designed, epidemiologic studies.

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Dr. Armand-Lefèvre is a clinical bacteriologist in the bacteriology laboratory of Bichat-Claude Bernard Hospital. Her current interest is the evolution of bacterial resistance in the natural ecosystems of humans.

**References**


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Human Pythiosis, Brazil

Sandra de Moraes Gimenes Bosco,* Eduardo Bagagli,* João Pessoa Araújo Jr.,* João Manuel Grisi Candeias,* Marcello Fabiano de Franco,† Mariangela Esther Alencar Marques,* Leonel Mendoza,‡ Rosangela Pires de Camargo,* and Silvio Alencar Marques*

Pythiosis, caused by *Pythium insidiosum*, occurs in humans and animals and is acquired from aquatic environments that harbor the emerging pathogen. Diagnosis is difficult because clinical and histopathologic features are not pathognomonic. We report the first human case of pythiosis from Brazil, diagnosed by using culture and rDNA sequencing.

Pythiosis is a cutaneous-subcutaneous disease of human and animals that occurs primarily in tropical and subtropical areas of the world. It is caused by the aquatic fungus-like organism *Pythium insidiosum* (kingdom Straminipila, phylum Oomycota, class Oomycetes) (1). Although pythiosis is not caused by a true fungus, the pathogen has some morphologic characteristics in common with fungal members of the order Zygomyces, mainly in histopathologic sections. This disease is a common cutaneous and intestinal disorder in horses, cattle, dogs, and cats (2,3). Human pythiosis may appear in a cutaneous-subcutaneous form with lesions on the limbs, periorbital and facial areas, and corneal ulcers. Pythiosis can also be a systemic disease involving the vascular system, which usually causes arterial occlusion. The systemic form has been documented in numerous patients, most from Thailand, with thalassemia. The remaining cases have been from Australia (2 patients), United States (2 patients), and 1 each from Haiti, Malaysia, and New Zealand (4,5).

*Pythium insidiosum* can be cultured on Sabouraud dextrose or brain heart infusion agar at 37°C from clinical material, such as pus, lesion exudates, and biopsy material. The characteristic asexual biflagellate zoospores, important for the diagnosis, can be induced in liquid media but not in solid cultures. In tissues, *P. insidiosum* stains well with Gomori methenamine silver and periodic acid-Schiff stain. The organism appears with broad, branched, and sparsely septate or nonseptate hyphae, often identified as fungal elements of the zygomycetes (6). Conventional diagnosis is based mainly on immunofluorescence and immunoperoxidase procedures, which have proved specific in tissues of persons, cats and dogs with pythiosis. Serologic tests, such as enzyme-linked immunosorbent assay (ELISA) and immunodiffusion, have been also used to diagnose pythiosis (7). Molecular diagnostic assays, such as nested polymerase chain reaction and a species-specific DNA probe from the ribosomal DNA complex, have been useful to identify *P. insidiosum* in the absence of culture (8,9). This article reports the first case of human pythiosis in continental Latin America in a patient from Brazil, and diagnosis was confirmed by molecular taxonomy.

The Study

A 49-year-old policeman was admitted on May 2002 to the dermatology division of the university hospital for the treatment of a skin lesion on his leg, initially diagnosed as cutaneous zygomycosis. The patient stated that a small pustule developed on his left leg 3 months earlier, 1 week after he fishied in a lake with standing water. The pustule was initially diagnosed as bacterial cellulitis; it was treated with intravenous antimicrobial agents with no improvement. A biopsy of the lesion showed a supplicative granulomatous inflammation associated with several nonseptated hyphae (shown by Gomori methenamine silver stain), a finding that led to the diagnosis of zygomycosis. The treatment was then changed to amphotericin B. After receiving 575 mg of accumulated dosage plus 2 surgical debridements of the lesion, the patient showed only slight improvement; he was then referred to our university hospital. At admission, the physical examination showed a tibial ulcer 15 cm in diameter with an infiltrating and nodular proximal border (Figure 1). Serologic testing showed the following laboratory values: leukocyte count 4,200/mm³ with 9% eosinophils, glucose 100 mg/dL, and negative serologic test results for HIV infection. Azotemia, hypokalemia, and normocytic anemia were observed as adverse effects of the previous amphotericin B treatment. Results of a second biopsy from the border of the ulcer again suggested zygomycosis (Figure 2).

Oral itraconazole, 400 mg/day, was prescribed. Although the initial response was encouraging, at the end of the first month of itraconazole administration, the lesions recurred. Potassium iodine, 4 g/day, was also prescribed, but no clinical improvement was detected after 2 months. Attempts to isolate the agent in the hospital laboratory yielded negative results. With the progression of the disease, an extensive surgical debridement was considered and a computed tomographic scan defined the limits of the infection. A course of amphotericin B was begun 1 week before the intervention, which included total removal of
The fascia lata. Amphotericin B was maintained until discharge from the hospital on day 103. A late skin graft produced an acceptable recovery. The excised tissue supplied enough material for culture and molecular assays in our laboratory, which led to a conclusive diagnosis.

The excised tissue was immediately washed in 70% alcohol and sterile saline, minced in small fragments (2 x 2 mm) and cultured on Sabouraud dextrose and potato dextrose agar (PDA, Oxoid Ltd., Basingstoke, Hampshire, UK), plus chloramphenicol and gentamicin (50 µg/ml), at 25°C. After the tissues underwent a period of growth, slide cultures were carried out with PDA medium.

The DNA extraction was performed according to the glass beads protocol proposed by van Burik et al (10). Polymerase chain reactions (PCRs) were carried out in 25 µL of reaction mixtures buffered with 20 mmol/L Tris-HCl (pH 8.4), containing 10 ng of genomic DNA, 20 pmol/L of each primer, 1.5 mM MgCl₂, 50 mmol/L KCl, 0.2 mmol/L of each deoxyribonucleoside triphosphate, and 0.2 U of Taq DNA polymerase (Amersham Biosciences Corp, Piscataway, NJ, USA). PCRs for elongation of rDNA-ITS region were performed in a thermocycler (MJ Research, Inc, Waltham, MA, USA), with an initial cycle at 94°C for 5 min, followed by 25 cycles at 94°C (1 min), 60°C (2 min), and 72°C (2 min) and a final extension of 7 min at 72°C. The ITS regions were amplified by using the universal ITS4 (5′–TCC GCT TAT TGA TAT GC–3′) and ITS5 (5′–GGA AGT AAA AGT CGT AAC AAG G–3′) set of primers. The amplicons were visualized in ethidium bromide-stained 1% agarose gel and purified in GFX column (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences).

Both strands were sequenced in an ABI Prism model 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were performed with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), 1.6 pmol/L of each ITS4 or ITS5 primer and 60 ng of purified DNA. Ultra pure water was used to complete a volume of 20 µL. The elongation of the ITS region was performed in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) with 40 cycles at 96°C (10 s), 50°C (5 s), and 60°C (4 min). The ClustalW program was used to align nucleotide sequences. The obtained sequences were submitted for analysis to GenBank by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Pure, colorless, membranous colonies grew on Sabouraud and PDA from almost all samples. On microscopic examination, slide cultures stained with lactophenol cotton-blue showed broad, branched, and sparsely septated hyphae, without fruiting bodies (Online Figure 3; available at http://www.cdc.gov/ncidod/EID/vol11no05/04-0943-G3.htm), which later were identified as colonies of *P. insidiosum*.

The first round of amplification with the generic primers for fungi (ITS4/ITS5) produced weak bands; in a second round, double PCR, defined and sharp bands of approximately 850 bp were seen (Online Figure 4; available at http://www.cdc.gov/ncidod/EID/vol11no05/04-0943-G4.htm). After being purified and sequenced in both directions, bands produced chromatograms with a resolved sequence of 593 bases. After BLAST analysis, the sequence showed 100% of identity with the deposited sequences of *P. insidiosum* (accession nos: AY151166, AY151165, AY151159), Schurko, et al. (11), with full annealing from base numbers 253 to 845, which include the almost complete gene 5.8S and the complete variable ITS2 region.

**Conclusions**

In the present case, a diagnosis of zygomycosis was initially suggested by the histopathologic examination carried out in 2 different laboratories. The patient had no evidence of immunologic disorders and no clinical manifestation of thalassemia. The infection was more likely acquired during water-associated leisure activities, the most common source of the infection among patients with pythiosis (12). Several antifungal drugs were used, but the lesions did not

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**Figure 1. Clinical aspects of the lesion when the patient sought treatment at the University Hospital. The figure depicts the wide extension of the lesion in a frontal (A) and in depth (B) medial views.**
improve. On the contrary, during the 14 weeks of chemotherapy the status of the lesion worsened, and amputation of the patient’s leg was considered. After extensive surgical debridement to remove the lesion, however, a cure was achieved. Although all mycologic features and epidemiologic data indicated possible infection by \textit{P. insidiosum}, only after sequencing rDNA ITS was the diagnosis of pythiosis definitively established. We believe this is the first case of human pythiosis described in South America, although similar cases might have been misdiagnosed as putative cases of zygomycosis in humans, as others have noted (6).

Cases of subcutaneous pythiosis with dissemination to internal organs have been described in equines in Brazil (13). Subcutaneous pythiosis in calves and equines seems to be common in the central and southeast regions of Brazil. More recently, an outbreak of cutaneous pythiosis was reported in 2 herds of crossbred wool sheep in the northeast region (14). This pathogen likely occurs in different regions of Brazil because of a prevalent tropical climate and abundant sources of water.

The fragment of rDNA sequenced in the present case included the complete variable ITS2 region and almost the complete coding sequence of 5.8S. This fragment showed 100% identity with the isolates M16, M12, and 339 that characteristically belong to the cluster I of the American isolates (11). The M16 isolate was obtained from a corneal lesion of a Haitian patient and the others were from Costa Rican equines. A strain of \textit{P. insidiosum} from a Brazilian equine (394) located in the same American cluster showed a similarity of 99% with our \textit{P. insidiosum} isolate.

Direct DNA sequencing, mainly of rDNA regions, proved to be an important and consistent tool for the taxonomic identification, of different groups of organisms (9). In this case, a molecular approach was decisive in identifying and diagnosing this life-threatening disease. Our experience indicates that other cases of pythiosis may have been misdiagnosed as cases of cutaneous zygomycosis. Although zygomycosis has a widespread distribution and is well known to physicians, this familiarity is not the case for human pythiosis. Health professionals, therefore, should be aware of the importance of an accurate diagnosis of this condition and know how to differentiate it from zygomycosis, since pythiosis has a completely different prognosis and requires different therapy.

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Travel-associated Rabies in Austrian Man

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Rabies developed in an Austrian man after he was bitten by a dog in Agadir, Morocco. Diagnosis was confirmed by reverse transcription–polymerase chain reaction and immunohistochemistry. The patient’s girlfriend was bitten by the same dog, but she did not become ill.

Rabies is an acute, progressive, fatal encephalomyelitis that can be prevented by vaccination. It is almost always transmitted by the bite of an infected animal and is still a public health problem in many countries in Africa and Asia. Travelers with extensive unprotected outdoor exposure, such as camping, in areas where rabies is endemic are also at high risk (1). We report a case of rabies associated with a dog bite in Agadir, Morocco, in an Austrian man.

The Case

Two Austrian tourists (a 23-year-old man and a 21-year-old woman) traveled to Morocco in May 2004. At the end of July, they were staying in Agadir, Morocco, where they played with puppies on the beach. One puppy was aggressive and bit the woman on the third finger of her right hand. While attempting to assist her, the man was also bitten on the right hand and leg. The wounds healed without further treatment, and the young people did not seek medical assistance. Three days after the bite, the dog died and was buried by the tourists. Four days later, the man became ill with a temperature up to 39°C, malaise, pain in the right arm, headache, feeling of extreme dryness in the mouth, and difficulty swallowing. Two days later, he was admitted to a hospital in the Spanish enclave of Ceuta. While attempting to assist her, the woman on the third finger of her right hand, the dog bit her and was admitted to the Medical University of Graz and received a thorough examination and psychological support. No abnormalities were found. Rabies vaccination, which had been started in Ceuta, was continued, and she was released from the hospital in healthy condition. One week after complete vaccination, her antibody titer was 118.53 U/mL. She remained healthy 4 months after the bite and is currently being monitored.

Since the female Austrian tourist was bitten by the same rabid dog, she was also admitted to the Medical University of Graz and received a thorough examination and psychological support. No abnormalities were found. Rabies vaccination, which had been started in Ceuta, was continued, and she was released from the hospital in healthy condition. One week after complete vaccination, her antibody titer was 118.53 U/mL. She remained healthy 4 months after the bite and is currently being monitored.
Conclusions

Our patient acquired rabies from a dog bite in July 2004 in Agadir, Morocco. On September 1, 2004, the World Health Organization announced that a rabid dog had been illegally imported into France from Agadir in July 2004. The dog was aggressive and bit several persons who were later contacted by French health authorities to assess their risk for infection and provide professional help, but no case of rabies associated with this dog has been reported to date (4,5). Our patient’s dog bite–associated case of rabies underlines the potential risk for infection by dogs from Agadir and other rabies-endemic areas (4,5). This case of rabies is the first to be diagnosed and treated in Austria since 1979. Because of official rabies vaccination policy, Austrian domestic and wild animals have been free of rabies since 1995, with rare exceptions. Rabid foxes occasionally immigrate to Austria, as was the case in 2002 on the Carinthian-Slovenian border, and transmit rabies to other animals (foxes, deer, dogs, cats, badgers) (6–8). The occasional reappearance of rabies in Austria usually leads to vaccination campaigns in relevant areas; these campaigns, in combination with routine animal vaccination, prevent the dissemination of rabies (6). The general public in Austria thus does not consider rabies to be a serious problem, and recommendations for vaccination for travelers to rabies-endemic countries are sometimes ignored.

Although preexposure rabies prophylaxis is recommended for travelers to Morocco (9), our patient and his girlfriend did not receive vaccination before their trip. They did also not seek medical assistance or receive postexposure prophylaxis immediately after the bites because they did not understand the enormous potential risk from a dog bite in a country where rabies is endemic. After the patient showed symptoms indicative of rabies 1 month later, he and his girlfriend were admitted to a hospital and received passive and active postexposure prophylaxis. Whereas the lethal course of the disease could not be prevented in the man, the woman remained healthy, and high levels of rabies antibodies developed. However, in 5% of rabies cases the incubation period exceeds 1 year (10). Since this young woman was bitten by the same dog that transmitted rabies to our patient, she is still being followed. Our case of travel-associated rabies in an Austrian man underscores the importance of adhering to vaccination recommendations and the need for providing detailed information to travelers.

Recently, authorities have recommended that molecular diagnostic methods with samples from several sources be performed and repeated until the diagnosis of rabies is established. However, molecular diagnostic methods, although useful and sensitive, may not always give positive results for patients with rabies (11). This lack of sensitivity may be due to the intermittency of virus shedding, the timing of sample collection, and the type of specimens collected. For this reason and in concordance with CDC recommendations, we suggest that immunohistochemical investigation of skin biopsy specimens should be performed for antemortem diagnosis of rabies, as we did with our patient (12).

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Figure 1. Immunohistochemical stain of neck biopsy specimen. Note positive cells with small intracytoplasmic granules at the border of stratum granulosum and stratum corneum of the epidermis. Bar = 20 μm.

Figure 2. Immunohistochemical stain of neck biopsy specimen. Note the positive cell in the center with small intracytoplasmic granules at the border of stratum granulosum and stratum corneum of the epidermis. On the right side, melanin-rich epidermal cells are seen. Bar = 10 μm.
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Antibody Testing and Lyme Disease Risk

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Lyme disease test results for >9,000 dogs were collected from participating veterinary clinics. Testing was conducted by using the IDEXX 3Dx kit, used widely by Maine veterinarians to screen clinically normal dogs during heartworm season. This study demonstrates how this test can be a valuable public health disease surveillance tool.

Lyme disease is the most commonly reported vector-borne disease in the United States; however, many experts believe that the number of cases is underreported. Lyme disease is often regarded as a routine condition or is frequently managed in high-volume settings (1). Few studies have assessed the accuracy of passive Lyme disease surveillance systems, but 1 study showed a 34% reporting rate (1). When tick identification services are offered, the identification data can show where disease vectors are found. In 1989, to determine the extent of the recently recognized infestation with *Ixodes scapularis*, the Maine Medical Center Research Institute’s Vector-borne Disease Laboratory offered free tick identification to physicians, hospitals, veterinarians, and the general public. Since that time, >20,000 ticks, representing 14 species, have been identified. Testing has documented *Borrelia burgdorferi* infection in *I. scapularis* from all Maine counties except 3.

Mapping of ticks submitted for identification is subject to certain biases, which limits its utility for predicting human risk. Submission rates vary depending on population, education, and local concern, and results show little about disease transmission, particularly in disease-emergent areas where infection rates may lag behind tick distribution. The limitations of passive Lyme disease surveillance and tick identification that provide geographic information about risk can be largely overcome by using canine seroprevalence studies. Dogs are sensitive indicators because they have greater exposure to ticks. In disease-endemic areas, ≥50% of unvaccinated dogs have been reported to be infected (2,3). The prevalence of Lyme borreliosis in dogs correlates with infection in humans (4,5), as well as entomologic indicators of disease transmission (6). A newly available enzyme-linked immunosorbent assay (ELISA) kit (SNAP 3Dx, IDEXX Laboratories, Westbrook, ME, USA) is used widely by veterinarians in Maine to screen dogs for *B. burgdorferi* and heartworm infection. This test is used as part of a health screen during the heartworm testing season and can potentially generate large volumes of unbiased test data for public health application.

The test kit detects antibodies directed against an invariable region (IR6) of the *B. burgdorferi* surface protein VlsE (Vmp-like sequence, Expressed) (2). The *C6* ELISA test is not cross-reactive with antibodies induced by vaccination with either recombinant *B. burgdorferi* outer-surface protein A (OspA) or whole-cell bacterin (2). This test has a very high accuracy rate, with 94.4% sensitivity and 99.6% specificity reported (7). In a clinical setting, when 18 dogs with known vaccination history were tested, the test results were 100% consistent with Western blot results (8).

The Study

One hundred sixty-four Maine clinics were contacted in February 2003 and invited to join the study; 69 of these agreed to participate. Clinics were instructed to record results of all IDEXX 3Dx Lyme disease tests that were conducted as part of a routine health screen, to record town of residence, and to record if a Lyme disease vaccine had ever been administered. Lyme disease vaccines can be highly effective (2); however, since vaccination rates are unevenly distributed, inclusion of vaccinated dogs would bias estimates of disease risk. This protocol was approved by the Maine Bureau of Health Institutional Review Board.

Canine seroprevalence rates were calculated for minor civil divisions, including towns and unorganized townships. Rates were calculated only for divisions that had results of 10 or more tests. The relationships between the canine prevalence rates and human Lyme disease reports to the Bureau of Health (217 division-matched reports) and tick submissions to the Vector-borne Disease Laboratory (12,482 division-matched submissions) for the 2 years before this study, 2001–2002, were tested with Spearman rank correlation. Canine *C6* antibodies persisted in experimentally infected, untreated dogs for ≥65 weeks, with no endpoint described (9); exposure status of the dogs in the present study could not be determined. Using data from 2 years allowed us to include sufficient numbers of human reports for meaningful statistic testing without sacrificing the ability to look at a “snapshot in time” of the Lyme disease spread.

Two maps were created. The first map (Figure 1) showed prevalence rates of minor civil divisions with ≥10 tests. The second map (Figure 2) showed pooled data from all divisions, including those with small sample sizes. For this map, an overlay of the state with 15-minute quadrangle

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gles was used. Each division from which data were collected was assigned to the quadrangle that contained the largest portion of its area. Seroprevalence rates for quadrangles were calculated by combining test results from all divisions within a quadrangle to find the average rate. Divisions were then assigned the average seroprevalence rate of their quadrangle for mapping. Quadrangles having a pooled total of <10 tests were not included in this map.

Test results from 9,511 dogs that had not been vaccinated for Lyme disease were submitted from 343 minor civil divisions. Tests were performed from March to July 2003. The overall seroprevalence rate was 8%. One hundred and eighty-three divisions met the criterion of a minimum sample size of 10 for calculating prevalence rates. At the division level, seroprevalence rates significantly correlated with the number of ticks submitted to the Maine Medical Center Research Institute’s Vector-borne Disease Laboratory from 2001 to 2002 \( (r = 0.41, p<0.001) \), and human Lyme disease reports to the Bureau of Health \( (r = 0.15, p<0.05) \) from 2001 to 2002.

Regional seroprevalence rates were calculated for 65 quadrangles representing 297 minor civil divisions. Seroprevalence rates ranged from 0% to 47%. Rates were highest along southern coastal Maine \((<47\%\)) , with regional rates of 11% as far east as Columbia and along the mid-New Hampshire border as far north as Upton. Forty-four divisions with \( \geq10 \) tests had prevalence rates of 0%; 12 of these had \( \geq30 \) tests and 3 had \( \geq60 \).

**Conclusions**

This study demonstrates how canine serosurveys using the IDEXX 3Dx test can serve as an active surveillance system for potential human Lyme disease risk. This method overcomes the limitations of human Lyme disease reporting systems by relying on routine screening of populations of healthy dogs to calculate true seroprevalence rates. In this study, a large volume of data from across the state was generated for the most extensive and detailed measure of regional Lyme disease risk in Maine to date. In contrast, passive human Lyme disease surveillance during the previous 2 years yields cases from <90 towns, approximately two thirds of which had only 1 or 2 cases.

Canine seroprevalence rates were congruent with *I. scapularis* submissions and human Lyme disease reports during a 2-year period when dogs could have been infected, reinforcing the effectiveness of this method for predicting geographic human risk. One previous study has calculated canine seroprevalence rates in Maine \( (6) \) , but a different assay technique was used \( (4) \) , which limited our ability to compare those rates to those of the current study. In spite of substantial agreement between canine seroprevalence and rates of tick submissions, mapping of canine seroprevalence data shows high-risk foci in inland areas that were not previously identified by 14 years of tick submissions to the Vector-borne Disease Laboratory or from human Lyme disease reporting to the Bureau of Health; this suggests that canine serosurveys may identify new areas of disease transmission. These are areas of low human population density, and repeat surveys may demonstrate the value of canine serosurveillance in detecting disease spread where human populations are low.

Mapping of pooled data on a regional scale allows geographic patterns of disease to be viewed. Most notably, our data show a concentration of infected dogs in southern and coastal areas. Patterns of infection are suggested in inland areas as well. The significance of these patterns with respect to environmental variables favoring disease transmission is unknown but could be clarified by comparing prevalence rates with patterns of land use, deer herd density, habitat, and other ecologic attributes.

The widespread acceptance of the IDEXX 3Dx test facilitates the use of canine serosurveys for public health. In many Maine veterinary offices, virtually every dog tested...
for heartworm in the spring is tested for *B. burgdorferi* antibody; however, well below 100% of canine patients are vaccinated against Lyme disease. Test results can be collected opportunistically from collaborating veterinarians with minimal effort. Previous serosurveys have involved much more intensive effort because of the need for veterinarians to collect extra blood samples. The ease of data collection based on this manner of testing enhances real-time as well as long-term monitoring of disease. Furthermore, the large volumes of test results generated from routine *B. burgdorferi* screening, and the ability to collect information on dog residence, make large-scale studies of disease geography possible. That we did not exclude in our analyses dogs that have traveled suggests that caution should be used when considering the importance of low prevalence rates or prevalence rates calculated from low sample sizes. However, our finding of dozens of towns with 0% prevalence suggests that the effect of dogs that have traveled on calculated seroprevalence rates is small.

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The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.
Incident Tuberculosis among Recent US Immigrants and Exogenous Reinfection

Ted Cohen*† and Megan Murray*‡

Mathematical models and molecular epidemiologic investigation support the argument that exogenous reinfection plays an important role in tuberculosis transmission in high-incidence regions. We offer additional data from tuberculosis cases among recent US immigrants which strengthen the claim that reinfection in areas of intense transmission is common.

As the incidence of tuberculosis (TB) in countries with the lowest rates of disease continues to decline, disease in these areas is increasingly borne by immigrants. Recently, researchers have examined patterns of incident disease among persons emigrating from high-incidence (generally >50 cases/100,000 persons/year) to low-incidence regions (generally ≤10 cases/100,000 persons/year) (1–5). While recent immigrants to these countries are at high risk for disease (6), more recent studies also find that elevated TB rates among immigrants to these countries persist for many years after relocation. Most researchers have assumed that TB cases among immigrants from high-incidence areas represent the reactivation of latent infections.

Studies of TB have been conducted to estimate the risk for disease among immigrants, to assess the risk for infection to the native-born population attributable to these immigrants, or to critique existing screening programs. We took an alternate approach and used these data to gain insight into the transmission dynamics that drive the epidemics in the high-incidence areas from which these persons have emigrated. We used data from the US national TB surveillance system to estimate age-specific incidence rates of TB among immigrants by time since arrival in the United States from each of 6 countries of origin: China, Haiti, India, South Korea, Philippines, and Vietnam. We explain that the patterns of incident disease are consistent with the current understanding of the natural history of TB and offer additional support for the argument that infection confers only partial immunity and reinfection plays a major role in areas with high TB incidence.

The Study

TB cases from all 50 states and the District of Columbia were recorded in the US national TB surveillance system (NTBSS). Report forms collected standard information at the time of diagnosis, including country of origin, age at diagnosis, and years since arrival in the United States (Centers for Disease Control and Prevention, unpub. data). US-born persons were defined as those either born in the United States (or its jurisdictions) or those born in a foreign country who had at least 1 US parent. Those who did not meet these criteria were considered foreign-born persons. Information on immigration status was not collected. Our analyses included all cases reported from 1999 to 2001 among persons who had emigrated from China, Haiti, India, South Korea, Philippines, or Vietnam within 10 years before their diagnosis in the United States. Case reports that were missing information on country of origin were not included in this analysis. While immigrants from Mexico are also at high risk for infection and disease, we excluded cases among this group because the large proportion of unauthorized immigrants precluded a reasonable estimation of population denominators for this subgroup.

We used US immigration statistics to document the number and ages of persons arriving from each of these countries in the years 1989–2001 (Available from http://uscis.gov/graphics/shared/aboutus/statistics/Immigs.htm). To estimate the appropriate population denominators from which these TB cases arose, we applied age-specific mortality rates (categorized as <1–4 years, 5–14 years, 15–24 years, 25–44 years, 45–64 years, and ≥65 years) for US immigrants and assumed that these populations were depleted only by death (no emigration from the United States). Confidence intervals for the country- and age-specific incidence rates were calculated by assuming that TB case counts were Poisson distributed.

Conclusions

From 1999 to 2001, a total of 5,198 cases of TB were recorded in NTBSS among persons who had emigrated from the study countries within the preceding 10 years. Figure 1 depicts the country-specific incidence rate of TB by time since arrival in the United States. Incidence rates among immigrants who have been in the country for <1 year at the time of their diagnosis are similar in relative rank and magnitude to the estimated incidence rates in the country of origin (Table 1) (7). TB incidence rates appear to decrease sharply over the first 2 years in the United States.

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Figure 2 depicts the age-specific incidence rates by time since arrival for US immigrants from each of 6 countries. When incidence rates during the first 2 years were compared with those after 5–10 years in the United States, significantly higher rates were found to occur immediately after arrival in all age groups examined (Table 2). Because incidence rates immediately after arrival will overestimate the true incidence if prevalent cases are misclassified as incident cases, we tested the sensitivity of these findings to the incidence rates calculated for the first 2 years after arrival. We found that this significant elevation in early (<1 year after immigration) versus late (years 5–10) incidence persists in almost every age group for all countries even if we assume that we overestimated incidence by a factor of 2 in the early years (Table 2).

Numerous studies have documented elevated rates of TB among groups migrating from high-incidence areas to low-incidence areas. Although some researchers have reported an immediate drop in incidence (6), most have found, as we did, that the highest rates of incident disease occur nearest to the time of emigration and that a declining, although still elevated, risk persists for at least a decade. One exception is a recent study from the Netherlands, which reported persistent elevations for a decade after emigration (4); of note, this study excluded all patients with cases occurring within 6 months of arrival on the basis that these early cases were likely to be prevalent cases.

Peri-immigration stress, malnutrition, and early overdiagnosis have been suggested as potential explanations for the fact that the highest TB incidence rates occur near arrival. The elevated risk that persists for decades has been attributed to the reactivation of latent infections (6), continued transmission among a relatively insular immigrant population (8), and disease secondary to migration back and forth from the country of origin (9). We believe that the pattern of early decline and late stability in incidence seen here and in most studies (and which may also have been seen by Vos et al. had they not excluded the cases occurring in the earliest interval) is consistent with the well-described 2-component phenomenon of disease occurrence after infection. The first phase (lasting ≈1–2 years) is dominated by the rapid progression of recent infections acquired in the country of origin, and the second phase (lasting perhaps decades) is defined by the relatively slow reactivation of latent infections acquired during a person’s lifetime before emigration (10). The fact that the oldest immigrants from each country continue to have high incidence of disease 10 years after arrival likely reflects

Figure 1. Tuberculosis incidence by time since arrival among recent US immigrants.

Figure 2. Tuberculosis incidence by time since arrival among recent US immigrants categorized by age group. Note that different scales are used for incidence.

| Table 1. Estimated incidence of tuberculosis among newest immigrants and disease incidence and infection prevalence in the countries of origin |
|-----------------|-----------------|-----------------|-----------------|
| Country         | Annual TB incidence in home country (7), cases/100,000 [rank] | TB incidence in first year after arrival in United States, cases/100,000 [rank] | % of population with latent infection in home country (7) |

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the increased lifetime risk for infection that they acquired while living in the high-incidence region. These dynamics, rather than stress, immune suppression, or malnutrition, may explain the patterns of disease occurrence after arrival.

The steep decline in incident disease among the oldest cohorts within the first 2 years after arrival deserves special attention. While the current incidence of disease in the countries of emigration is very high, the annual risk for infection with TB over the past several decades has steadily declined. Thus, a large proportion of those in the oldest age groups would have been infected with *Mycobacterium tuberculosis* early in their lives. The observed decline in incidence after arrival is strikingly similar to observed patterns of declining risk for disease by time since infection (10) and provides compelling evidence that recent infection in the country of emigration is likely to be responsible for much early incident disease in the oldest age groups. The fact that most of these persons would have also been infected early in their lives argues that exogenous reinfecction plays a key role in TB dynamics in high-incidence countries. This conclusion is consistent with other epidemiologic studies that report simultaneous decreases in incident disease across all age groups as the force of infection declines (11); molecular studies that document reinfection in high- (12), moderate- (13), and low-incidence
settings (14); and theoretical work that supports the role of reinfection as necessary to explain historical trends of disease (15). Together, these studies argue against the notion that previous infection imparts immunity to future infection and disease. The degree to which natural infection protects a person from subsequent exposures remains an important unanswered question.

Our data support the assertion that persons living in areas with intense transmission are likely to be infected multiple times throughout the course of their lives and, if they progress to disease, are most likely to do so because they were recently infected. Although the importance of exogenous reinfection to TB epidemics has been debated, our data reinforce arguments that reinfection likely plays a major role in high-incidence areas. This finding has numerous implications for designing and evaluating treatment programs in areas of high incidence. For example, in areas of intense transmission, persons receiving treatment who have intermittent or persistent smear-positive sputum may not be treatment “failures” but rather patients with multiple infections. Those who plan empiric treatment may not be treatment “failures” but rather patients with multiple infections. Those who plan empiric treatment strategies for patients with active disease should consider the drug-resistance profiles of currently circulating strains rather than those of strains observed in the past.

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Dr. Murray is an assistant professor of epidemiology at the Harvard School of Public Health and an infectious disease physician at Massachusetts General Hospital. Her research focuses on the transmission dynamics of emerging infectious diseases and in particular on multidrug-resistant TB.

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Emergency Survey Methods in Acute Cryptosporidiosis Outbreak

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In August 2003, a communitywide outbreak of cryptosporidiosis occurred in Kansas. We conducted a case-control study to assess risk factors associated with *Cryptosporidium* infection by using the telephone survey infrastructure of the Behavioral Risk Factor Surveillance System. Using existing state-based infrastructure provides an innovative means for investigating acute outbreaks.

Internet-based computerized questionnaire administration has been increasingly used in epidemiologic investigations and can reduce the resources and workload required for these studies (1,2). Computer-assisted telephone interview (CATI) surveys have been used for a variety of population and national health surveys, but their use in acute infectious disease outbreak investigations has not been reported. These surveys have the advantage of computerized data entry and trained interviewers, which minimizes data entry errors and interview time. These surveys also provide a readily accessible computerized database for data analysis. CATI systems facilitate telephone survey administration, provide sophisticated record management, and allow close monitoring of study progress.

*Cryptosporidium* spp. are chlorine-resistant, protozoan parasites that cause prolonged watery diarrhea. Outbreaks of cryptosporidiosis have historically been associated with recreational water (3–7), day camps (8), and daycare facilities (9–11). In August 2003, a communitywide cryptosporidiosis outbreak occurred in Douglas County, Kansas, USA. We conducted a community-based epidemiologic investigation to determine the risk factors associated with *Cryptosporidium* infection by using the infrastructure of the Health Risk Studies Program at the Kansas Department of Health and Environment (KDHE). This program conducts the Behavioral Risk Factor Surveillance System (BRFSS) and other telephone surveys within the state health department rather than through a contract with an external research organization. We assessed the feasibility of using a CATI system to conduct a case-control study in an acute outbreak investigation and identified areas to improve the use of this system.

The Study

In late August 2003, local, state, and federal health officials began an investigation to determine the risk factors associated with an outbreak of cryptosporidiosis and to develop interventions to control it. The epidemiologic investigation resulted in 96 laboratory-confirmed cases of *Cryptosporidium* infection and >600 clinical cryptosporidiosis cases.

BRFSS is an established nationwide population-based telephone survey system that primarily measures behavioral risk factors associated with leading causes of death. It is currently the largest continuous telephone survey in the world; it expanded to all 50 states in 1993 (http://www.cdc.gov/brfss). In Kansas, the Health Risk Studies Program conducts the BRFSS in-house and provides the capacity and expertise to design and implement special surveys. During this outbreak investigation, KDHE, the Lawrence-Douglas County Health Department, and the Centers for Disease Control and Prevention (CDC) used the BRFSS infrastructure, which consisted of a fully networked, computer-assisted telephone interviewing system (WinCATI Sawtooth Technologies, Northbrook, IL, USA) to conduct a case-control study.

We conducted a matched case-control study to identify specific risk factors for infection. Laboratory-confirmed case-patients were identified through laboratory surveillance. Clinical cryptosporidiosis patients were identified during the case ascertainment portion of the study, by surveying households of elementary school children and persons who had sought healthcare for diarrheal symptoms. All laboratory-confirmed patients were enrolled, as were a random selection of clinical cryptosporidiosis patients within 4 age strata. Two controls were matched to each patient, and each control was asked the same questions for the specific exposure period of the patient to whom they were matched. A maximum of 1 case-patient or control-patient per household was enrolled.

The CATI system relies on a networked central server with both interviewer and supervisory stations (Figure). The system allows for questionnaire programming, record management, scheduling of calls, and monitoring the disposition of calls. Telephone numbers for patients were programmed into the CATI system; controls were identified by random digit dialing. Telephone numbers, which included all telephone exchanges represented in the
100,000-person county, were purchased from a commercial survey research sample provider. This sample was prescreened to remove both business and nonworking numbers but did include unlisted numbers. All residential numbers in the community were eligible, and the CATI system released the telephone numbers randomly. Frequency matching controls to patients was performed by inquiring for a person at the residence within a certain age range. The questionnaire was programmed into the CATI system by using both range and logic checks, to minimize data entry errors, as well as skip patterns, which allow the respondent to answer only those questions that pertain to them. Twenty BRFSS personnel were trained in two 1-hour training sessions on the use of the outbreak instrument and conducted pilot testing of the questionnaire for appropriate wording and skip patterns.

This case-control study was initiated within 8 days of finalizing the questionnaire. Approximately 11,400 telephone calls were made, and 770 interviewer hours were used in a 41-day period to complete 151 case-patient and 302 control interviews. The average interview length for completion of the case questionnaire was 28 minutes, and the average interview length for completion of the control questionnaire was 16 minutes (Table). Data from the investigation showed multiple risk factors associated with Cryptosporidium infection, including exposure to several recreational water venues.

**Conclusions**

This study highlights the feasibility and potential benefits of a coordinated effort between chronic and infectious disease sections at local, state, and federal public health agencies in responding to an acute infectious disease outbreak. The BRFSS program at KDHE has facilitated the development of the internal expertise and infrastructure necessary to design and implement large-scale and complex telephone surveys. This program includes providing a cohort of trained interviewers who could efficiently collect data to allow a comprehensive assessment of the risk factors associated with Cryptosporidium infection in this outbreak.

With the WinCATI system, interviewers were able to enter questionnaire data directly into the computerized system in real time, thus creating a database that could be easily converted into a variety of statistical programs for data analysis. This system obviated the need for paper questionnaires and subsequent data entry. The questionnaire was programmed to require certain data before proceeding (logic checks) or to warn the user of an incorrect entry (data checks), thus decreasing the possibility of missing or including incorrect data. Use of an existing infrastructure did not require immediate recruitment and training of volunteer interviewers, the traditional method for outbreak investigations, but provided a trained interviewing staff. Additionally, this mechanism liberated the professional public health staff to focus their efforts on the multifaceted public health interventions required in a communitywide outbreak.

The use of existing CATI systems may be of value in several circumstances. As demonstrated here, in large, communitywide outbreaks, CATI systems can provide substantial resources and personnel capacity that may substantially enhance investigation efforts in responding to a public health threat. Additionally, CATI systems, similar to BRFSS, are well-suited for performing long-term studies, for on-going studies attempting to determine the source of sporadic infectious disease cases, and for public health surveillance. They can also provide a practical means of obtaining controls for case-control studies.

Nevertheless, several limitations should be noted about the use of population-based telephone surveys in responding to acute outbreak scenarios. Unlike traditional communicable disease control programs, community telephone survey efforts, such as BRFSS, were not created for immediate response, and therefore their use in this context has some limitations. These include the time required to program the questionnaire into a CATI system and the organization.

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**Table. Utilization of CATI system for case-control study**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case-patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interview period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. enrolled</td>
<td>151</td>
<td>302</td>
</tr>
<tr>
<td>No. calls made</td>
<td>1,357</td>
<td>10,101</td>
</tr>
<tr>
<td>No. refusals</td>
<td>56</td>
<td>330</td>
</tr>
<tr>
<td>Average interview</td>
<td></td>
<td></td>
</tr>
<tr>
<td>length (min)</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>Interviewer hours</td>
<td>263</td>
<td>508</td>
</tr>
</tbody>
</table>

*CATI, computer-assisted telephone interview.
zation of professional staff time in an outbreak situation. The preprogramming of generic infectious disease outbreak questionnaire modules (e.g., demographics, clinical symptoms, or foodborne or waterborne exposures) into a CATI system may help decrease the start-up time required for questionnaire implementation.

CATI surveys may also be less useful in several circumstances. These include smaller focal outbreaks in which the use of many resources and lengthy start-up times would be disadvantageous; particularly when these investigations are within the capacity of existing communicable disease programs. CATI surveys also have the standard limitations and biases inherent in telephone surveys. These include the following: selection bias, inclusion of only those who have a home telephone number; and response bias. In addition, those who participate may be different from those unwilling to participate, and declining response rates have been noted among telephone surveys (12). Moreover, the regular use of a CATI infrastructure, like BRFSS, for acute outbreak situations needs to be further assessed to prevent it from detracting from standard BRFSS activities. CATI systems, therefore, may not replace existing disease investigation programs but have the potential to supplement these programs.

Using existing state-based infrastructure in the chronic disease arena should be considered as a potential response strategy for future public health emergencies, and state health departments should consider developing plans and identifying financial resources for implementing similar strategies when performing large-scale investigations. Because many state health departments may contract with a survey research firm to perform population-based telephone surveys, including reference to special studies related to urgent public health needs should be included in these contract negotiations. Using CATI systems provides an innovative and potentially valuable adjunct to current outbreak investigation methods and should be considered as a viable addition or alternative for conducting acute outbreak investigations, particularly during large-scale, emergency situations when resources are limited.

Acknowledgments
We thank Mona Arnold and Ginger Taylor for their contributions to this study. We are indebted to the BRFSS personnel involved in conducting the interviews, as well as the health department staff at the Lawrence-Douglas County Health Department and KDHE for assisting in this investigation.

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Emergency Survey Methods in Cryptosporidiosis Outbreak

References


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Media Effects on Students during SARS Outbreak

Sheri L. Bergeron* and Ana L. Sanchez*

A few months after the 2003 severe acute respiratory syndrome (SARS) outbreak, a sample of Canadian undergraduate university students completed a questionnaire that showed that, despite believing media coverage of the outbreak was excessive, they had little anxiety about acquiring SARS. Additionally, 69% of participants failed a SARS-specific knowledge section of the questionnaire.

The 2003 outbreak of severe acute respiratory syndrome (SARS) underlined the importance of fast and accurate risk communication to the public. Several studies have attempted to evaluate the media’s performance during the outbreak (1,2), and the general consensus is that the media coverage was excessive, sometimes inaccurate, and sensationalist (1–3). Whether this excessive coverage had a beneficial or detrimental effect on the public remains unknown. A logical assumption would be that, in response, the public would not only have high anxiety of acquiring SARS, but also would be more informed about the cause, symptoms, and other aspects associated with the syndrome. The purpose of this study was to determine, in an undergraduate university student population, preferences and use of various types of mass communication media, anxiety levels of acquiring the infection, and general knowledge of SARS.

The Study

Following ethics approval by the Brock University Research Ethics Board in October 2003, a pilot survey of 30 students was conducted to validate a questionnaire and determine the sample size for the study. Results showed that approximately 30% of participants may have had some anxiety of acquiring SARS during the outbreak. Therefore, in a population of 13,000 students, a sample of 310 was calculated to obtain a measurable anxiety level (Epi Info 6.04b, Centers for Disease Control and Prevention, Atlanta, GA, USA).

The questionnaire consisted of 25 questions in different formats (multiple choice, 7-point scale, open-ended, and follow-up streamed questions) and collected information on the following topics: demographics; access to and use of the Internet, radio, television, magazines, and newspapers for general use and as a source for SARS information; perceived amount of media coverage; anxiety of acquiring SARS; and SARS-specific knowledge.

The study was conducted from October 2003 to January 2004 in 2 consecutive phases; pen and paper questionnaires were used in the first and a Web-based version was used in the second. For phase 1, students were randomly approached between classes on 2 different days and invited to participate in the study. For phase 2, flyers were distributed inviting students to visit a Web site that contained all pertinent information and a link to an interactive questionnaire accessible only to Brock students. The Web site was available until the target sample size was reached. Phases were designed in such a way that students could only participate once.

Data were compiled by using Microsoft Excel 2000 (Microsoft Corp., Redmond, WA, USA) and transferred into SPSS Version 11.1 (SPSS Inc., Chicago, IL, USA). Statistical analyses included frequencies, proportions, and t tests/analyses of variance to evaluate differences among stratified media groups and results from the SARS knowledge section.

In total, 314 students enrolled in the study (186 in phase 1 and 128 in phase 2), but 14 incomplete questionnaires were excluded, resulting in a final sample of 300 students. Of the 300 participants, 219 (73%) were women, and 213 (71%) were majoring in nonhealth-related areas. The average age was 21.1 years (SD = 4.7), with 93% between 18 and 23 years of age.

Assessment of access and usage of 5 forms of mass media communication showed that 89% used the Internet, 88% television, 77% radio, 56% newspapers, and 28% magazines. The daily use of television, radio, and Internet was categorized as previously reported (4), and participants were grouped into light (<2 h/day), medium (2–4 h/day) or heavy users (>4 h/day). Most students were light users of all 3 media types; however, Internet users were more likely to be heavy users than the other 2 groups combined (odds ratio = 3.55, 95% confidence interval 1.8–7.0, p = 0.0000357).

The perceived amount of media coverage was measured on a 7-point scale. Stratified results showed that most students (92.5%) considered the media coverage excessive.

Levels of anxiety of acquiring SARS were measured on a 7-point scale; the average anxiety level was 3.2, with a median of 3. Furthermore, when results were aggregated into low anxiety (score ≤3) and high anxiety (score ≥4) groups, 57% of students reported low anxiety, and 43% reported high anxiety. Anxiety levels were similar between health and nonhealth majors and were not associated with use intensity of any type of media.

Of the 300 participants, 206 (69%) failed the SARS-specific questions section (average 1.97, SD = 1.1); for which passing was defined as ≥3 correct answers of 4
questions, 1 each on cause, transmission, symptoms, and treatment of SARS. Health majors had a higher average score than nonhealth majors ($t$ [degrees of freedom, df = 152] = -3.5, $p = 0.001$), particularly in regard to questions about the cause and treatment of SARS (Figure 1).

The average SARS-specific knowledge score was not statistically associated with access to and use of any type of media. However, a passing score was more common for those who used the Internet to obtain SARS-related information ($t$[df = 149] = 1.7, $p = 0.088$).

**Conclusions**

Mass communication media are valuable resources for efficiently communicating risk information to the public. However, extensive collaboration among public health departments and media outlets is essential to deliver health information to all sectors of society (5,6). During the SARS outbreak, a deficient communication strategy among national and international public health agencies led to conflicting messages that created confusion and uncertainty in both the media and the general public (6). In reporting the events as they unfolded, the media communicated this confusion to the public. This study was undertaken only 5 to 8 months after the last known viral transmission (7) and sampled a particular sector of the population, young students attending a university in southern Ontario, 105 km from Toronto, the epicenter of the outbreak in Canada.

Most participants reported access to and use of several forms of media; the Internet as the most used, followed by television and radio. Newspaper and magazines were the least popular, which suggests that these forms of media are less appealing to young populations.

Overall knowledge about the cause, transmission, symptoms, and treatment associated with SARS was very low for this population. As expected, knowledge was higher among health majors but was not associated with any other variable. However, Internet use seemed to increase baseline SARS knowledge. A possible explanation for this observation is that, in contrast with television and radio, in which passive communication occurs, the Internet requires more participation, attention, and information processing as the user must search and choose to read the information. For a young population that prefers the Internet, this medium could be a great tool for delivering health messages.

When anxiety levels of acquiring SARS were assessed, the results did not support the assumption that the media created anxiety in this young population. The only predictors of high anxiety levels were sex (women) and area of residence in the greater Toronto area. Although anxiety levels for older age groups have not been studied, this finding may suggest that younger persons have different per-
ceptions of health risks and that health messages should be designed with these differences in mind.

In summary, this study showed that predetermined assumptions did not hold true for a young population. Despite believing that media coverage had been overdone, they reported low anxiety of acquiring SARS and showed poor knowledge of this emerging infectious disease (Figure 2). The discrepancy between the amount and type of information dispersed by the media and what was actually absorbed by the young population suggests that mere exposure to copious information is not enough to strengthen knowledge or elicit feelings that would induce persons to modify behavior.

Acknowledgments

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In 1972, noroviruses (previously called “Norwalk-like viruses”) were discovered as the first viruses definitively associated with acute gastroenteritis. During the next 2 decades, researchers were unable to develop simple methods to detect these common viruses or to find the etiologic agents of nonbacterial gastroenteritis outbreaks and hospitalizations. Indeed, of >2,500 foodborne outbreaks reported to the Centers for Disease Control and Prevention from 1993 to 1997, <1% were attributed to noroviruses, and 68% were of “unknown etiology” (1). As a result, noroviruses were out of sight and mind and thus relegated to a minor role as agents of gastroenteritis at a time when high-profile outbreaks of *Salmonella* Enteritidis (2) and *Escherichia coli* (3) had focused attention and budgets on preventing foodborne bacterial illnesses.

The development of reverse transcription–polymerase chain reaction in the early 1990s provided the breakthrough needed to facilitate diagnosis of norovirus infection. Today, noroviruses are recognized as the most common cause of infectious gastroenteritis among persons of all ages (4). They are responsible for ≤50% of all foodborne gastroenteritis outbreaks in the United States (5) and are a major contributor to illness in nursing homes (6) and hospitals (7). Noroviruses have been detected in 35% of persons with sporadic gastroenteritis of known cause (8) and in 14% of all children <3 years old hospitalized for gastroenteritis (9). Norovirus infection has put apparently healthy people in intensive care (10) and has been associated with chronic diarrhea among transplant patients (11).

In addition, we now know of a myriad of strains of norovirus, which have been classified into 5 genogroups, distinguishable from sapoviruses, a separate genus of human caliciviruses, but also in the *Caliciviridae* family. This diversity represents a dramatic increase from the single calicivirus strain discovered >30 years ago. Moreover, the known host range of noroviruses has expanded: the virus is found in mice (12), cows (13), and pigs (14), and antibodies to bovine strains have been found in humans, which has stimulated speculation about zoonotic transmission (15). However, a fundamental question remains—is the increased detection of norovirus the result of better application of improved diagnostics or does evidence exist that norovirus disease is an emergent problem?

Recent reports have established that norovirus strains can periodically emerge either globally or nationally, displace other strains, and increase disease incidence (16,17). In winter 2002, a new virus variant was attributed to a well-publicized surge of norovirus outbreaks on cruise ships and in nursing homes in the United States (18,19) and in healthcare facilities in Europe (20). Why these strains emerge into prominence is unclear, but they often belong to genogroup II, cluster 4 (Bristol virus). Whether these strains cause different or more severe symptoms than other noroviruses, are more transmissible, or can better evade the host immune response is not known. The periodic emergence of strains is likely to have always been a feature of noroviruses, but we do not know whether norovirus infections are more frequent now than in 1929, when Zahorsky first described “winter vomiting disease” (21). Despite a lack of consistent retrospective data to definitively answer this question, several factors suggest that norovirus disease may actually be more common today.

First, the rates of bacterial foodborne illnesses are declining, in large part because of measures such as improved refrigeration and use of Hazard Analysis and Critical Control Point systems to reduce contamination of food of animal origin (22). Most of these measures, however, will be ineffective against noroviruses, which are resistant to chlorination and freezing, persist in the environment, and require only very low inoculums to infect. Thus, the relative contribution of noroviruses to foodborne disease is likely to be increasing. Second, modern lifestyles make us more vulnerable to norovirus infection than when these viruses were discovered. Since 1972 in the United States, more elderly people live in communal settings, with the number of beds in nursing homes increasing >75% (23). In addition, we now eat more foods that have been handled by a variety of potentially infected people; 46% of household food expenditures is now spent...
COMMENTARY

on eating out, compared with 32% in 1972 (24). We also eat more of the foods that are likely to be contaminated with norovirus; consumption of fresh vegetables and fruit has risen >20% in the last 30 years (25), and this produce is often grown in countries where crops are still irrigated with sewage-contaminated water. Finally, more people than ever are traveling and have an increased risk for norovirus infection through exposure to hotels, airplanes, and cruise ships. From 1993 to 1998, for example, the number of cruise ship passengers in the United States increased by 50% (26). Faced with these trends, how should the public health community respond?

First, research on the disease prevalence of noroviruses is only beginning. If noroviruses are an increasingly common cause of infectious gastroenteritis, with some cases resulting in diarrhea-related deaths and hospitalizations, then substantially greater investments are required in their diagnosis. Increased use of diagnostics along with improved surveillance, such as in sentinel sites, will permit identification of new strains and shifts in the epidemiology of norovirus disease. The development of easy-to-use, sensitive assays for use by clinical and public health laboratories should also have a high priority.

Second, we do not know how to stop norovirus transmission. Foods can be contaminated with norovirus either at the source (27) or at the point of service by infected food handlers. Noroviruses can spread by water, direct person-to-person contact, or airborne droplets of vomitus (28), and they can persist in the environment as a source of continuing infection despite efforts at disinfection (29). Recent advances in finding a cell culture system for noroviruses may allow for assessing the efficacy of various disinfectants (30), but only by full epidemiologic investigation of viral gastroenteritis outbreaks and by application of molecular tests will transmission routes be determined, differences in epidemiology between strains be detected, and targeted control measures implemented.

Norovirus infections are common and likely to become more so. Effective prevention strategies must now be designed and implemented.

Acknowledgments

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References

Are Noroviruses Emerging?

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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The disease dengue fever (DF; also known as break-bone fever, dandy fever, and by other names) can be caused by any of 4 viruses within the virus family Flaviviridae, genus Flavivirus, i.e., dengue virus types 1–4 (DENV-1–4). Dengue fever is a short-duration, nonfatal illness characterized by sudden onset of headache, retroorbital pain, and high fever, joint pain, and rash. Whereas uncomplicated DF usually is the case, the picture can be much darker than that. Through a mechanism known as immune enhancement, sequential infections with certain dengue viruses set the stage for a far more serious complication, dengue hemorrhagic fever (DHF) and dengue shock syndrome, so that having uncomplicated DF can presage having DHF (1).

DHF is characterized by high fever, vascular permeability, bleeding, enlargement of the liver, and circulatory failure (dengue shock syndrome). In mild or moderate cases, signs and symptoms subside after the fever subsides, but in severe cases the patient’s condition suddenly deteriorates, body temperature decreases, and the circulatory system begins to fail. The patient then may quickly go into shock and die within a day, or quickly recover, if volume therapy is instituted (2).

Dengue viruses are transmitted from person to person or from monkey to monkey through infected female mosquitoes of the genus *Aedes*. The mosquito acquires the virus by taking a blood meal from an infected human, the principal amplifying host for these viruses, or from an infected monkey. Humans circulate these viruses in their blood (viremia) for 7 to 10 days after infection, allowing ample time for mosquitoes, often many mosquitoes, to feed and become infected. After an intrinsic incubation period of 1 week to 10 days, the mosquito is capable of transmitting the virus to a new host while blood feeding.

During epidemics of dengue, attack rates may be 80%–90% in susceptible persons. Although, it is not usually recognized, more than half the people who are infected with a dengue virus may be asymptomatic, which would indicate a substantial underreporting of infections. These comprise a substantial number of people who may have been primed for more serious illness at a later date and are unaware of their situation.

The global prevalence of dengue has increased substantially recently. Dengue is endemic in ≥100 countries in Southeast Asia, Africa, the Western Pacific, the Americas, Africa, and the eastern Mediterranean area (available from http://www.who.int/mediacentre/factsheets/fs117/en/), with imported cases essentially everywhere tourists, business people, and military personnel travel, whether dengue is recognized there or not. More than 2 billion of the approximately 6.5 billion inhabitants of this planet are at risk of acquiring dengue, and the World Health Organization has estimated that “there may be 50 million cases of dengue infection worldwide every year” (available from http://www.who.int/mediacentre/factsheets/fs117/en/). However, this is a misstatement. Either there are 50 million dengue infections (some with illness, some not) or there are 50 million people sick with dengue each year. Infections are not the same as illnesses.

The mild form of dengue is a serious annoyance and often is painful for those with it. However, DHF is the major international public health concern. Before 1970, a total of 9 countries had reported DHF epidemics; by 1995, >4 times that number reported such outbreaks. Most of these countries are in Southeast Asia and the Western Pacific, but with the worldwide spread of all dengue types, this disease threatens residents in tropical and subtropical regions, predominantly in urban and semiurban areas. In 2001, ≥600,000 cases of dengue were reported in the Americas, of which 15,000 were cases of DHF, more than twice the number of DHF cases in the Americas in 1995 (available from http://www.who.int/mediacentre/factsheets/fs117/en/). In 2001 alone, Brazil reported nearly 400,000 cases, including 670 cases of DHF. Not only is the
geographic distribution of dengue spreading, but the seriousness of its complications is being recognized (available from http://www.cdc.gov/search.do?action=search&query=Text=dengue). An estimated 500,000 persons with DHF require hospitalization each year, a substantial proportion of whom are children. Tragically, DHF is a leading cause of hospitalization and death of children in several Asian countries. Case-fatality rates can exceed 20%, are usually 2.5%, but can be reduced to <1% with rapid recognition and proper treatment.

In countries that are prepared for dengue and its complications, diagnostic services are available. We are able to sequence these viruses and determine their origins and evolutionary determinants. We can, to some degree, control the vector mosquitoes (*Aedes aegypti* and *Ae. albopictus*). Our knowledge of the pathophysiology of DHF is quite sophisticated (2,3). A vast literature is available about these viruses and the diseases they cause. Why, then, does dengue continue to spread? If we cannot eradicate dengue (and its vector *Ae. aegypti*) from populations on islands, from where can we eradicate it? Politics or misdirected funding, as always, has something to do with this, but the situation is much more complicated than that. Unless transovarial transmission (passage of virus from female to offspring through the egg) is much more important than it appears to be, other mechanisms are at play. Univalent vaccines for these viruses have been prepared but, for the most part, health authorities are (justifiably) unwilling to use such vaccines because they have the potential to stimulate the production of antibodies, which would prime vaccines for DHF by immune enhancement. Fortunately, novel approaches (development of incompetent mosquitoes), development of modern tetravalent vaccines, and development of chimeric vaccine viruses (4), using classic as well as molecular approaches will soon be available and hold out promise of tools we need to eliminate or eradicate this scourge.

This issue of Emerging Infectious Diseases includes some very interesting reports on dengue and its clinical complications, dengue diagnosis, and dengue epidemiology. These add considerably to the scientific record.

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Dengue is epidemic or endemic in virtually every country in the tropics; it is even cited in the Guinness World Records, 2002, as the world's most important viral hemorrhagic fever and the most geographically widespread of the arthropodborne viruses. As illustrated in this issue of Emerging Infectious Diseases, dengue epidemics are expanding rapidly, as is the literature on the subject. That dengue was transmitted in the United States for nearly 1 year in 2001 (1) should serve as a wake up call to Americans, most of whom are ignorant of the threat of this disease. Both the major dengue vectors, Aedes aegypti and Ae. albopictus, are widely distributed in the continental United States.

This emerging disease continues to baffle and challenge epidemiologists and clinicians. Despite endemicity of 3 or more different dengue viruses, why does severe dengue occur in some populations and not in others? Why are children principally affected in some areas and adults in others? How can severe dengue reliably be recognized early enough to permit appropriate therapy to be applied? Recent studies point in the direction of answers to these questions.

During an infection with any of the 4 dengue viruses, the principal threat to human health resides in the ability of the infecting virus to produce an acute febrile syndrome characterized by clinically significant vascular permeability, dengue hemorrhagic fever (DHF). However, because at onset vascular permeability exhibits only subtle changes, how can a diagnosis be made early enough to begin life-saving intravenous treatment? In persons with light skin color, the standard sphygmomanometer cuff tourniquet test has been widely used to screen children in outpatient settings; a positive test result is an early warning of incipient DHF. Because of genetic diversity among humans, the tourniquet test as a screening tool requires widespread evaluation and validation. In a prospective study of 1,136 Vietnamese children with serologically confirmed overt dengue infections, the tourniquet test had a sensitivity of 41.6%, a specificity of 94.4%, a positive predictive value of 98.3%, and a negative predictive value of 17.3% (2). A positive result should prompt close observation, but a negative result does not exclude an ongoing dengue infection.

A more robust screening test could result from the studies of Wills et al., who measured the size and charge characteristics of proteins leaking through the endothelial sieve in DHF patients (3). Such changes are caused, presumably, by a cytokine cascade, as yet incompletely identified. Their observations suggest that capillaries leak in most overt dengue infections, but fluid loss is not at levels that alter cardiovascular status. Quantitative differences in duration and amount of protein leak demarcate DHF from dengue fever. Importantly, the authors found increased amounts of serum proteins, increased differential protein excretion, and increased amounts of heparan sulfate in the urine of DHF patients. Detecting protein or heparan sulfate in acute-phase urine could provide early evidence of increased vascular permeability.

Humans are not uniformly susceptible to the DHF syndrome. HLA gene distribution correlates with increased susceptibility as well as with increased resistance (4). In addition, a powerful resistance gene is found in blacks (5). Importantly, susceptibility to vascular permeability during a dengue infection is age-related. This conclusion comes from a study of the age distribution of patients hospitalized during the 1981 DHF epidemic in Cuba. In that epidemic, persons 2–50 years of age were exposed to infections with dengue 1 virus (DENV-1) from 1977 to 1979 and DENV-2 virus in 1981 at similar rates (6). Thus, age-specific hospitalization and death rates were a measure of intrinsic susceptibility to vascular permeability during secondary DENV-2 virus infections. The youngest children were found to be at greatest risk; rates fell rapidly and were lowest in older teenagers and young adults, rising again.
somewhat in older patients. The susceptibility of young children to DHF precisely paralleled age-related changes in microvascular permeability measured in normal children and adults.

That cytokines, not virus, damage endothelial cells during the course of a dengue infection could be inferred from a recently published immunopathology study of human tissues from DHF patients (7). Despite in vitro evidence that dengue viruses infect human endothelial cells, dengue virus was found to have replicated in vivo in monocytes, macrophages, and B lymphocytes. While endothelial cells stained for dengue antigens, this staining was concluded to be the result of in vivo deposition of virus-antibody complexes but not cellular infection.

Differences in intrinsic virulence are widely believed to explain the ability of some dengue virus strains to cause DHF while others do not. Indeed, phenotypic attributes of avirulent American DENV-2 genotype viruses appear to correlate with differences in disease severity (8). A different explanation was provided by a prospective fever study in Iquitos, Peru. In 1990, DENV-1 virus entered Iquitos and became endemic. In 1995, widespread, mostly silent infections with American genotype DENV-2 were detected. Many persons infected with DENV-2 had antibodies elicited by an earlier DENV-1 infection. In contrast to Cuba, DHF did not develop in any of these secondarily infected persons. Antibodies to DENV-1 in the sera of Iquitos residents strongly neutralized American genotype DENV-2 viruses, but not virulent Asian genotype DENV-2 viruses isolated from DHF epidemics in the Americas (9). DENV-2 antigenic differences, reflected in heterotypic neutralization by antibodies to DENV-1, appeared to result in downregulated infection severity and corresponding clinical expression.

In the American tropics, with the exception of Cuba in 1981, most dengue cases were observed initially in adults. An illustration of this phenomenon is exhibited by the recent outbreak in Hawaii, where primary infections with DENV-1 virus produced dengue fever syndrome in adults. Dengue fever syndrome in susceptible adults may be contrasted to the innate susceptibility of children for vascular permeability syndrome during a secondary dengue virus infection. In Southeast Asia, the epicenter of DHF epidemics in children, dengue infection rates are falling, resulting in changing epidemiologic patterns of DHF. In Thailand, for example, the modal age at which children are hospitalized for DHF has steadily increased over the past several decades (A. Nisalak, pers. comm.). In addition, because an increasing number of persons experience their first dengue infection at an older age, dengue fever cases are now appearing in adults.

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References

Autochthonous dengue infections were last reported in Hawaii in 1944. In September 2001, the Hawaii Department of Health was notified of an unusual febrile illness in a resident with no travel history; dengue fever was confirmed. During the investigation, 1,644 persons with locally acquired denguelike illness were evaluated, and 122 (7%) laboratory-positive dengue infections were identified; dengue virus serotype 1 was isolated from 15 patients. No cases of dengue hemorrhagic fever or shock syndrome were reported. In 3 instances autochthonous infections were linked to a person who reported denguelike illness after travel to French Polynesia. Phylogenetic analyses showed the Hawaiian isolates were closely associated with contemporaneous isolates from Tahiti. *Aedes albopictus* was present in all communities surveyed on Oahu, Maui, Molokai, and Kauai; no *Ae. aegypti* were found. This outbreak underscores the importance of maintaining surveillance and control of potential disease vectors even in the absence of an imminent disease threat.

Dengue viruses cause a wide range of illness, including dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). Four dengue serotypes, known as DENV-1, -2, -3, and -4, can cause severe and fatal disease. Dengue typically occurs in tropical and subtropical areas in the world and is transmitted by *Aedes* mosquitoes; *Aedes aegypti* is the principal vector worldwide (1). DF and DHF are the most important arboviral diseases of humans; ≈50–100 million dengue infections and several hundred thousand cases of DHF occur annually (2).

The first large-scale dengue fever epidemic in Hawaii occurred in the late 1840s; a second outbreak occurred at the turn of the century, with an estimated 30,000 cases (1,3). During those periods *Ae. aegypti* was widespread in Hawaii (4). Epidemic dengue occurred again on Oahu in 1943 to 1944, when 1,498 infections were reported, mostly in urban areas of Honolulu (5). *Ae. albopictus* had been introduced into Hawaii at the beginning of the century, and by 1940 it was the dominant day-biting *Stegomyia* mosquito species in the islands (4,5).

After the Second World War, no confirmed autochthonous dengue infections were reported in Hawaii. Nevertheless, dengue illnesses were occasionally identified among travelers to Hawaii who had been infected overseas. The annual number of imported cases was low, with 20 infections recorded during the 10-year period from 1991 through 2000 (P. Effler, unpub. data).

On September 12, 2001, the Hawaii State Department of Health (HDOH) received a call from a physician in Hana, Maui, who had seen a patient with febrile illness and rash 1 week earlier. The physician indicated that several of the patient’s family members had become symptomatic; none had a history of recent foreign travel. On investigation by HDOH staff, dengue fever was suspected, and clinical specimens were collected and forwarded to the Centers for Disease Control and Prevention (CDC) for diagnosis. On September 21, CDC confirmed recent dengue infection in the index patient. We report the results of an investigation into the first outbreak of dengue fever in Hawaii in 56 years.

**Methods**

**Case Finding**

From September 23 to 28, 2001, HDOH contacted all licensed physicians in the state by email or facsimile to request that any patient with a denguelike illness (DLI) be
tested for dengue, regardless of travel history. DLI was defined as fever or chills plus 2 or more of the following symptoms (6): myalgia, headache, arthralgia, eye or retroorbital pain, rash, or hemorrhagic manifestation (e.g., petechiae, hematuria, hematemesis, menorrhagia, melena).

On September 24, 2001, active surveillance was established at 51 clinical settings across the state. All acute-care hospitals and major clinics were contacted daily to determine the number of clinically compatible illnesses seen in the previous 24 hours and to arrange for laboratory evaluation of suspected cases. Although HDOH recommended dengue testing only for patients meeting DLI criteria, it was performed whenever requested by a physician.

HDOH staff interviewed persons with suspected dengue infection to obtain symptom and travel histories. Visits to residences and work sites were conducted. Patients’ household contacts or co-workers with a history of illness were urged to be tested for dengue.

**Laboratory Surveillance**

All clinical laboratories in Hawaii were asked to report any requests for dengue diagnostic testing and to forward aliquots of serum samples obtained for dengue testing to the HDOH State Laboratories Division. Laboratory analyses to detect anti-dengue immunoglobulin (Ig) M and IgG and to isolate and identify the virus were performed by methods previously described (7–12).

RNA was extracted by using QIAmp Viral RNA Mini kits (Qiagen GmbH, Hilden, Germany). Sequencing was performed by using the Taq DyeDeoxy Terminator Cycle Sequencing kits (Applied Biosystems, Foster City, CA, USA). Sequencing products were cleaned by using agarose gel electrophoresis and silica gel adsorption (Qiagen PCR purification columns) and analyzed on an ABI PRISM 377 DNA sequencer (Applied Biosystems). Sequences were assembled and aligned with Lasergene software (DNASTar, Madison, WI, USA), and phylogenetic trees were generated with PHYLIP v. 3.5c (University of Washington, Seattle, WA, USA).

**Case Definition**

Laboratory-positive recent dengue infection was defined as a person who had 1) dengue virus isolated from serum, 2) a positive dengue IgM antibody test result, or 3) a positive IgG antibody test result in a person initially tested for dengue ≥60 days after onset of DLI and who was epidemiologically linked to another person with recent dengue infection identified by virus isolation or positive IgM serologic test result.

Persons were classified as negative for dengue infection if they had at least 1 specimen collected 6–60 days after illness onset that was IgG negative. Persons were classified as indeterminate for dengue infection if all specimens were collected <6 days after illness onset and were negative for virus isolation and for anti-dengue IgM. Imported dengue was defined as illness in a person with laboratory evidence of recent dengue infection and a history of international travel within 14 days of illness onset.

**Entomology**

During the outbreak investigation, a CDC entomology team conducted spot checks of potential breeding sites in 29 communities (at least 20 sites per community) on all islands except Hawaii and Lanai. From March to May 2002, HDOH vector-control staff placed ovitraps at 295 sites throughout the state; local vector-control staff relied on prior experience to select sites with known populations of day-biting mosquitoes. In both surveys, larvae were collected from breeding sites and identified to species. In the second survey, eggs were reared to the fourth larvae or adult stage before speciation. Adult mosquitoes attracted to humans were also captured and identified at many of these sites; in the outbreak areas; landing counts were obtained by recording the number of mosquitoes landing on a stationary person during a 5-minute period.

**Statistical Analysis**

Univariate analyses were conducted by using EpiInfo Version 6.4c (CDC, Atlanta, GA, USA). A difference in proportions was considered significant if the chi-square p value was <0.05.

**Results**

From September 12, 2001, to April 30, 2002, a total of 1,644 persons in Hawaii without a history of recent foreign travel were tested for possible dengue infection. Of these, 122 (7%) had laboratory evidence of a recent dengue infection: 15 (12%) were positive by virus isolation; 99 (81%) had anti-dengue IgM; and 8 (7%) had a history of DLI, anti-dengue IgG, and an epidemiologic link to a patient with recent infection (Table 1). Testing was indeterminate for 422 (26%) persons, and the remaining 1,100 (67%) did not have dengue infection. The median age was 41 years (range 1–77), 35 years (range 0–89), and 29 years (range 0–81) for persons who were laboratory positive, negative, and indeterminate for dengue infection, respectively.

Autochthonous dengue infections were identified on 3 of 6 islands (Table 1). Exposures on Maui, Oahu, and Kauai accounted for 76%, 21%, and 3% of all recent dengue infections, respectively. Eighty (66%) of the laboratory-positive infections were from persons who stayed in the Hana area of Maui, an area with <2% of the island resident population (Figure 1). On Oahu, 20 (77%) of the
infections occurred among residents of 2 nearly adjacent communities on the windward side with a combined population of 25,709 (<3% of the island’s total). The heavily affected areas of Maui and Oahu both have thick vegetation and heavy precipitation (average annual rainfall >177 cm/year, 4 times the annual rainfall in Honolulu).

The outbreak spanned >8 months, with a peak incidence in late September 2001. (Figure 2) The first suspected dengue illness was reported with an onset date September 5, 2001; subsequent investigations identified an additional 31 laboratory-positive patients with illness onset before that date, and the earliest was May 27, 2001.

Of laboratory-positive cases, 89% met the clinical criteria for DLI (Table 2). Patients with recent dengue infection reported a greater number of symptoms than those who did not have dengue. One or more hemorrhagic manifestations were reported in 42 (34%) persons with dengue infection. Myalgia, chills, arthralgia, and rash were significantly more common among patients with laboratory-positive dengue infection than in persons with negative or indeterminate results.

No cases of DHF or DSS, as defined by the World Health Organization, were reported, and no deaths occurred (14). Three patients with laboratory-positive dengue infection were hospitalized for their illness.

Eighty-one (66%) of the recent infections were initially reported by physicians treating acutely ill patients, while the remaining 41 (34%) were identified through HDOH field investigations. Thirteen household clusters accounted for 53 (43%) of the 122 patients.

One-hundred and fifteen (95%) of the 122 persons with laboratory-positive infection were residents of the state of Hawaii. All 7 visitors with dengue stayed at rental properties in the Hana area of Maui. Another 70 nonresidents with possible dengue infections who visited Hawaii during the outbreak were reported to HDOH; 30 of these nonresidents were serologically tested, and results for all were negative.

From January 1, 2001, to April 30, 2002, a total of 43 cases of imported dengue infection were reported to HDOH (Figure 3). Oahu had the greatest number of imported infections (31 infections), followed by Maui (6 infections), Hawaii (4 infections), and Kauai (2 infections). Eighteen (42%) of the imported dengue infections were from the Society Islands, 13 (30%) were from American or Western Samoa, 7 (16%) were from the Philippines, and 1 each was from Cambodia, Easter Island, Indonesia, Thailand, and Vietnam. Imported dengue

![Oahu Dengue Cases](image1)

![Maui Dengue Cases](image2)

Figure 1. Autochthonous dengue infections, Maui and Oahu, Hawaii, 2001–2002.
peaked in July and August 2001; exposures in the Society Islands accounted for the largest proportion of cases during this time (n = 9, 47%).

All 15 dengue virus isolates obtained from patients with exposure in Hawaii were DENV-1. Phylogenetic analysis of envelope glycoprotein sequences showed that the Hawaiian isolates belonged to a group composed primarily of Pacific Island isolates from recent years (Figure 4). High bootstrap values showed the Hawaiian isolates were associated more closely with contemporaneous Tahiti and subsequent Easter Island isolates than with a 2001 isolate from American Samoa.

In entomologic surveys conducted during the outbreak, Ae. albopictus was present in all 29 communities surveyed on Oahu, Maui, Molokai, and Kauai, but no Ae. aegypti were found at any site. In drier areas, on the leeward sides of the islands, container indices were high (>50%), but landing rates were generally low. However, in Nahiku, a small community in densely vegetated woodland near Hana, Maui, that was heavily affected during the outbreak, adult Ae. albopictus populations were high, with landing rates of 70 to 90 mosquitoes per person in 5 minutes. In the surveys conducted at 300 sites in 2002, Ae. albopictus larvae were ubiquitous on all islands, including Lanai and Hawaii, but Ae. aegypti was only found in 3 communities in the southern part of the island of Hawaii.

**Discussion**

This report describes the first outbreak of dengue fever in Hawaii since the mid-1940s. Understanding the factors...
that contributed to the reemergence of dengue after such a prolonged absence and to the cessation of transmission will help public health authorities develop future prevention and control strategies.

At the time of the 2001 Hawaii outbreak, a large DENV-1 epidemic was occurring in the Society Islands, 4,400 km south of Hawaii. More than 33,000 dengue illnesses were recorded in the Society Islands from February to November 2001, and of the 1,400 persons hospitalized, DHF was diagnosed in 45%, and 20% had symptoms of DHF or DSS. *Ae. aegypti* was identified as the vector (15,16).

Virologic and epidemiologic data strongly suggest that the Hawaii dengue outbreak was directly linked to the one in French Polynesia. Travelers are a potential source for dengue outbreaks; many epidemic introductions are thought to result from the arrival of a single viremic person into an *Ae. aegypti*– or *Ae. albopictus*–infested area (17). DENV may have been introduced to Maui when a group of >30 persons from Hana visited Tahiti during April–May 2001. One of the travelers (patient A) became ill shortly after returning to Hana and later tested positive for anti-DENV IgM and IgG. Patient A was a close associate of the first known autochthonous case-patient in the Hawaii outbreak, whose illness onset occurred ≈2–3 weeks later.

Although patient A may have been the source for the Hana outbreak on Maui, available information suggests that additional separate virus introductions led to independent foci of autochthonous cases on the other 2 affected islands. In Kauai, only 1 of 4 dengue case-patients had any known exposure to persons from Maui. Moreover, the first identified case-patient in Kauai shared accommodations with a person in whom a febrile illness developed shortly after the patient returned from Tahiti. On Oahu, none of the 26 confirmed infections could be epidemiologically linked to exposures on Kauai or Maui. Furthermore, during an investigation of an autochthonous cluster on Oahu, the likely index patient was as an IgM-positive family member who had a DLI 4 days after returning from a trip to Tahiti.

* Ae. albopictus* was the vector responsible for the 2001 Hawaii outbreak. Both entomologic surveys support that *Ae. albopictus* is ubiquitous, often common on all the islands, whereas *Ae. aegypti* is restricted to a few small foci on the relatively sparsely inhabited island of Hawaii.

Several factors may explain why the outbreak in Hawaii followed a much different course than the concurrent epidemic caused by an apparently similar DENV-1 strain elsewhere in the Pacific. First, differences in mosquito species, behavior, and ecology are critical to understanding why the Hawaii outbreak was less severe than that described in the Society Islands, where *Ae. aegypti* was the principal mosquito vector. *Ae. aegypti* females are highly anthropophilic and often feed on several persons before obtaining enough blood to complete a gonotrophic cycle. This tendency towards multiple feeding may contribute to the explosive nature of dengue outbreaks in areas where *Ae. aegypti* is present. Compared with *Ae aegypti*, *Ae. albopictus* is considered to be an inefficient epidemic dengue vector because it is less anthropophilic and not as well adapted to urban domestic environments (18). *Ae. albopictus* will readily feed on humans, but usually only...
on a single person, and it also feeds on other animals, which decreases the probability of human contact (19,20).

Lifestyle factors may also help explain why Hawaii’s dengue outbreak was limited (21). Residences in many affected areas often had dense, uncultivated vegetation near housing and, not infrequently, an abundance of items that could serve as suitable *Aedes* breeding sites: tires, buckets, and discarded vehicles. Furthermore, dwellings in these areas often lacked window screens and doors. The combination of ample mosquito breeding sites and relatively unrestricted access to residents in some sections of windward Oahu and Hana, Maui, probably enhanced opportunities for mosquito-human contact beyond levels that existed in Hawaii’s major population centers.

Public health measures may also have helped mitigate the spread of Hawaii’s outbreak. This response consisted of 4 simultaneous, integrated initiatives: 1) enhanced surveillance to detect new foci of transmission; 2) rapid education of healthcare providers to improve the diagnosis and treatment of dengue; 3) health promotion activities directed toward the general public, including visitors; and 4) vector-control efforts, which included a combination of source reduction activities, limited use of larvicides, and area spraying (Appendix).

Worth noting is that most of the illnesses in the Hawaii outbreak were mild, given that an apparently similar DENV-1 strain caused a major epidemic of DHF and DSS in French Polynesia. One possible explanation for the difference in illness severity observed between these locations is that the number of cases in Hawaii was too small to manifest the extremes of the clinical spectrum. A second explanation is that a history of dengue infection, i.e., antibody-dependent enhancement, may have been important in French Polynesia (22). A third explanation is that the Hawaiian virus had changed genetically and became less virulent or lost its epidemic potential. This loss of epidemic potential occurred in the 1970s when both DENV-1 and DENV-2 were reintroduced into the Pacific after an absence of 25 years (23). Despite close similarities in the envelope protein sequences of the 2001 Tahiti and Hawaii viruses, important differences may exist in other areas of the genome that could influence these properties. Recent studies in Sri Lanka and Puerto Rico suggest that the genetic changes associated with epidemic potential occur in the nonstructural virus genes and not the envelope gene commonly used to show genetic relatedness between dengue viruses (24,25). Full-length genomic sequencing of DENV-1 viruses is pending.

The Hawaii experience demonstrates the potential of *Ae. albopictus*, under suitable conditions, to transmit small outbreaks of dengue within the United States. During the last 15–20 years, this mosquito has expanded its geographic range within the United States and now is found in at least 24 states on the mainland (26,27). From 1986 to 2000, a total 516 laboratory-confirmed and 2,128 suspected dengue infections were imported into the United States (28–33). The true incidence of imported dengue infection is probably higher, since dengue may often go undiagnosed in areas where the virus is not endemic (4,20,23,34,35). Given the high volume of travel between the US mainland and dengue-endemic areas of the world (an estimated 14 million passengers to and from the Caribbean, Central and South America, and Oceania in 2001), we recommend that health officials keep local clinicians informed of dengue activity in these regions and that clinicians consider the possibility of autochthonous transmission when evaluating febrile rash illnesses, particularly when local vector surveillance indicates high populations of *Ae. aegypti* or *Ae. albopictus* mosquitoes (36,37).

This investigation has several limitations. First, despite extraordinary efforts to obtain specimens, ≈25% of all persons initially evaluated for dengue did not submit a convalescent-phase specimen (>5 days after illness onset) required for definitive case classification. During follow-up attempts to obtain convalescent-phase sera, we often learned that patients or their physicians had decided that dengue was unlikely and no further testing was necessary; however, some dengue infections may have been missed. Secondly, because persons acquire dengue from mosquitoes that feed during the daytime, infection might have occurred at a location other than where the patient lived. We mapped the distribution of residences, however, because this information is not subject to recall bias. Thirdly, when investigating newly reported cases, we did not routinely elicit the number of household members and obtain serum samples from them in a standardized manner. Therefore, we cannot calculate the proportion of close contacts who were infected.

The Hawaii dengue experience is another example of how readily pathogens can cross great expanses of ocean to cause outbreaks in new territory (1,38–40). Important lessons learned from this episode include the need to closely monitor and respond to disease developments in the global community and the need to maintain surveillance and control of potential disease vectors even in the absence of an imminent disease threat.

**Appendix**

**The Public Health Response to Dengue in Hawaii, 2001–2002**

Enhanced surveillance involved 1) conducting active surveillance at >50 medical facilities statewide, 2) providing free laboratory testing for all patients with suspected dengue, 3) providing assistance with phlebotomy and obtaining convalescent-phase serologic specimens, and 4) providing details of an imminent disease threat.
samples, 4) creating a patient-tracking system, and 5) notifying all state epidemiologists though Epi-X to identify any possible dengue cases exported from Hawaii.

Provider education included 1) issuing medical alerts to physicians, 2) conducting grand rounds and other lectures on dengue at local medical centers, and 3) distributing CDC video tapes on dengue diagnosis and treatment to physicians.

Health promotion efforts included 1) conducting door-to-door source reduction campaigns by repellent.

and others who distributed educational materials and mosquito Maui rental car agencies and hotels; and 7) establishing check-

lates on dengue diagnosis and treatment to physicians.

HDOH and Department of Education health educators; 4) distrib-

which provided the public and officials with information on the latest developments; 6) distributing educational brochures to

uting >600,000 dengue brochures through high-volume stores and other venues; 5) developing a dengue education Web site, which provided the public and officials with information on the latest developments; 6) distributing educational brochures to Maui rental car agencies and hotels; and 7) establishing check-

points along the Hana Highway staffed by public health nurses and others who distributed educational materials and mosquito repellent.

Vector control efforts included 1) inspecting private and public properties for mosquitoes, larvae, and potential breeding sites; 2) conducting door-to-door source reduction campaigns by HDOH staff and community volunteers in Hana and windward Oahu; and 3) treating >2,500 residences statewide with insecticides or larvicides.

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Dengue viruses cause a range of clinical illness, including dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. Dengue is transmitted by infected *Aedes* mosquitoes and is endemic in many areas in the world (1). The occurrence of dengue is rising worldwide, particularly in the Americas, where the reported incidence more than tripled from 1996 to 2002 (2). At the beginning of the 21st century, dengue fever and dengue hemorrhagic fever are the most important arboviral diseases of humans, with an estimated 50–100 million dengue fever cases and several hundred thousand dengue hemorrhagic fever cases occurring each year (3).

In September 2001, the Hawaii Department of Health (HDOH) identified the first autochthonous case of dengue fever in 56 years. The ensuing dengue virus 1 (DENV-1) outbreak, transmitted by *Aedes albopictus* mosquitoes, spanned 9 months. A total of 122 laboratory-positive infections cases were identified from 3 islands: Maui (n = 92), Oahu (n = 26, and Kauai (n = 4) (Figure 1). Seven (6%) of the dengue infections were documented among nonresidents, all of whom had stayed in the Hana area of Maui.

Despite the relatively high rates of dengue in many tropical destinations frequented by tourists and numerous case reports of dengue in travelers, limited information is available on the risk for infection among short-term visitors (4–8). An estimate of 1 case of dengue illness per 1,000 travelers has been reported recently; however, this figure was derived from surveys of soldiers and expatriates living abroad, i.e., persons whose exposure risks may be quite different from those of recreational travelers (9). Given that Hawaii receives >7 million visitors each year, even low rates of dengue transmission to visitors during an outbreak could result in substantial numbers of infections being exported to the US mainland and elsewhere. We studied the risk for dengue infection among visitors to Hawaii during the peak of the outbreak in 2001.

**Methods**

State law requires passengers and families to submit a “Plants and Animals Declaration Form” on arrival in Hawaii. The declaration also solicits information regarding the nature of the passengers’ visit to the state, including

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*Hawaii State Department of Health, Honolulu, Hawaii, USA*
proposed destinations and intended length of stay. After review at the port of entry, the declaration forms are forwarded to Department of Business and Economic Development and Tourism for data entry and storage. We selected the 4 weeks for which the incidence of illness onset for laboratory-positive dengue infections in Hawaii was the highest (September 12–October 10, 2001) and identified 99,766 parties with a declaration who arrived in Hawaii during that time frame (Figure 2). The person who completed the form was defined as the “visitor” for our study. To be eligible for inclusion, visitors had to 1) supply a complete mailing address within the United States, excluding Hawaii; 2) be visiting Hawaii, as opposed to an in-transit layover (<24 hours) to another destination; and 3) express an intention to visit just 1 of the following islands during their stay—Maui, Oahu, or Hawaii. By sampling visitors who intended to visit only 1 island, we hoped to reduce the number of respondents reporting multiple exposures so that any dengue infections identified could be attributed to a specific island.

A total of 43,161 (43%) visitors fit the criteria. From these, a stratified, random sample of 4,000 visitors was selected: 2,000 visitors to Maui and 1,000 visitors each to Oahu and Hawaii. (The island of Hawaii is commonly called the Big Island and is referred to as such for the remainder of this article to avoid confusion with the state of Hawaii.) Because most dengue infections identified during the outbreak were in residents of Maui, visitors to this island were oversampled to increase the probability of detecting dengue infections among travelers. In contrast, visitors to the Big Island were used as a comparison group because no dengue infections had been identified from exposures on that island during the outbreak (Figure 1).

On December 11, 2001, a questionnaire packet was mailed to the 4,000 visitors in our sample. The packet contained a letter explaining the purpose of the study, a self-addressed stamped envelope, and a 14-question survey. Visitors were asked demographic information, dates of travel to Hawaii, knowledge of the dengue outbreak, changes made to vacation plans due to the outbreak, actions taken to reduce mosquito exposures, which islands were visited, and specifically whether the visitor had traveled to the Hana, Maui, area. In addition, the questionnaire asked if the visitor became ill during or up to 14 days after the trip, and if so, what symptoms were experienced. Finally, visitors were asked to provide a name, phone number, or email address so they could be contacted by HDOH.

Dengue-like illness (DLI) was defined as fever and/or chills with at least 1 additional symptom, including the following: headache, body aches, eye pain, muscle aches, joint pain, rash, bleeding gums, blood in the stool, or nosebleed. Our description of clinical DLI was intentionally less restrictive than that used for surveillance by the World Health Organization (WHO), which uses fever plus 2 other compatible symptoms. We also allowed a history of chills to serve as an indicator of fever when no temperature was taken because we wanted to increase the probability of identifying any dengue infections that had occurred in visitors (1,10).

Visitors reporting DLI were contacted. After a brief description of the outbreak, and risks and benefits of participating, they were asked to consent to having blood drawn for serologic testing. HDOH arranged to have blood drawn in conjunction with the visitor’s local health department, doctor, or diagnostic laboratory at no cost to the patient. All specimens were tested for anti-dengue immunoglobulin (Ig) G antibodies at the Division of Vector-Borne Infectious Diseases, Centers for Diseases Control and Prevention (CDC), by methods previously described (11,12).

Statistical Evaluation

Statistical analyses were performed by using SAS (version 8.2, Cary, NC, USA) and SAS-Callable SUDAAN release 8.0 (Research Triangle Institute, Research Triangle Park, NC, USA). Responses were weighted to reflect the visitor’s port-of-entry (which island they originally flew into from the mainland) and their intended island destination; the survey weights were created by SMS Research and Marketing Services, Inc (Honolulu, HI, USA). In the following text, numbers are presented unadjusted, followed by the weighted percentage unless otherwise noted; chi-square and relative risks are weighted.

The incidence of dengue infection among participants was calculated as the number of visitors who were anti-dengue IgG positive, divided by the total number of person-days in Hawaii for all those serologically tested. The
upper 95% confidence limit (CL) for dengue incidence was calculated on the basis of the Poisson distribution as Maximum risk = \(-\ln (0.05)/t \times (365)\), where \(t\) is the number of person days of exposure \((13,14)\). The state of Hawaii’s HDOH Institutional Review Board approved the study through expedited review on March 12, 2002.

Results

A total of 3,064 (77%) visitors responded to the survey (Figure 3). Respondents were 47% male and 53% female. The median age was 44 years \((\text{range } 14–94\text{ years})\). Respondents were similar to nonrespondents in terms of sex and the number of days spent in Hawaii. Most respondents and nonrespondents were 30–59 years of age \((69\%\text{ and } 67\%, \text{respectively})\).

The median length of stay in Hawaii was 8 days \((\text{range } 2–188\text{ days})\). Resort hotels were the most common accommodation \((60\%)\), followed by condominiums \((22\%)\), private residences \((16\%)\), bed and breakfast hotels \((1\%)\), and campgrounds \((0.4\%)\). The distribution of island destinations among respondents is shown in Figure 4. A total of 2,729 \((88\%)\) of the respondents visited only the island they had indicated on the agricultural declaration upon arrival in Hawaii; 94 \((4\%)\) went to the island indicated on the declaration form as well as to other islands; and 241 \((8\%)\) visited an island(s) other than the one indicated on the declaration. Sixteen percent of all respondents \((n = 655)\) reported going to Hana, Maui, of whom 74 \((12\%)\) stayed overnight.

Knowledge of the Outbreak

Only 238 \((8\%)\) of respondents reported that they knew about the dengue outbreak before they traveled to Hawaii; 1,467 \((45\%)\) learned of it while in Hawaii; 244 \((9\%)\) first heard of it after leaving Hawaii but before receiving the survey questionnaire, and 1,071 \((37\%)\) learned of it after leaving Hawaii and before receiving the questionnaire packet, >80% first learned of the outbreak from the news media (newspaper, radio, or television) (Figure 5).

Of the 1,705 respondents who learned of the outbreak either before or during their visit to Hawaii, 483 \((27\%)\) took personal precautions to reduce their exposure to mosquito bites, and 213 \((10\%)\) changed their vacation plans because of the outbreak. Among those who changed their plans, 146 \((66\%)\) did not travel to Hana, Maui; 73 \((35\%)\) skipped activities such as camping, hiking, or golfing that they had originally planned; 9 \((5\%)\) changed the types of places they stayed at while in Hawaii; 5 \((5\%)\) decided not to travel to Maui; and 7 \((5\%)\) shortened their vacation to Hawaii. Determining how many deferred coming to Hawaii because of the dengue outbreak, of course, is not possible. Of those who took personal precautions to avoid mosquito bites, 332 \((66\%)\) used repellent, 118 \((24\%)\) left places if mosquitoes were seen, 83 \((18\%)\) limited their time outdoors, 80 \((17\%)\) wore long pants and long-sleeved shirts when outdoors, and 38 \((9\%)\) used mosquito coils, sprayed insecticide in or around the places that they stayed, or both.

A total of 232 \((8\%)\) visitors reported becoming ill either during their trip or within 14 days of leaving Hawaii. The reported illness in 138 \((59\%)\) of the ill respondents did not meet criteria for DLI (Table). Respondents with illness that did not meet DLI criteria reported a median of 2 symptoms; headache, nausea, and diarrhea were the most frequently reported among this group.

Ninety-four visitors \((3\%\text{ of all respondents and } 43\%\text{ of those ill})\) met criteria for DLI, of which 12 reported at least 1 hemorrhagic manifestation. Respondents with DLI reported a median of 7 symptoms; chills, headache, body pain, muscle aches, and fever were each reported by more than half of respondents with DLI.

Of visitors with DLI, 38 \((40\%)\) became ill while still vacationing in Hawaii, 34 \((36\%)\) became ill 1–10 days after leaving Hawaii and 16 \((18\%)\) became ill 11–14 days after leaving the islands. The median duration of illness was 7 days \((\text{range } 1–60)\). Thirty-four \((34\%)\) of the visitors with DLI saw a doctor for their illness; in 2 patients, dengue was diagnosed by their physician, and 2 others stated that they had been hospitalized, once for suspected meningitis and once for a respiratory infection.

The proportion of visitors reporting any illness \((7\%–8\%)\) or DLI \((3\%)\) was very similar for each of the 3 islands \((\text{Oahu, Maui, and the Big Island})\). Visiting the epicenter of outbreak, Hana, Maui, was not significantly asso-

![Figure 3. Participation rates in the survey and serologic testing to assess the risk for dengue transmission to visitors during an outbreak in Hawaii, 2001. See text for further details.](image-url)
Dengue Risk among Visitors to Hawaii

Associated with reporting DLI. However, staying overnight in Hana was associated with reporting DLI symptoms when compared to visitors without illness (relative risk = 2.78, 95% CI 1.04–7.43). The type of accommodation used in Hawaii (hotel, condominium, bed and breakfast) was not significantly associated with reporting DLI.

Nine (9%) of the 94 persons with DLI did not provide a telephone number or email address for follow-up. Of the remaining 85 (91%) respondents with DLI, 27 (32%) provided a serum specimen for testing through their local department of health or physician; 18 (23%) could not be contacted after repeated attempts; and 40 (45%) refused testing or did not show up for their scheduled appointment to draw blood.

The 27 visitors who had blood drawn stayed in Hawaii for a median of 8 days (mean 13 days). The median interval from illness onset to collection of the serum was 224 days (range 153–310). All samples from the 27 persons were negative for anti-dengue IgG antibodies. These 27 visitors contributed a total of 358 person-days of exposure during the outbreak; the point estimate of dengue incidence is therefore zero infections per 358 person-days of exposure (0 per person-year) with an upper 95% CL of 3.0 cases per person-year (unweighted data).

Discussion

The state of Hawaii currently receives 5.3 million domestic and 2.2 million international visitors each year. In light of this, the recent reemergence of dengue fever in Hawaii represents a potential threat to populations across the US mainland and elsewhere. The current study was undertaken to assess the risk of visitors acquiring dengue infection during the outbreak. This information is needed because of the sheer number of visitors and the high likelihood that dengue will be reintroduced into Hawaii in the future.

Our investigation indicated that the risk for dengue among domestic travelers to Hawaii during the peak of the outbreak was low. Although 3% of the visitors surveyed reported experiencing DLI, none of the persons who underwent anti-dengue IgG antibody testing had evidence of dengue infection. The major limitation of this study is that only approximately one quarter of persons with DLI provided a serum specimen. However, these persons were similar to those with DLI who did not provide a specimen in regard to age, sex, islands visited, and number of days spent in Hawaii. In addition, the median number of symptoms reported by those with DLI who provided a blood specimen and those who did not was the same for both groups, and no significant differences were found between groups with regard to individually reported symptoms. In aggregate, these data suggest that the results from the subset who provided a specimen for testing may be representative of all who reported DLI.

Two additional pieces of information corroborate our assessment that the risk for dengue to travelers in this outbreak was low. First, we found that the proportion of travelers reporting DLI was the same for visitors to each of 3 islands, Maui, Oahu, and the Big Island. This finding is in stark contrast to the incidence of dengue infection among Hawaii residents during the outbreak, where the rates per 100,000 persons were dramatically different, 73, 7, and 0 for Maui, Oahu, and the Big Island, respectively. If DLI were a specific indicator of dengue infection, one might have expected the proportion of visitors reporting DLI by

![Figure 4. Island visited for 3,064 survey respondents, by weighted and unweighted frequencies. Big Island is the term used for the island of Hawaii to avoid confusion with the state of Hawaii. More than 1 island could be listed for each respondent, but most visitors went to only 1 island, that is, 3,384 island visits were reported by 3,064 respondents.](image-url)

![Figure 5. Source of information through which 1,949 visitors to Hawaii first learned of the dengue outbreak in 2001. HDOH, Hawaii Department of Health. *Other sources of information included family, friends, or co-workers who learned of the outbreak from the media or Internet; signs posted in and around Hana, Maui; and other tourists or Hawaii residents who informed the respondent.](image-url)
island to roughly parallel the risk profile observed for island residents. In other words, the lack of concordance between the trends in dengue incidence rates among residents of the various islands during the outbreak and the proportion of visitors with DLI suggests that the reported DLI illnesses, including those not evaluated serologically, were not dengue fever.

Second, during the outbreak investigation conducted before this survey, we made a concerted attempt to identify travelers with dengue with little success. These efforts included a request to all state epidemiologists to consider dengue infection among persons returning ill from Hawaii, communication through CDC’s Epi-X system, and extensive outreach through venues frequented by visitors (car rental agencies, hotels, tourist information centers). Only 7 (6%) of the 122 persons with laboratory-positive dengue infection were nonresidents. All 7 nonresidents with dengue stayed at rental homes in the epicenter of the outbreak (Hana, Maui), and 6 of the 7 had occupied the same house. Another 70 nonresidents who visited Hawaii during the outbreak were reported to HDOH by other states as having possible dengue infections; 30 agreed to be serologically tested—all results were negative.

While imperfect, the most effective way of alerting visitors about the outbreak appears to be through the media, including radio, television, and newspapers. Among visitors who learned of the outbreak before or during their visit, almost 90% stated that they first heard about it through these channels. Other sources of information such as travel agents, Web pages, hotel staff, car rental agencies, and information centers appear to have been less informed, but they can still play an important role in providing important supplemental information. Since many people on vacation purposefully avoid television and newspapers, new strategies should be explored for increasing the proportion of travelers who can be reached with important health protection messages if needed in the future.

We caution that the low risk for dengue infection among travelers to Hawaii may not be applicable to other settings where dengue is endemic, where the predominant mosquito vector is *Ae. aegypti*, or when outbreak-associated attack rates are much higher than that observed in Hawaii during 2001 to 2002. However, relatively few studies have attempted to quantify the risk for dengue infection among short-term travelers, and the data available from other cohorts encompass a wide range. One cohort study in Puerto Rico identified no recent dengue infections in 153 relief workers, and the investigators estimated the upper limit of risk to be 1.7 dengue infections per person-year exposure (13). A study of Swedish travelers estimated the risk for dengue fever to be highest among visitors to the Indian subcontinent and the Malay Peninsula (30–58 infections per 100,000 travelers) (15). However, another study among Israeli travelers to

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Reported (% frequency) by participants with DLI, n = 94</th>
<th>Reported (% frequency) by participants without DLI, n = 138</th>
<th>Chi square†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chills</td>
<td>80 (88)</td>
<td>7 (3)</td>
<td>114.0†</td>
</tr>
<tr>
<td>Headache</td>
<td>74 (79)</td>
<td>58 (47)</td>
<td>17.8†</td>
</tr>
<tr>
<td>Body pain</td>
<td>59 (62)</td>
<td>29 (18)</td>
<td>32.1†</td>
</tr>
<tr>
<td>Muscle aches</td>
<td>58 (64)</td>
<td>27 (19)</td>
<td>32.2†</td>
</tr>
<tr>
<td>Fever</td>
<td>58 (56)</td>
<td>7 (4)</td>
<td>51.2†</td>
</tr>
<tr>
<td>Extreme tiredness</td>
<td>51 (49)</td>
<td>34 (22)</td>
<td>13.4†</td>
</tr>
<tr>
<td>Nausea</td>
<td>44 (45)</td>
<td>31 (25)</td>
<td>6.9†</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>38 (37)</td>
<td>31 (25)</td>
<td>2.4</td>
</tr>
<tr>
<td>Joint pain</td>
<td>28 (31)</td>
<td>20 (14)</td>
<td>6.9†</td>
</tr>
<tr>
<td>Vomiting</td>
<td>23 (20)</td>
<td>18 (15)</td>
<td>0.7</td>
</tr>
<tr>
<td>Pain behind the eyes</td>
<td>22 (29)</td>
<td>16 (12)</td>
<td>6.6†</td>
</tr>
<tr>
<td>Itching</td>
<td>15 (14)</td>
<td>10 (8)</td>
<td>1.5</td>
</tr>
<tr>
<td>Rash</td>
<td>13 (13)</td>
<td>18 (13)</td>
<td>0.0</td>
</tr>
<tr>
<td>Shortness of breath</td>
<td>10 (15)</td>
<td>7 (7)</td>
<td>2.1</td>
</tr>
<tr>
<td>Dark stool</td>
<td>7 (7)</td>
<td>4 (3)</td>
<td>1.2</td>
</tr>
<tr>
<td>Bloody nose</td>
<td>4 (7)</td>
<td>3 (3)</td>
<td>1.1</td>
</tr>
<tr>
<td>Bleeding gums</td>
<td>4 (4)</td>
<td>0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*DLI, dengue-like illness, was defined as fever and/or chills, plus any of the following symptoms: headache, body aches, eye pain, muscle aches, joint pain, rash, bleeding gums, melena (dark stools), or nosebleed. Figures are exact; percentages and chi-square values were calculated by using survey weights.

†p<0.05.
Southeast Asia estimated the infection rate to be nearly 1,000-fold higher (3–5 per 1,000 travelers) (16). Moreover, a seroconversion rate of 6.7% was reported among 104 younger Israeli travelers on extended trips (3–16 months) to the tropics (8). The lack of homogeneity of these data suggests that factors such as location of travel, the intensity of dengue activity at the time, the length of stay, and the type of travel engaged in are likely to be important determinants of the risk for dengue among visitors in any particular setting.

While a precise estimate of the risk may not be obtainable, >500 laboratory-confirmed and 2,000 suspected dengue infections reported in returning US travelers from 1986 to 2000 indicate that the risk for infection among visitors to dengue-affected areas is not insignificant (17–22). In light of this, we recommend that US clinicians consider the possibility of dengue transmission when evaluating febrile rash illnesses among travelers to areas with a history of dengue activity, including the Pacific Islands.

This study has several limitations. First, as noted above, only approximately one fourth of all persons reporting DLI underwent serologic testing. The most frequent reasons for declining to have blood drawn were inconvenience, an aversion to needles, or sense that since the illness was in the past (on average, 7 months before) and had resolved, testing had little benefit. If we were to conduct this type of study in the future, we would make a concerted attempt to shorten the time between departure from Hawaii and follow-up contact.

A second limitation is that we sought to test only persons who had symptoms compatible with dengue fever; therefore, our efforts would not have identified infections that were very atypical in presentation or asymptomatic. However, we used a fairly inclusive definition of DLI, and there is no reason to believe the rate of dengue infection would have been appreciably higher among those who were asymptomatic as compared to those with compatible symptoms. Third, illness and exposure histories were self-reported and therefore potentially subject to recall bias.

In summary, our findings suggest that the risk for dengue for travelers to Hawaii during the 2001–2002 outbreak was limited. Given Hawaii’s interconnectedness to areas of the world with endemic and epidemic dengue, Hawaii will likely experience another outbreak in the future. Should that occur, the most effective means currently available for informing visitors is through the media; the message to tourists should be that the risk for dengue infection is likely to be low, but it still exists.

Acknowledgment

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References


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We describe the spread of a dengue virus during an outbreak in Saint Martin island (French West Indies) during winter 2003–2004. Dengue type 3 viruses were isolated from 6 patients exhibiting clinical symptoms. This serotype had not been detected on the island during the preceding 3 years. Genome sequence determinations and analyses showed a common origin with dengue type 3 viruses isolated in Martinique 2 years earlier.

Dengue virus (DENV) infection is recognized as a major public health problem; >50 million persons are infected each year worldwide (1), and the incidence of severe, sometimes lethal, forms of the disease is increasing (2). Dengue viruses are mosquito-borne flaviviruses with a single-stranded, nonsegmented, positive-sense RNA genome ≈11 kb in length. Four antigenically distinct serotypes, DENV types 1 to 4, exist (3). Infection with any serotype can lead to disease, ranging from mild infection, dengue fever (a generally mild disease with complete recovery), to severe forms (dengue hemorrhagic fever and dengue shock syndrome). Molecular epidemiologic studies have investigated the possibility of a link between particular DENV genotypes or clusters and particular clinical forms of disease (4,5). Consequently, finding new viral genotypes in areas where they had been absent could be of epidemiologic and clinical interest. A recent work described the emergence and the global spread of DENV-3 subtype III (5). Originating from the Sri Lankan/Indian subcontinent, the new variant likely spread to eastern Africa in the 1980s, then to Latin America in the mid-1990s (5). Previous work in our laboratory identified a DENV-3 subtype 3 of the same origin in Martinique in 1999 (6).

Whereas other Caribbean islands had annual dengue epidemics, the last outbreak reported in Saint Martin (French West Indies) (Figure 1) was in 1977 (7). Only DENV-1 was isolated during this epidemic, while...
DENV-1, DENV-2, and DENV-3 circulated in Puerto Rico at the same time (8). In 2000, during an interepidemic period, 1 isolate of DENV-3 was reported in Saint Martin (http://www.carec.org/annrep00/index.html), but to our knowledge its sequence was not determined. During the last 3 years, no further DENV isolates have been reported on the island. However, in December 2003, an outbreak of febrile illness was reported after heavy rains. An estimated 108 persons were infected, and DENV was suspected to be the causative agent by Saint Martin Lepers Laboratory (9). During this period, blood samples collected at early and late stages of infections were examined at the Tropical Medicine Institute of the French Armed Forces Medical Service (Institut de Médecine Tropicale du Service de Santé des Armées [IMTSSA]) tropical virology laboratory in Marseille to identify the etiologic agent. DENV-3 was isolated on C6/36 cells (Aedes albopictus). Partial genomic sequences were determined for each isolated virus to evaluate the origin and diversity of its spread. This approach should help clarify the geographic distribution of isolates in the Caribbean islands as well as the virus’ circulation in and transmission to humans.

Materials and Methods

The Outbreak

In December 2003, an outbreak of febrile illness was reported (9) after heavy rainfalls in Saint Martin (29,000 inhabitants in the French part and 36,000 in the Dutch part). The outbreak seemed to start first in the northeastern part of the island (“Baie orientale” and “Cul de sac” neighborhoods), then spread through the island (Figure 1). The temporal distribution of the outbreak is shown in Figure 2 (10). We used a commercial enzyme-linked immunosorbent assay (ELISA) (Eurobio, Courtaboeuf, France) in Saint Martin Lepers Laboratory to determine that, from October 2003 to April 2004, a total of 108 persons were infected; a DENV was suspected as the etiologic agent (10). Twelve persons manifesting thrombocytopenia without hemorrhagic signs were hospitalized (9).

Collection and Transport of Samples

Sera from 26 patients with dengue-like syndromes were collected at a local laboratory and transported (by air, at 4°C) to the IMTSSA laboratory in Marseille for serologic diagnosis and etiologic agent identification. All these samples were collected from December 2003 to January 2004, the peak of the outbreak.

Serologic Diagnosis

In-house immunoglobulin (Ig) M-capture enzyme immunoassays (MAC-ELISA) and IgG sandwich ELISA were used to detect serum IgM and IgG antibodies to Toscana virus, dengue viruses, West Nile virus, and St. Louis encephalitis virus. Briefly, IgM antibodies were captured with rabbit anti-human IgM antibodies (Interchim, Montluçon, France). Toscana, dengue, West Nile, and Saint Louis antigens, prepared on Vero cells and inactivated by betapropiolactone (Sigma-Aldrich, St Quentin Fallavier, France), were added. Specific binding was demonstrated by using a Toscana virus, dengue viruses, West Nile virus, and St. Louis encephalitis virus mouse hyperimmune ascitic fluid virus (11) and a goat anti-mouse peroxidase-labeled conjugate (Interchim). For IgG detection, antibodies to Toscana virus, dengue viruses, West Nile virus, and St. Louis encephalitis virus were captured by goat anti-mouse IgG antibodies; viral antigens were followed by test sera; and specific binding was demonstrated by using a peroxidase-labeled goat anti-human IgG conjugate. Serum samples were considered positive if the optical density at 450 nm was >3-fold the mean of negative sera with an OPTImax spectrophotometer (Molecular Devices, Saint Gregoire, France).

Virus Isolation and Propagation

Work with the infectious virus was carried out in a biosafety level 3 laboratory. Virus isolation was attempted when the first sample could be collected <5 days after onset of illness and when enough serum was collected for serologic analysis (12). DENV-3 strains (see Online Appendix, available from http://www.cdc.gov/ncidod/EID/vol11no05/04-0959_app.htm) were all isolated from leukocytes of patients. Leukocytes of the samples were directly coincubated with C6/36 cells (Ae. albopictus) grown at 28°C in Leibowitz’s L15 medium (BioWhittaker Europe, Verniers, Belgium) supplemented with 1% L-glutamine and 2% tryptose phosphate broth. Fetal bovine serum (5% final) was added 1 h later. Supernatants were collected on day 5 after infection.

Figure 2. Temporal distribution of the outbreak (adapted from [10]). Bars indicate the suspected dengue patients observed by sentinel physicians; dark squares (curve) indicate laboratory-confirmed cases.
Indirect immunofluorescence (IF) was performed on C6/36 cells with DENV-1, -2, -3, and -4 serotype–specific monoclonal antibodies (kindly provided by Nick Karabatsos, Centers for Disease Control and Prevention, Fort Collins, Colorado); on mouse hyperimmune ascitic fluids for West Nile virus and Bunyamwera virus; and on grouping fluids for alphaviruses, phleboviruses, and California serogroup viruses. After isolation attempts, remaining sera were used for serologic studies but not for DENV-3–specific reverse transcription–polymerase chain reaction (RT-PCR) because too little serum was available after serologic tests.

RNA Preparation and cDNA Synthesis

Viral RNA was extracted from 200-µL aliquots of infected cells supernatants by using the High Pure Viral RNA kit (Roche Diagnostics, Meylan, France) following manufacturer’s protocol. Two overlapping viral cDNA fragments were generated by RT with Superscript II reverse transcriptase (Invitrogen/Life Technologies, Cergy Pontoise, France) according to the manufacturer protocols with D3/C/863/18 and D3/C/1419/18 primers (Table). Specific primers (Table) designed from the nucleotide sequence of the reference DENV-3 H87 (GenBank accession no. L11423) (13) were used for PCR amplification by using AmpliTaq DNA Gold (Applera, Courtaboeuf, France).

DNA Sequencing

PCR products were purified from 1.5 % agarose gels, by using the QIAquick gel extraction kit (Qiagen, Courtaboeuf, France) and directly sequenced with the direct and reverse primers (Table) and the Big Dye Sequencing kit (Applera). Sequencing was carried out with an automatic sequence analyzer (ABI PRISM 3100, Applera) following the manufacturer’s protocol.

Sequences of the Saint Martin DENV isolates were compared to GenBank database DENV sequences (complete list in Online Appendix). Alignments of nucleotide and amino acid sequences of the prM/M and partial E nucleotide sequences (nucleotide numbers 437–1144 by reference to the sequence of the D3H87 strain) were performed with ClustalW1.7 software (14). Phylogenies were constructed with the MEGA 2 program (15), and tree drawing used the Jukes-Cantor algorithm for genetic distance determination and the Neighbor Joining method. The robustness of the resulting tree was tested by 1,000 bootstrap replications.

Results and Discussion

From October 2003 to April 2004, DENV-3 circulated on the island, and 108 cases of dengue fever were serologically or virologically confirmed in both the Lepers Laboratory and the IMTSSA laboratory, and 12 persons were hospitalized (10). If one takes into account the 180 suspected cases observed during this period, the incidence of dengue fever during the epidemic was \( \approx 0.62\% \) (180/29,000) on the French part of the island; this value was consistent with values already observed in the Caribbean region (http://www.carec.org/data/dengue/1998/table2.html). No data were available from the Dutch half.

During this period, an additional 26 blood samples from patients with dengue-like syndromes at early stage of the infection were received in IMTSSA Tropical Virology Laboratory in Marseille. Among them, 13 were positive for dengue infection by presence of IgM, elevation (4-fold) of specific IgG, or both. Virus was isolated from 6 other patients, and DENV-3 was identified by IF using serotype–specific monoclonal antibodies. No fluorescence was observed when DENV-1, DENV-2, or DENV-4 monoclonal antibodies were used. Moreover, IF tests were negative when antibody to West Nile virus, Toscana virus, Bunyamwera virus, alphaviruses, phleboviruses and California serogroup viruses was used. These cases were considered confirmed dengue fever; all the patients recovered fully.

DENV-3 was genetically identified in the 6 IF positive isolates (D3StMart1–D3StMart6, referred to as GenBank accession no. AY750713–AY750718) by using BLAST-N/CBI software. Comparison of partial sequences showed a high degree of identity between the strains isolated from patients on Saint Martin: paired identity at the nucleotide level ranged from 99.3% to 100%. When compared to 00PuertoR1 strain of the Latin American cluster defined by Messer (5), the values were 99.5%–99.6%. Nucleotide comparison gave the following values: 98.3%–98.4% for East African cluster (5), 98.8%–98.9% for Group B (5), and 96.8%–96.9% for Group A (5). This comparison indicated a close relationship between Saint Martin and Latin American isolates.

The strains from Saint Martin shared an 11-nucleotide insertion between position 10,275 and position 10,276 in the 3′UTR (AGTGAAAAAGA). The same insertion was found in isolates from Martinique 2 years earlier (6) and from DENV-3 Sri Lanka (6), indicating a probable com-
mon ancestor. This finding was consistent with a Sri Lankan origin for DENV-3 subtype III circulating in the Latin American and Caribbean regions (5) and with extension of the virus from a single importation event.

A phylogenetic tree was constructed based on partial sequences of the prM/M-E gene region (position 437–1144) of the genomes, including the strains from Saint Martin and several other previously characterized strains from different origins (16–19). DENV isolated from Saint Martin was grouped in subtype III, as defined by Lanciotti (17) (data not shown). Another phylogenetic tree was constructed with the strains analyzed by Messer (5) (Figure 3). Despite the high overall similarity of the subtype III sequences, distinct groups could be distinguished. The 6 isolates from Saint Martin clustered together. The small lineage difference of D3StMart2 is supported by a 95-bootstrap value, but no evidence suggests that such difference implies multiple introductions or a longer transmission period. However, Saint Martin DENV-3 fell in the Latin American cluster defined by Messer (5), which also includes isolates from Guatemala, Nicaragua, and Mexico. Isolates from Saint Martin were particularly close to Martinique isolates (6) and the 00PuertoR1 isolate, which were the geographically and temporally closest isolates of the cluster. The analysis of the deduced amino acid sequences generated a similar phylogram. Altogether, these results indicate that the Saint Martin viral isolate belongs to the genotype Puerto Rico 2000 previously reported (5). These results also confirm a common origin for all DENV-3 circulating in the Caribbean and Latin American region; their ancestor probably originated from Sri Lanka. Additional American DENV-3 strain sequences, encompassing the insertion site, should help to confirm this hypothesis.

The question arose whether the introduction from Sri Lanka was a single event in the Latin American region, first appearing in Panama (5), then spreading through the Caribbean subregion and South America. In fact, viruses belonging to subtype III were first reported in Nicaragua and Panama in 1994 (20). They were then identified in Guatemala from 1996 to 1998; in Puerto Rico, Barbados, and Jamaica in 1998 (18); and in Martinique in 1999 (6). The introduction of DENV-3 in Saint Martin was reported in 2000 (http://www.carec.org/annrep00/index.html), but the subtype was not determined. No virus isolation was reported for 3 years, until the viruses we characterized were isolated. The rapid extension of DENV-3 belonging to subtype III, which have almost completely replaced preexisting DENV of serotype 1 and 2, is indicative that these particular viruses have adapted to Caribbean and Latin American conditions. Together with previous studies (5,6), identification of viruses belonging to subtype III in another area of the Caribbean region confirms the remarkable epidemiologic success of this particular lineage of dengue viruses.

Comparable substitution of local DENV by a new genotype was previously described in the Caribbean region (12). New viruses, often originating from Southeast Asia, arise as successive waves, replacing previous ones. In this regard, sampling of DENV-3 subtype III virus nucleotide sequences from all countries in the Caribbean and the peri-Caribbean area need to be expanded so that we can understand DENV circulation in the region.

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reflecting the views of the French Armed Forces Medical Service or the French Army at large.

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Dengue Antibody Prevalence in German Travelers

Ole Wichmann,* Annekathrin Lauschke,* Christina Frank,† Pei-Yun Shu,‡ Matthias Niedrig,† Jyh-Hsiung Huang,‡ Klaus Stark,† and Tomas Jelinek*

We studied 2,259 German citizens after they returned from dengue-endemic countries from 1996 to 2004. Serotype-specific dengue antibodies indicated acute infections in 51 (4.7%) travelers with recent fever and 13 (1.1%) travelers with no recent fever, depending largely on destination and epidemic activity in the countries visited.

The number of dengue cases reported worldwide and the number of countries with endemic dengue activity has increased dramatically in recent decades (1). The disease is endemic in most tropical parts of the world, many of which are popular tourist destinations. Because of this popularity, and probably also because of heightened awareness, diagnoses of dengue in international travelers have increased as well (2–4).

Four dengue virus serotypes (DENV-1 to -4) are known to cause a wide range of clinical features (5); these viruses are transmitted mainly by Aedes aegypti mosquitoes. As in indigenous populations, a large proportion of asymptomatic infections are found in travelers (6,7). Recovery from an infection provides lifelong immunity against that serotype but confers only transient protection against heterogeneous infections (1), and sequential (secondary) infections may increase the risk of developing the more severe manifestation, dengue hemorrhagic fever (DHF) (8). DHF seems to occur rarely in European travelers, but several cases have been reported (9).

Previous studies demonstrated that the risk of acquiring dengue is highest when traveling in Southeast Asia (3,4,6). In that region, the disease is most prevalent during and after the rainy season, when vector breeding is maximal (10).

Only in a few industrialized countries is imported dengue fever a notifiable disease. In addition, substantial underdiagnosis persists because the disease is not well known to general practitioners in Western countries, and adequate diagnostic tests are available only in specialized clinics. Thus, little is known about dengue incidence in travelers over time. As dengue transmission may vary not only by season, but also from year to year, data derived from short-term observations may overestimate or underestimate the risk for travelers.

Determining the incidence of dengue in travelers is hampered by the fact that dengue immunoglobulin (Ig) G antibodies are broadly cross-reactive with other flaviviruses and vaccines against them (e.g., yellow fever and Japanese encephalitis). Previous studies to determine antibodies to dengue viruses by using commercially available enzyme-linked immunosorbent assay (ELISA) kits carry a risk for overestimation because of this cross-reactivity. Recently developed tests like the envelope/membrane (E/M) and nonstructural protein NS1 serotype-specific IgM ELISAs and NS1 serotype-specific IgG ELISA therefore offer new opportunities for a more reliable diagnosis and for determining the infecting serotype (11,12).

The Study

To study the influence of increased worldwide dengue activity on international travelers, 2,259 patients were studied retrospectively for dengue antibodies after returning from dengue-endemic countries. A 36-month period from January 1996 to December 1998 was compared with a 27-month period from January 2002 to March 2004. We recruited travelers who came to the travel clinic of the Berlin Institute of Tropical Medicine, Germany, with fever (n = 1,091) or diarrhea without fever (n = 1,168) and for whom serum samples were available. Thus, 2,259 patients’ serum samples were tested for anti-dengue IgM and IgG by using an IgM-capture ELISA and an IgG indirect ELISA (PanBio Pty Ltd., East Brisbane, Queensland, Australia). A probable acute infection was defined according to manufacturer’s instruction as having a sample:calibrator absorbance ratio of IgM ≥1.0. Acute probable primary infection was characterized by the elevation of IgM ≥1.0 with IgG ≤4.0, and acute secondary infection was characterized by the elevation of IgG ≥4.0 (13). For a more specific diagnosis, all serum samples from patients with probable dengue infections were then investigated by using E/M and nonstructural protein NS1 serotype-specific IgM ELISAs and NS1 serotype-specific IgG ELISA as described previously (11,12). Furthermore, an additional confirmatory testing was performed by using immunofluorescence assays (Euroimmun AG, Luebeck, Germany), and if these results were contrary, sera collected during the acute phase of illness were processed by using polymerase chain reaction assays to detect viral nucleic acid.

Among the recruited patients, 1,163 (51.5%) were male. The median age was 33 years (range 2–79 years). Antibodies were detected by the screening test in 127 (5.6%) serum samples, indicating probable acute dengue infection. The more specific analysis confirmed infection...
in 64 cases (2.8% prevalence), including 8 (12.5%) patients with secondary immune response. One of these 8 secondary and none of the 56 primary infections led clinically to DHF. Among 1,091 patients with fever and 1,168 diarrhea patients without fever, 51 (4.7%) and 13 (1.1%), respectively, had an acute dengue infection.

The highest prevalence of dengue antibodies (4.6%), indicating acute infection, was found in patients returning from Asia (n = 1,020) (Table 1), including Southeast Asia (7.4% of 500 total travelers and 11% of 310 febrile travelers) and the Indian subcontinent (1.8%). Traveling in Southeast Asia was associated with a significantly higher risk compared to other disease-endemic areas in Africa and Latin America (odds ratio 5.3, 95% confidence interval 3.2–9.0). Comparing patients with and without acute dengue infection, no significant difference was seen in the median length of travel (28 vs. 24 days, respectively, p = 0.083, Mann-Whitney test) or the median age of the patients (32 vs. 33 years, respectively, p = 0.58, Mann-Whitney test). Patients 30–44 years of age had the highest antibody prevalence (37 [3.8%] of 966).

When patients from 1996 to 1998 (n = 1,073) were compared with those from 2002 to 2004 (n = 1,186), a slight increase was seen in the overall prevalence, from 2.7% to 3.0%, although this finding was not significant (p = 0.63). The Figure shows annual dengue prevalence among travelers to Thailand and to the Indian subcontinent, highlighting that infection rates fluctuate strongly between years and between quarters within years. In the last quarter of 1997 and 1998, 64 travelers returned from Thailand, and 14 (22%) acquired an acute dengue infection. Among those, 5 were infected by the serotype DENV-1, 3 by DENV-2, and 4 by DENV-3 (Table 2). In 2 cases the serotype was undetermined.

Conclusions

In this study population, 4.7% of all febrile patients returning from different areas of the tropics had dengue antibodies that indicated acute infection. This number underlines the effect on international travelers to dengue-endemic areas. As long as no dengue vaccine is commercially available, the single-most effective preventive measure is avoiding mosquito bites. This advice should be included in every medical pretravel consultation. Among patients without fever, 13 (1.1%) had detectable dengue antibodies compatible with acute dengue infection, which underscores that symptoms commonly associated with dengue, such as fever, myalgia, arthralgia, and exanthema, are helpful for diagnosis when present, but the absence of typical symptoms does not exclude infection.

<table>
<thead>
<tr>
<th>Years</th>
<th>Southeast Asia and Indian subcontinent</th>
<th>Sub-Saharan Africa</th>
<th>Latin America and the Caribbean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996–1998</td>
<td>22/491 (4.5)</td>
<td>2/309 (0.6)</td>
<td>4/271 (1.5)</td>
</tr>
<tr>
<td>2002–2004</td>
<td>25/529 (4.7)</td>
<td>3/376 (0.8)</td>
<td>8/279 (2.9)</td>
</tr>
<tr>
<td>Total</td>
<td>47/1,020 (4.6)</td>
<td>5/685 (0.7)</td>
<td>12/550 (2.2)</td>
</tr>
</tbody>
</table>

*Four patients are not included in this analysis since they traveled to >1 continent.
A study on 483 cases of imported dengue infections in Europe showed that DHF developed in 2.7% of the patients; immigrants from dengue-endemic countries returning to Europe after visiting their home country were at higher risk for more severe disease than Europeans (9). The immunologic status of those patients was unknown. However, immigrants from dengue-endemic countries have a higher prevalence of dengue antibodies from previous infections. In our population, all 54 patients with primary dengue infection had classical dengue fever, and 1 of 8 patients with secondary dengue infection had DHF. The patient with DHF was born in Sri Lanka and immigrated to Germany 2 decades ago. These observations might be taken as more evidence for the importance of letting patients know that they have been infected with dengue and should, therefore, protect themselves from infection with subsequent serotypes.

Fluctuations in prevalence between years, especially the maximal prevalence in 1998, correspond to similar observations on record. In a study conducted among Israeli travelers to Southeast Asia from 1994 to 1998, a sharp increase in incidence was noted in 1998 compared with previous years (14). Similar to the worldwide increase of cases reported to the World Health Organization, the number of Swedish travelers returning from Southeast Asia with dengue fever was considerably higher in 1998 than during previous years (3). Decreasing resources for vector-borne infectious disease prevention and control (15) might have contributed to this epidemic in Southeast Asia, which followed the economic crisis in 1997.

The risk among a cohort of Dutch short-term travelers to dengue-endemic areas in Asia from 1991 to 1992 showed marked seasonal variation for the Indian subcontinent (7). In our population, such seasonal variations were not detectable. The infection rates were more influenced by major outbreaks, such as the one in India in 2003 or in Southeast Asia in 1997 to 1998. Similar findings have been described for Israeli travelers during their trip to Thailand in 1998 (14) and in German travelers to Brazil and Thailand in 2001 and 2002, respectively (4).

To screen our samples, ELISA-based tests for IgM and IgG were combined. In a study performed with paired serum samples, combining IgG and IgM ELISAs had a sensitivity of 100% in primary infections and 99% in secondary infections. The specificity was 100% in non-flavivirus infections and 80% in Japanese encephalitis virus infections when an IgG sample:calibrator absorbance ratio of 3.0 was used as a cutoff (13). However, because our study was retrospective, only single serum samples were available from each traveler. To increase specificity, all serum samples that indicated probable infections were further investigated with more specific ELISA techniques. By using virus-infected culture supernatants as the source of viral antigens, the E/M-specific capture IgM has been found to differentiate reliably between Japanese encephalitis, dengue, West Nile virus, and yellow fever (12). Furthermore, an NS1 isotype- and serotype-specific ELISA can reliably differentiate Japanese encephalitis virus infection, Japanese encephalitis virus vaccination, and primary and secondary dengue virus infection (11). In primary infection, IgM is detectable 3–8 days from the onset of symptoms (8); thus, some of our travelers might have had false-negative test results if samples were taken during the acute phase of illness. Therefore, the true infection rates in our study might have been higher than the numbers indicated by single-sample serology.

Overall, we demonstrated an almost stable rate of dengue infections among Berlin Institute of Tropical Medicine patients returning from all tropical regions when recent years are compared with the mid-1990s. Large outbreaks like those in 1997–1998 in Southeast Asia (especially Thailand) and 2003 in major cities of India, all popular tourist destinations, contributed to the numbers. Quarterly and annual fluctuations might lead to misinterpretation of probable trends if data are derived only from short-term observations. In addition, this variability underscores the importance of tourists’ seeking information before traveling to dengue-endemic areas.

**Acknowledgments**

We thank Ingo Möhl and Shengül Erdal for their support in performing the laboratory investigations and data collections.

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**Table 2. Frequencies of dengue serotypes that caused infections in German travelers according to year of travel and country of origin**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-1</td>
<td>D-2</td>
</tr>
<tr>
<td>Thailand</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Indonesia</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Indian subcontinent</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Rest of Asia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>The Americas and the Caribbean</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Africa</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Seychelles.
† Angola and Kenya.
Dr. Wichmann works at the Berlin Institute of Tropical Medicine, Germany, as a physician in the outpatient department and in the research group on imported infectious diseases. His research interests include the epidemiology, pathogenesis, and treatment of vectorborne diseases.

References


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Leptospirosis during Dengue Outbreak, Bangladesh

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We collected acute-phase serum samples from febrile patients at 2 major hospitals in Dhaka, Bangladesh, during an outbreak of dengue fever in 2001. A total of 18% of dengue-negative patients tested positive for leptospirosis. The case-fatality rate among leptospirosis patients (5%) was higher than among dengue fever patients (1.2%).

Leptospirosis is a zoonotic infection caused by spirochetes of the genus *Leptospira*. Infection usually results when water or soil contaminated with the urine of an infected animal comes in contact with human skin or mucous membranes (1). Clinical manifestations of leptospirosis can range from a self-limited febrile syndrome to a fatal illness (Weil disease) characterized by hemorrhage, renal failure, and jaundice. In tropical settings, leptospirosis can be indistinguishable from other febrile illnesses such as scrub typhus, malaria, or dengue.

Although leptospirosis has been reported in neighboring areas of Southeast Asia (2,3), the disease is not recognized in Bangladesh, where diagnostic tests for leptospirosis are not available. However, environmental factors, such as floods, humidity, and water contamination, are amenable to spread of the disease in Bangladesh.

An epidemic of dengue fever and dengue hemorrhagic fever began in Bangladesh in 2000 (4), and a surveillance system was established to identify patients with dengue-like illness at 2 major hospitals in Dhaka. Approximately three-quarters of patients meeting surveillance criteria had laboratory evidence of dengue infection. We hypothesized that leptospirosis might be a cause of illness among febrile patients who did not have dengue fever. To assess this, we retrospectively analyzed acute-phase serum samples from all dengue-negative patients by using real-time polymerase chain reaction (PCR) for *Leptospira*. We used data collected as part of the surveillance program to identify distinguishing clinical characteristics of leptospirosis.

The Study

In 2000, the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) worked with staff from Dhaka Medical College and Holy Family Red Crescent Hospital to initiate surveillance for dengue as part of an emergency response to an epidemic of dengue and dengue hemorrhagic fever. Physicians at Dhaka Medical College and Holy Family Red Crescent Hospital were trained in the clinical diagnosis and management of dengue and dengue hemorrhagic fever according to World Health Organization guidelines. Patients hospitalized with fever and in whom a physician suspected dengue were enrolled in the surveillance program. Clinical and epidemiologic information, as well as acute-phase serum specimens, were systematically collected from surveillance patients. Acute-phase serum specimens were assessed for dengue virus antibodies by using a commercial immunoglobulin (Ig)G and IgM capture enzyme-linked immunosorbent assay (ELISA) (PanBio Dengue Duo, PanBio Ltd., Brisbane, Queensland, Australia) (5). In addition, serum samples collected from a subset of patients during the first 5 days of illness were evaluated for dengue virus RNA by using reverse transcriptase–polymerase chain reaction (RT-PCR), as described previously (6). Serum samples from patients with no dengue infection shown by antibody or RT-PCR testing were retrospectively assessed for leptospirosis by using a real-time PCR that amplifies the LipL32 gene (7), a virulence factor that is conserved among pathogenic *Leptospira* strains (8). Microplate *Leptospira* IgM ELISA testing (PanBio Ltd) was conducted on all *Leptospira* PCR-positive serum specimens of sufficient quantity (9).

Specimens from 1,297 patients hospitalized at Dhaka Medical College and Holy Family Red Crescent Hospital between January 1 and December 31, 2001, were evaluated for dengue infection by using capture ELISA; 55 acute-phase serum samples were additionally evaluated by using RT-PCR for dengue. A total of 938 (72%) patients were diagnosed with dengue fever by serology (932 patients), RT-PCR (3 patients), or both (3 patients). Acute-phase serum specimens from the 359 patients without laboratory evidence of dengue were evaluated for leptospirosis; 63 (18%) had *Leptospira* detected by using PCR. Sixty-one of the PCR-positive samples were tested for *Leptospira*-specific IgM; 18 (30%) showed positive results and 5 (8%) showed equivocal results.

Patients with leptospirosis diagnosed by using PCR were 6 to 70 years of age (mean 28, SD 13); 74% were
male. Patients with leptospirosis were of similar age and sex as patients with dengue (Table 1). Patients with leptospirosis had less education and came from households with lower income than patients with dengue. The peak occurrence of leptospirosis was in October and November, shortly after the monsoon season in Bangladesh. This overlapped with the period of highest dengue activity (July through December) (Figure).

Patients with leptospirosis reported a slightly longer duration of fever than patients with dengue (Table 2). While most patients with dengue or leptospirosis had continuous fever, an intermittent fever was more likely with leptospirosis. Reports of rash were more common with dengue fever. Aside from fever and rash, the symptoms of patients with leptospirosis and dengue were similar (Table 2): headache, myalgia, nausea, and vomiting were most common.

The median temperature and heart rate at physical examination were higher in leptospirosis patients than in dengue patients. Evidence of bleeding, including petechial rash, positive tourniquet test result, and gum bleeding, were more common in patients with leptospirosis. Subconjunctival hemorrhage, which may have been confused with conjunctival inflammation, was more commonly reported in patients with leptospirosis. Hepatomegaly and jaundice were more common in leptospirosis patients, but this difference was not statistically significant.

On laboratory examination, total leukocyte counts were similar in patients with dengue and patients with leptospirosis; however, lymphocytes were more likely to be predominant in patients with dengue. Hemoconcentration and thrombocytopenia were associated with dengue fever.

Of the patients whose outcome was known, 3 (5%) patients with leptospirosis died, compared with 11 (1.2%) patients with dengue (p = 0.048). Antimicrobial therapy for leptospirosis was not provided, and data on the cause of death were not available. The patients who died of leptospirosis were younger than those who died of dengue (24 ± 6 years of age vs. 36 ± 9 years, p = 0.05).

Conclusions

This is the first description of disease caused by *Leptospira* in urban Bangladesh. Our findings indicate that leptospirosis causes serious febrile illness in the densely populated city of Dhaka.

Studies conducted in other dengue-endemic areas have shown that leptospirosis can be confused with dengue fever (10–12). Most clinical symptoms of leptospirosis patients in Dhaka were nonspecific and not distinguishable from symptoms associated with dengue fever or other viral illnesses. Although fever in leptospirosis patients was higher and of longer duration than in dengue patients, there is sufficient overlap of clinical findings to suggest that clinicians caring for patients in Bangladesh should maintain a high index of suspicion for both diseases, especially during the peak incidence seasons that follow the monsoons.

![Figure. Proportion of dengue and leptospirosis patients at 2 major hospitals in Dhaka, Bangladesh, by month, 2001.](image)

Table 1. Demographic characteristics of dengue patients compared with those of leptospirosis patients, Dhaka, Bangladesh*

<table>
<thead>
<tr>
<th>Demographic characteristic</th>
<th>Dengue patients (n = 936)</th>
<th>Leptospirosis patients (n = 63)</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>27.5 ± 11.1</td>
<td>27.9 ± 13.3</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>694 (74)</td>
<td>46 (73)</td>
<td>NS</td>
</tr>
<tr>
<td>Monthly household income (US$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>174 (19)</td>
<td>20 (32)</td>
<td>0.015</td>
</tr>
<tr>
<td>60–119</td>
<td>205 (22)</td>
<td>17 (27)</td>
<td></td>
</tr>
<tr>
<td>120–200</td>
<td>127 (14)</td>
<td>7 (11)</td>
<td></td>
</tr>
<tr>
<td>&gt;200</td>
<td>432 (46)</td>
<td>18 (29)</td>
<td></td>
</tr>
<tr>
<td>Household size</td>
<td>5.4 ± 2.7</td>
<td>4.8 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>Level of education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illiterate</td>
<td>83 (9)</td>
<td>16 (25)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Primary</td>
<td>144 (15)</td>
<td>13 (21)</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>424 (45)</td>
<td>22 (35)</td>
<td></td>
</tr>
<tr>
<td>University</td>
<td>214 (23)</td>
<td>8 (13)</td>
<td></td>
</tr>
<tr>
<td>Other or unknown</td>
<td>73 (6)</td>
<td>4 (6)</td>
<td></td>
</tr>
</tbody>
</table>

*Data are mean ± SD or no. (%).
†By Pearson chi-square test or analysis of variance. NS, not significant.
Recognition of leptospirosis is especially important since antimicrobial agents can reduce its severity and duration (13).

Leptospirosis patients identified in Dhaka were impoverished and poorly educated. This may reflect more frequent exposure to environments contaminated with urine from rodents or other animals. In contrast, dengue patients came from households with higher incomes and levels of education. Whether these socioeconomic differences reflect differing patterns of disease can only be determined by future population-based studies, which may in turn shed light on optimal prevention strategies.

Most of the patients hospitalized with leptospirosis and dengue virus infection in Dhaka during the period of this study were male. A recent seroprevalence study in Bangladesh did not demonstrate a sex difference in dengue seropositivity (14), but leptospirosis has been reported predominantly in men in other regions (15). Further prospective research would be useful to better define the clinical spectrum and gender distribution of disease in Bangladesh.

This study has a number of limitations. Only acute-phase serum samples were obtained as part of the hospital-based dengue surveillance program in Bangladesh. Serologic diagnosis of leptospirosis with a single specimen obtained early in infection is limited; hence, we used Leptospira-specific PCR for diagnosis in our study population. Although this molecular technique is highly sensitive and specific for the presence of leptospiremia (7), more cases may have been detected through the use of microscopic agglutination testing on paired serum samples. Notably, less than one-third of the patients with a diagnosis of leptospirosis by PCR had detectable levels of Leptospira-specific IgM. This is likely due to the sampling of acutely ill patients before seroconversion. In support of this, leptospirosis patients who had detectable levels of IgM exhibited a trend toward longer duration of fever compared with those who did not have detectable levels (6.5 vs. 5.5 days, p = 0.12).

Some cases of leptospirosis resulted in death, and the case-fatality rate among leptospirosis patients was significantly higher than among dengue fever patients. The differ-

### Table 2. Clinical characteristics of dengue patients compared with those of leptospirosis patients, Dhaka, Bangladesh*

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Dengue patients (n = 938)</th>
<th>Leptospirosis patients (n = 63)</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symptom</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of fever (days)</td>
<td>5.2 (1–40)</td>
<td>5.8 (1–12)</td>
<td>0.04</td>
</tr>
<tr>
<td>Characteristic of fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td>894 (95)</td>
<td>53 (85)</td>
<td>0.001</td>
</tr>
<tr>
<td>Intermittent</td>
<td>44 (5)</td>
<td>9 (15)</td>
<td></td>
</tr>
<tr>
<td>Rash</td>
<td>562 (60)</td>
<td>24 (39)</td>
<td>0.001</td>
</tr>
<tr>
<td>Headache</td>
<td>846 (90)</td>
<td>51 (82)</td>
<td>0.05</td>
</tr>
<tr>
<td>Myalgia</td>
<td>836 (89)</td>
<td>53 (85)</td>
<td>NS</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>444 (47)</td>
<td>24 (39)</td>
<td>NS</td>
</tr>
<tr>
<td>Pruritus</td>
<td>202 (22)</td>
<td>6 (11)</td>
<td>0.05</td>
</tr>
<tr>
<td>Rhinitis</td>
<td>10 (1)</td>
<td>3 (5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Nausea</td>
<td>899 (96)</td>
<td>60 (97)</td>
<td>NS</td>
</tr>
<tr>
<td>Vomiting</td>
<td>780 (83)</td>
<td>52 (84)</td>
<td>NS</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>337 (38)</td>
<td>23 (37)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Physical finding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°F)</td>
<td>96.6 (94–106)</td>
<td>101.4 (99–108)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart rate</td>
<td>82 (48–160)</td>
<td>90 (60–160)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>79 (8)</td>
<td>7 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>Jaundice</td>
<td>17 (2)</td>
<td>3 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>Petechial rash</td>
<td>348 (38)</td>
<td>12 (20)</td>
<td>0.005</td>
</tr>
<tr>
<td>Positive tourniquet test result (&gt;20 petechiae/inch²)</td>
<td>699 (75)</td>
<td>20 (33)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gum bleeding</td>
<td>331 (35)</td>
<td>12 (20)</td>
<td>0.01</td>
</tr>
<tr>
<td>Subconjunctival hemorrhage</td>
<td>299 (32)</td>
<td>31 (51)</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Laboratory finding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte count</td>
<td>6.9 ± 5.3</td>
<td>7.0 ± 5.7</td>
<td>NS</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>47 ± 14</td>
<td>31 ± 15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelet count (× 10³/μL)</td>
<td>85 ± 74</td>
<td>128 ± 83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>41 ± 5.8</td>
<td>37 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Outcome of hospitalization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovered or left against medical advice</td>
<td>918 (98.8)</td>
<td>57 (95)</td>
<td>0.048</td>
</tr>
<tr>
<td>Death</td>
<td>11 (1.2)</td>
<td>3 (5)</td>
<td></td>
</tr>
</tbody>
</table>

*Data are median (range), no. (%), or mean ± SD.
†By Pearson chi-square test for categorical data and Mann-Whitney U test or analysis of variance for continuous data. NS, not significant.
different case-fatality rates may be related, however, to the intensive training in dengue case management that occurred during this epidemic period, or to the lack of specific antimicrobial therapy for unrecognized cases of leptospirosis. Our findings underscore the need for greater awareness of leptospirosis in the Indian subcontinent, more data on its incidence in Bangladesh, and optimal treatment regimens for leptospirosis that can be applied in resource-poor settings.

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Retinal Hemorrhages in 4 Patients with Dengue Fever

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We report 4 patients with retinal hemorrhages that developed during hospitalization for dengue fever. Onset of symptoms coincided with resolution of fever and the nadir of thrombocytopenia. Retinal hemorrhages may reflect the rising incidence of dengue in Singapore or may be caused by changes in the predominant serotype of the dengue virus.

Dengue is usually a self-limiting viral fever spread by the Aedes aegypti mosquito. Minor bleeding frequently complicates dengue fever. Hemorrhagic complications are usually mild and limited to gum bleeding, epistaxis, hematuria, or menorrhagia (1). However, cases of severe and life-threatening bleeding have been reported in the medical literature. Rarely, retinal hemorrhages affecting patients with dengue fever are reported. We report 4 patients with dengue fever complicated by retinal hemorrhages who were hospitalized in our institution in June and July 2004.

The Study

In June and July 2004, a total of 1,620 cases of dengue fever and dengue hemorrhagic fever were reported in Singapore; 621 case-patients were admitted to our hospital. In the same period, retinal hemorrhages were diagnosed in 4 dengue fever patients in our hospital.

As part of our protocol, we use polymerase chain reaction (PCR) (while a patient is still febrile) and serology (after resolution of fever) to confirm clinical diagnoses of dengue fever. Real time-PCR is performed daily with commercial reagents (Artus GmbH, Hamburg, Germany) on a Light Cycler (Roche Diagnostics, Penzberg, Germany). Serology is performed with the Dengue Duo IgM and IgG Rapid Strip Test (PanBio, Brisbane, Queensland, Australia). Dengue serotypes were determined by immunofluorescence, with type-specific monoclonal antibodies, on virus cultured from serum taken early in the disease. The diagnosis of retinal hemorrhage was made by an ophthalmologist after dilated fundoscopic examination. Fundal photography was also performed for all patients for baseline recording.

Four patients (3 women and 1 man) had visual symptoms, which developed during hospitalization for dengue fever. The mean age of the patients was 34 years (range 21–49). Four patients had reduced visual acuity. Two patients noticed the visual problems in the morning upon waking up. One patient complained of metamorphopsia. All denied any associated eye pain, photophobia, or increased tearing. Except mild myopia in 1 patient, no patient had a history of medical or ocular problems. The average duration of fever before visual symptoms developed was 6.25 days (range 5–8). The onset of visual symptoms was usually observed within 1 day from the resolution of fever and the nadir of the thrombocytopenia. The average platelet count on the day of onset of symptoms was 36 × 10⁹/L (range 33–44). The lowest platelet count was recorded 1 day before the onset of symptoms in 1 patient, on the day of onset of symptoms in 2 patients, and 1 day after the onset of symptoms in 1 patient. The average platelet count at the nadir of thrombocytopenia was 33 × 10⁹/L (range 26–40).

Fundoscopic examination showed bilateral blot hemorrhages within the vascular arcades in all 4 patients. All patients received standard supportive care, and 2 patients received platelet transfusion.

The clinical diagnosis of dengue fever was confirmed by PCR in 2 cases, by immunoglobulin (Ig)G and IgM in 1 patient, and by IgM in the remaining patient. Dengue virus was isolated from all 4 patients. All were serotype 1. In 3 patients, visual symptoms resolved completely within 2 days, with full recovery of the patients’ visual acuities. Improvement in visual symptoms was delayed and incomplete in patient 4, and she had reduced visual acuity and metamorphopsia even after 2 months.

Conclusions

Singapore has experienced a steady rise in the incidence of dengue in recent years. In 2002, a total of 3,937 cases of dengue were reported, followed by 4,785 cases in 2003. As of September 11, 2004, a total of 4,985 cases were reported (2).

Hemorrhagic diathesis is a common complication of dengue fever. Such complications typically occur toward the end of the febrile period, when the symptoms of classic dengue fever resolve. Clinically, bleeding is usually mild and manifests itself as epistaxis, gum bleeding, hematuria, and menorrhagia. Only occasional cases of severe gastrointestinal or retroperitoneal bleeding are reported. However, retinal hemorrhages are exceedingly rare as a complication of dengue fever.

We performed a Medline search using dengue and retinal hemorrhage as key words. It showed only 4 case reports describing retinal hemorrhages associated with
dengue fever (3–6). Only 1 case was reported in the English medical literature. Haritoglou et al. (3) described a patient with dengue fever who had bilateral loss of vision. Fundoscopic examination indicated exudative maculopathy and small hemorrhages located in the nerve fiber layer. Initially, this patient had a visual acuity of 20/250 in both eyes. It increased to 20/100 in the right eye and 20/32 in the left eye 8 weeks after the patient was initially seen. The residual deficit was assumed to be due to intraretinal lipid deposits. Wen et al. (4) described 24 patients with visual disturbances complicating dengue fever. The mean age of these patients was 32 (range 16–45); 19 of them had blurring of vision, while others complained of central scotoma, floaters, photophobia, or halo vision. Five patients reported unilateral symptoms. Visual symptoms developed in most patients 5–8 days (range 2–15) after the onset of fever. Fundoscopic examination indicated retinal hemorrhages in 15 patients. Seven of the remaining 9 patients were seen a long time after the onset of symptoms, and fundoscopic examination showed only mild, atypical maculopathy. Optic nerve atrophy developed in 1 patient, and another patient was diagnosed with optic neuritis.

Our observations are similar to those described in the above reports. Our patients were also young, and visual symptoms developed 5–8 days after the onset of fever, consistent with current theories on the immunopathogenesis of dengue (7). All our patients complained of decreased visual acuity, and fundoscopic examination showed macular hemorrhages and exudative maculopathy. Careful review of clinical records allowed us to make additional observations, which have not been previously reported. In all our patients, visual symptoms developed shortly (within 1 day) before or after resolution of fever. The onset of symptoms was also associated with the nadir of thrombocytopenia.

All patients were evaluated by an ophthalmologist. They were treated conservatively, and 2 received platelet transfusion. Although 3 patients improved symptomatically within 2 days, recovery was slower and incomplete in the fourth patient. Although we see a considerable number of patients with dengue in our hospital, these patients are the first we have seen with dengue fever complicated by retinal hemorrhages. We postulate 2 explanations for this new clinical manifestation. First, the recent, sharp rise in the incidence of dengue fever may have allowed us to observe this otherwise rare manifestation. Or, their infection could have been caused by a particularly virulent type of dengue virus.

We cultured virus from acute-phase serum samples from all 4 patients; all were serotype 1. In 2003, 72% of 68 viruses isolated from sequential sera were serotype 2, and 21% were serotype 1. This proportion has changed dramatically over the last year. In 2004, 70% of 37 sequential isolates belonged to serotype 1 and only 22% to serotype 2 (Barkham T., unpub. data). Although it is tempting to presume that the recent increase in incidence of retinal hemorrhages is associated with the unique virulence of serotype 1 virus, the fact that the dengue virus in all 4 patients belonged to serotype 1 may have been pure chance and merely reflect the fact that serotype 1 became predominant in 2004. We are planning to clarify this issue by conducting a prospective study and would be pleased to hear from other centers with similar experiences.

Only a small minority of patients admitted with dengue fever reported ocular symptoms. All of them had retinal hemorrhages involving the macula. Hemorrhage involving peripheral parts of the retina is probably much more common. However, such patients may remain asymptomatic or have only minimal symptoms. The true incidence of retinal hemorrhage in dengue fever may be substantially higher. Further studies will be necessary to determine the true incidence of retinal hemorrhage in patients with dengue fever.

Retinal hemorrhage is a rare but potentially serious complication of dengue fever. The onset of symptoms appears to coincide with the resolution of fever and the nadir of thrombocytopenia. Patients with dengue fever who report visual symptoms should be evaluated promptly. Although there is no specific therapy, retinal hemorrhage may be an indication for early and aggressive correction of thrombocytopenia.

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Dengue Virus Type 3, Cuba, 2000–2002

To the Editor: In 1994, dengue virus type 3 (DENV-3) was reintroduced into Latin America after an absence of 17 years, and was isolated almost simultaneously in Nicaragua and Panama. In September 2000, DENV-3 was isolated for the first time in Cuba, producing a small outbreak that affected 3 areas of Havana City. In total, 138 cases of dengue fever were confirmed, with either DENV-3 or DENV-4 as the etiologic agents. No dengue hemorrhagic fever cases were observed, and the outbreak was brought under control within 6 weeks (1). At the end of June 2001, dengue was again reported in Havana. Through the end of February 2002, a total of 14,443 dengue cases were reported for the entire country, including 81 cases of dengue hemorrhagic fever and 3 fatalities (2).

To understand the molecular epidemiology of DENV-3 in Cuba, and particularly to determine whether the 2000 and 2001 outbreaks were caused by the same viral genotype, the complete envelope (E) gene sequences of isolates from both outbreaks were determined. To assist in this analysis, we also sequenced a DENV-3 strain representing the 1994 Nicaraguan epidemic, the first one isolated in Latin America in 1994 (3).

Three DENV-3 viruses isolated during the 2000–2002 Cuban outbreaks were studied. Two were obtained from acute-phase sera and the other from a spleen section sample from a patient who died. Serum samples and macerated spleen fragments were spread onto C6/36 mosquito cells (grown at 33°C) by using the rapid centrifugation assay (4).

Viral RNA was extracted from 200 μL of supernatant medium of virus-infected cells by using the RNAgent Total RNA Isolation system (Promega, Madison, WI, USA). The E gene was amplified by using reverse transcription–polymerase chain reaction (RT-PCR) as described previously (5). Double-stranded sequencing of the E gene was conducted on an ABI sequencer according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). All sequences determined in this study have been deposited in GenBank (accession nos. AY702030–AY702033).

The E gene sequence of the 3 Cuban DENV-3 isolates and the single strain from Nicaragua isolated in 1994 were aligned with the E gene sequences (1,479 bp in length) of 60 DENV-3 isolates deposited in GenBank, representing the global genetic diversity of DENV-3. Phylogenetic trees were produced with a maximum likelihood method incorporating the GTR+Γ+I model of nucleotide substitution, with the general time-reversible (GTR) substitution matrix, the base composition, the gamma (Γ) distribution of among-site rate variation, and the proportion of invariant sites (I) all estimated from the data. To explore the robustness of particular phylogenetic groupings, a bootstrap resampling analysis was undertaken. All analyses were performed with the PAUP* package (6).

The tree (Figure) showed 5 major groups or genotypes of DENV-3: 1) a
Pacific/Asian group (genotype I); 2) an Asian group, containing a large array of viruses sampled from Thailand and Malaysia (genotype II); 3) a Latin American group that includes the Cuban viruses and isolates from Venezuela, Martinique, and Nicaragua, as well as those from Samoa, India, and Sri Lanka (genotype III); 4) a small group of Asian viruses (genotype IV); and 5) a final, most divergent group containing virus samples from Puerto Rico (genotype V).

A similar genotype structure of DENV-3 has been observed in previous studies of this virus (5, 7–9). The 3 Cuban viruses clearly fall into genotype III. Indeed, strain D3.Nicaragua.24/94 falls at the base of the Latin American clade within genotype III, as would be expected if it was the first DENV-3 strain isolated from this region in 1994. Moreover, the most divergent strains from genotype III (with the exception of a single strain from Samoa) come from Asia, which suggests that this group of viruses was exported from Asia to Latin America in the early 1990s. Finally, the Cuban viruses fall into 2 distinct groups: the 116/00 strain isolated during the 2000 outbreak, is very closely related to the Brazilian/Martinique viruses isolated in the same year, whereas strains 580/01 and 21/02, isolated in 2001–2002, appear to have diverged distinctly to form a separate cluster (99% bootstrap support).

A total of 15 nucleotide differences were distributed throughout the E gene. Of these, 12 were synonymous and 3 were nonsynonymous substitutions resulting in amino acid changes (E19 Thr/Pro, E226 Ile/Thr, and E329 Ala/Val). Moreover, 2 different nucleotide substitutions were found for the 2 isolates obtained during the 2000 outbreak (2001–2002). These substitutions resulted in a nonconservative amino acid change (E22 Asp/Val). The ABI chromatograms of these sequences showed populations in this position, but the predominant population for each strain differed.

By using appropriate phylogenetic methods, deducing the most likely dispersal pattern for closely related dengue virus strains with different patterns of spatial and temporal sampling is possible. The current study strongly suggests that an Asian DENV-3 virus assigned to genotype III has evolved in situ in the Caribbean region since 1994.

The nucleotide differences observed in the Cuban isolates support the hypothesis that DENV-3 was introduced twice into Cuba from the wider Latin American region. Further evidence to support this hypothesis is that, during the interepidemic period, from January to June 2001, no immunoglobulin (Ig) M–positive samples from patients with fever and suspected dengue cases were observed, which suggests that the virus was not circulating. Therefore, these data are consistent with the idea that, rather than in situ evolution, a second introduction of the virus occurred in 2001.

By comparing the amino acid sequences of the Cuban isolates with other DENV-3 strains assigned to genotype III, we confirmed that several distinct amino acid replacements had occurred. In particular, the nonconservative substitution Ala/Val was seen in the Nicaragua 1994, Mexico 1996, Cuba 2001–2002, and Venezuela 2001–2002 isolates. However, the Cuba 2000, Brazil 2000, and Martinique 1999–2001 isolates preserved the Ala at this position, as was also the case for viruses sampled from the putative Asian source (from India and Sri Lanka). The functional significance of amino acids at this position has not been determined.

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Nosocomial Dengue by Mucocutaneous Transmission

To the Editor: Wagner and colleagues report nosocomial dengue transmitted by needlestick and note that it is the fourth case of nosocomial dengue to their knowledge (1). In the same issue of Emerging Infectious Diseases, Nemes and colleagues report a separate case of nosocomial dengue also transmitted by needlestick (2). Three other cases of nosocomial dengue transmission by needlestick have previously been published (3–5).

We have recently published a case of nosocomial dengue infection that was transmitted by mucocutaneous exposure to blood from a febrile traveler who had recently returned from Peru (6). During phlebotomy, a healthcare worker was splashed in the face with the traveler’s blood. Both the traveler and the healthcare worker were subsequently found to have dengue fever with dengue virus type 3. This route of infection is biologically plausible because infection through mucosal surfaces (intranasal and oral routes) has been shown possible for arboviruses (7). In our review of the literature, we also found a report of dengue virus transmission by bone marrow transplantation (8). Other cases of transmission of dengue virus without a mosquito vector have occurred in 5 reported instances of infection in newborns as a result of perinatal transmission (9). Other cases of transmission of dengue were subsequently found to have dengue virus–infected mosquitoes outside the clinical setting.

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Barriers to Creutzfeldt-Jakob Disease Autopsies, California

To the Editor: The recent article by Louie et al. underscores a more general disparity between the need for autopsies in potential infectious disease deaths and our present national capacity (1). In addition to confirming Creutzfeldt-Jakob disease (CJD) and allowing the differentiation of classic and variant CJD, autopsies identify previously undetected infections, discover causative organisms in unexplained infectious disease deaths, and provide insights into the pathogenesis.
of new or unusual infections (2,3). This information is essential for public health and medical interventions.

As outlined by Louie et al., hospital autopsy rates have dropped to single digits, and concerns by pathologists about occupational risks and biosafety have likely contributed to this decline. Currently, the last stronghold of autopsy expertise is forensic pathology (4). However, the medicolegal death investigative system does not have jurisdiction over all potential infectious disease deaths nor is it adequately supported to assume the cases that are missed by our present hospital autopsy system. Additionally, many medicolegal and hospital autopsy facilities with outdated or poorly-designed air flow systems are ill suited to handle autopsies when infectious disease is suspected (5). Air-handling systems can be expensive to fix.

Reference centers such as the National Prion Disease Pathology Surveillance Center, while providing diagnostic expertise, fail to surmount the biosafety obstacles (real and perceived) that prevent pathologists from enthusiastically performing autopsies on those who died of potential infectious diseases, including prion diseases. One potential solution is the creation of regional centers of excellence for infectious disease autopsies that could operate in conjunction with a mobile containment autopsy facility (5,6). Such centers could provide diagnostic expertise as well as biosafety capacity.

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Q Fever Wildlife Reservoir

To the Editor: To the list of zoonotic infections with wildlife sources reported by Kruse et al. (1), I would add Coxiella burnetii infection because of its global impact, extensive presence in the animal kingdom, and potential for use as an agent of bioterrorism (2). C. burnetii causes Q fever, a self-limited disease that usually appears as undifferentiated fever, pneumonia, or hepatitis, but which may progress into chronic disease, especially endocarditis, among susceptible persons. Q fever is endemic worldwide in domestic mammals, especially ungulates (cattle, sheep, and goats), but also has been found in wild mammals, birds, and arthropods. The transmission of Q fever to humans from wild rabbits was documented in the 1980s (3). More recently, a study showed seroprevalence of Q fever ranging from 7% to 53% in brown rats (Rattus norvegicus) in Oxfordshire, which suggests that they are a possible reservoir for C. burnetii in the United Kingdom. The study also speculated why cats, as frequent predators of rats, are important in maintaining the transmission cycle of the disease (4).

A case-control study published in 2001 (5) attempted to define the risk factors for an increase in the incidence of Q fever in French Guiana in 1996. The study found no link between Q fever and domestic ungulates, the usual source of outbreaks. The role of pets, basically dogs and cats, as a reservoir was also excluded. Multivariate analysis showed that living in close proximity to the forest, exposure to wild animals (including bats), and working in public trade or public works were all associated with infection. A strong correlation between large amounts of rainfall and higher incidence of Q fever was found also. All of these findings suggested a wild reservoir as a potential source of the epidemics, although the researchers could not identify a particular species as the specific source.

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References


Vaccine-derived Poliovirus, Thailand, 2003

To the Editor: The Polio Eradication Campaign was started in Thailand in 1990, and the last polio case was reported in April 1997. Although no new cases have been reported, the Polio Eradication Campaign continues with 4 prevention strategies: high coverage with 3 doses of oral polio in children <1 year of age, acute flaccid paralysis surveillance, acute flaccid paralysis case investigation and response, and National Immunization Day. Also, the Ministry of Public Health is prepared for a national emergency response to polio importation and circulated vaccine–derived poliovirus (1).

In April 2003, a case of acute flaccid paralysis was reported from Phakhao district, Loei province. The patient was an 18-month-old boy with normal physical development and nutritional status. He had a history of mild asthma and had received bronchodilator drugs occasionally during upper respiratory tract infections in the past. The patient had been fully vaccinated. He had received a total of 5 doses of oral polio vaccine: a dose at 2, 4, and 6 months of age, and 2 doses on National Immunization Day in December 2002 and January 2003.

On March 27, 2003, while visiting his grandmother in Phoowiang district, Khonkaen province (80 km from his residence), the patient became ill. Pneumonia was diagnosed; injected medications were administered into his left hip once a day for 3 days. The patient fully recovered.

On April 1, 2003, cellulitis of finger developed in the patient. The affected finger was incised and drained, and oral antimicrobial drugs were administered. The inflammation extended to his elbow but later subsided.

On April 7, fever, cough, and dyspnea developed in the patient. Two days later, the patient’s left leg became weak. He was admitted to Phakhao Hospital with a diagnosis of pneumonia and with weakness in his left leg. He was later referred to Loei Provincial Hospital and acute flaccid paralysis was diagnosed on April 11. The muscle weakness progressed until he could not sit.

On April 14, the patient was referred to Khonkaen Regional Hospital with weakness in both legs and arms (grade 0–1). Chest radiograph showed perihilar pneumonia. Cloxacillin, gentamicin, and immunoglobulin (Ig) (6 g/day × 4 days, patient weight 12 kg) were administered intravenously to the patient. He was discharged on April 30 with a diagnosis of Guillain-Barré syndrome and bacterial pneumonia. The muscle tone in his right leg and both arms was grade 3; however, he could not move his left leg.

Stool samples were collected on April 11 and 14 and tested for polio at the Department of Medical Science (reference laboratory for polio in Southeast Asia). Poliovirus type 2 was isolated in the samples; however, the results were inconclusive for strain differentiation. The isolates were sent to the Centers for Disease Control and Prevention (CDC), USA, for genetic sequencing, and the result showed poliovirus type 2 with 1.6% difference from Sabin strain poliovirus. Without evidence of recombination with other nonpolio enterovirus, the pattern of genomic change was similar to the change that occurs in immunodeficient persons. Immune system testing of the patient on August 13 showed IgG = 205.9 mg/dL (normal 800–1,700), IgA <5.5 mg/dL (normal 100–490), IgM <16.8 mg/dL (normal 50–320), and IgE <18.0 mg/dL (normal 0–100). Antibodies to poliovirus type 1, 2, and 3 were 1:16, 1:32, and 1:8, respectively. Testing of the follow-up stool samples showed P1/Sabin on August 10. Test results were negative on October 13, and results showed nonpolio enterovirus on November 10 and December 14.

Before the large-scale outbreak response immunization was conducted, 339 serum samples were collected from children <5 years of age who lived in the same district as the patient or in the same subdistrict as his grandmother. Among 153 children who brought their vaccination records, the median dose of oral polio vaccine was 7 (range 2–15). All had antibody >1:8 to poliovirus types 1, 2, and 3. Approximately 2,000 stool samples were collected from children <5 year of age who lived in the same district as the case-patient or his grandmother. However, after the immunodeficiencies vaccine–derived poliovirus was identified, isolation of the virus was attempted only from stool samples from children who lived in the same subdistrict as the patient. From 223 stool samples, 4 Sabin strain poliovirus and 32 nonpolio enteroviruses were isolated. In addition, 2 of 18 stool samples collected in July from close contacts of the case-patient were positive for Sabin strain poliovirus and negative for vaccine–derived poliovirus.

The Loei Provincial Health Office initially did a small-scale response immunization in 3 adjacent villages (128 of 129 children) on the day that the case of acute flaccid paralysis was reported. Coverage of third dose of oral polio vaccine in these villages was 100%. No response immunization was conducted at the village in Khonkaen. On August 8, genetic
sequencing results showed vaccine–derived poliovirus; the decision was made to launch an outbreak response immunization for 175,000 children <5 years of age living in Loei, Khonkaen, and Nongbualamphoo provinces (visited by the patient from March to August 2003). Two-round campaigns were conducted in August and September. The estimated vaccine coverage was >95%.

Considering the rate of 1% genomic diversity per year and the immunodeficient status of the patient (2), he should have harbored the vaccine strain virus since he received the first dose of routine oral polio vaccine immunization at 2 months of age, and the virus was replicated in his gut. However, why the virus disappeared in subsequent stool specimens is unknown. Circulating vaccine–derived poliovirus is unlikely in this event, as we found no evidence of recombination with other nonpolio enterovirus, high oral polio vaccine coverage in the community, and no vaccine–derived poliovirus in other children.

Although immunoglobulin levels in this case were low but still detectable, whether the patient’s illness was agammaglobulinemia or hypogammaglobulinemia is uncertain. The detected immunoglobulin levels, as well as the antibody level to poliovirus, may be due to intravenous immunoglobulin (IVIG) the patient received while hospitalized 4 months before testing. Since August 2003, the patient has been on IVIG replacement therapy after prolonged and repeated respiratory tract infections.

In retrospect, problems surrounded this event. First, because of several attempts to confirm the result, identification of strain differentiation was delayed. Second, genetic sequencing was delayed because of a communication gap associated with new bioterrorism regulations in the United States during specimen transfer. Third, knowledge of a possible immune deficiency in the previously healthy child was lacking, testing for the patient’s immune status was delayed.

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Toscana Virus and Acute Meningitis, France

To the Editor: Sandfly fever viruses, Sandfly fever Sicilian virus, and Toscana virus (family Bunyaviridae, genus Phlebovirus) have been recognized as etiologic agents of human illnesses in European countries bordering the Mediterranean Sea. These viruses are responsible for rapidly resolving diseases with nonspecific symptoms such as fever and myalgia. However, infection with Toscana virus may involve the central nervous system; severity may range from aseptic meningitis to meningoencephalitis (1). In most cases, illnesses caused by Toscana virus mimics a flulike syndrome with fever, photophobia, headache, red eyes, and stiff neck. Recently, 2 cases of Toscana virus meningoencephalitis in patients with unusual symptoms and life-threatening complications were described in Italy (2). However, sequelae have never been reported.

Toscana virus infection is now epidemic in Italy and Spain (1,3). Furthermore, sporadic cases have been reported in travelers returning from Italy, Spain, Greece, Portugal, and the South of France (4–6). The epidemiology of Toscana virus in France is still unknown. Although infections with this virus have been diagnosed by serologic tests in French patients and in tourists residing in southeastern France, this pathogen has reportedly never been isolated in France (7,8). Here we describe the clinical and biologic features of autochthonous meningitis due to Toscana virus.

On July 9, 2004, a 57-year-old woman who had never left the southeastern coast of France reported malaise and vomiting. On hospital admission, her body temperature was 38.5°C, and clinical examination showed photophobia and stiff neck.
Skin and abdomen were normal. Cardiopulmonary and neurologic functions were also normal. Analysis of hematologic and biochemical blood tests revealed mild hyperglycemia (6.88 mmol/L) and elevated γ-glutamyltransferase (104 IU/L) and C-reactive protein levels (57 mg/L). Cerebrospinal fluid (CSF) analysis showed 3,500 leukocytes/µL (70% lymphocytes, 30% neutrophils), and glucose and protein levels of 2.5 mmol/L and 2.749 mg/L, respectively. Blood and CSF cultures were bacteriologically sterile. Polymerase chain reaction (PCR) assays of CSF for herpes simplex virus were also negative. The patient received intravenous amoxicillin and acyclovir for 3 days. The patient recovered after 6 days without sequelae.

Serum and CSF samples collected during the acute phase were tested for immunoglobulin (Ig)M and IgG antibodies to a battery of arboviruses. These samples contained no antibodies (optical density [OD] ratio <1.5) to flaviviruses, dengue virus, West Nile virus, and tickborne encephalitis, Toscana virus, or Sandfly fever Sicilian virus (Table). However, the IgM OD ratios (≥2.5) obtained against Toscana virus antigen were high. A second serum sample tested 1 month later showed seroconversion to Toscana virus with OD ratios >3 for both IgM and IgG (Table).

Virus isolation was attempted by incubation of peripheral blood mononuclear cells and CSF collected on the day of onset with C6/36 (Aedes albopictus) and Vero (E6 clone) monolayers. Toscana virus was found only on Vero cells by indirect immunofluorescence using mouse hyperimmune ascitic fluid against Toscana virus (Langat) (Table). However, the IgM OD ratios (≥2.5) obtained against Toscana virus antigen were high. A second serum sample tested 1 month later showed seroconversion to Toscana virus with OD ratios >3 for both IgM and IgG (Table).

Virus isolation was attempted by incubation of peripheral blood mononuclear cells and CSF collected on the day of onset with C6/36 (Aedes albopictus) and Vero (E6 clone) monolayers. Toscana virus was found only on Vero cells by indirect immunofluorescence by using mouse hyperimmune ascitic fluid against Toscana virus. In contrast, no fluorescence was found by using mouse hyperimmune ascitic fluids against Rift Valley fever and Sandfly fever Sicilian virus.

S segment of Toscana virus genome was partly amplified from culture supernatants by reverse-transcription PCR and sequenced (9). Nucleotide and peptide sequences obtained (GenBank accession no. AY766034) displayed 87% and 100% identity, respectively, with Toscana virus sequences available on GenBank database, thus confirming the infection by Toscana virus.

Toscana virus, transmitted to humans by Phlebotomus vectors, has been recognized as a major cause of aseptic meningitis in Italy and Spain. P. perniciosus, proven to be a vector of Toscana virus (10), is abundant along the French Mediterranean coast. The isolation of an autochthonous Toscana virus strain shows that the conditions of an efficient transmission cycle were combined in southern France. Until now, human infection by Toscana virus was fortuitously detected by serologic means, suggesting that subclinical infection may also occur (8). Thus, Toscana virus infection in France likely has been underestimated. Moreover, meningitis caused by Toscana virus has been underestimated and other diseases caused by Toscana virus may have also been underestimated. The requirement for virus growth in cell culture delays a diagnosis based on viral isolation, which is limited by the transitory presence of the virus in blood or CSF. As reported here, Toscana virus infection was only confirmed after the patient relapsed. Considering that signs and symptoms of Toscana virus meningitis are not pathognomonic, this case highlights the need for rapid and specific diagnostic tools, such as PCR assays, to identify infections caused by Toscana virus and other neurotropic viral agents. Moreover, a systematic serologic study of recovered meningitis patients may help to better characterize viral meningitis of unknown etiology.

Finally, this work suggests that, in addition to West Nile virus, Toscana virus should now be considered as a potential etiologic agent of acute meningitis in the southeastern part of France. Entomologic and epidemiologic surveys, however, will have to be conducted in the near future to determine the risk for the people living in that area.

Acknowledgments

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Table. Arbovirus antibody investigation of samples*

<table>
<thead>
<tr>
<th>Viral antigens</th>
<th>CSF†</th>
<th>Serum 1†</th>
<th>Serum 2‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dengue</td>
<td>1.26</td>
<td>1.00</td>
<td>1.59</td>
</tr>
<tr>
<td>West Nile</td>
<td>1.06</td>
<td>1.00</td>
<td>1.59</td>
</tr>
<tr>
<td>Toscana</td>
<td>2.50</td>
<td>1.00</td>
<td>1.59</td>
</tr>
<tr>
<td>Sandfly fever Sicilian</td>
<td>0.96</td>
<td>1.00</td>
<td>1.59</td>
</tr>
<tr>
<td>Tickborne encephalitis (Langat)</td>
<td>0.96</td>
<td>1.00</td>
<td>1.59</td>
</tr>
</tbody>
</table>

†CSF, cerebrospinal fluid; Ig, immunoglobulin; MAC-ELISA, immunoglobulin M antigen capture enzyme-linked immunosorbent assay. Samples were considered positive if the ratio is over 3. Bold values indicate positive results.

‡Serum obtained 30 days after the onset of the disease. $MAC-ELISA.

Sandwich-ELISA.
LETTERS

Helicobacter pylori, Republic of Georgia

To the Editor: Helicobacter pylori infection is a principal cause of chronic active gastritis and peptic ulcer disease as well as gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (1). Poverty and crowding have been associated with infection epidemiologically (2,3). The Republic of Georgia has a per capita annual income of US $591, making it one of the poorest countries in the world (4,5). Georgia also reports a high annual incidence of gastric cancer, 17/100,000 population in 2002 (National Center for Disease Control, Tbilisi, unpub. data), which suggests an elevated prevalence of H. pylori infection. Testing and treatment for H. pylori are not practiced in this country, and diagnostic capacity for H. pylori is nonexistent. In October 2003, we conducted an exploratory pilot study of H. pylori infection to begin characterizing prevalence and risk factors for infection.

We studied a convenience sample of adults residing in or near the capital city of Tbilisi. Urban participants were recruited in 11 of Tbilisi’s 12 residential districts and in 1 district of Rustavi, a city 20 miles south of Tbilisi. Rural participants were recruited from 3 villages within 10 miles of Tbilisi. In each district or village, we nonsystematically selected 10 households and recruited 1 adult per household. Exclusion criteria included age <18 years; reported allergy to omeprazole, clarithromycin, or amoxicillin; or treatment with any antimicrobial agent within the preceding 2 weeks. This protocol was reviewed and approved by the Human Subjects Review Board at the National Center for Disease Control, Tbilisi. Active infection with H. pylori was measured by a validated, point-of-care 13C-urea breath test (Meretek Corporation, Lafayette, CO, USA) (6). Participants responded to a questionnaire that requested information about gastrointestinal symptoms during the preceding 12 months; diagnosis of gastritis, peptic ulcer disease, and gastric cancer made by a physician; family history of peptic ulcer disease or gastric cancer; and knowledge about H. pylori. Low, medium, or high socioeconomic status categories were designated on an ecologic basis, according to average real estate prices and common perception of the living standard of the participant’s district or village of residence. Analyses were conducted with SAS (SAS Institute, Cary, NC, USA) version 9.0. Measures of inference are not reported because participants did not constitute a rigorously selected population sample.

Of 136 persons eligible to participate in the study, 135 (99%) consented to take part. Median age was 39 (range 19 – 79); 82 participants (61%) were women. Twenty-seven (20%) reported having some knowledge of H. pylori, but none had been tested or treated for the infection. Ninety-seven (72%) participants had active H. pylori infection: 58 (71%) of 82 participants residing in neighborhoods of medium- or high-socioeconomic status were infected compared to 74 (69%) of 50 participants <50 years of age tested positive for H. pylori infection. One hundred five (71%) of 147 participants ≥50 years of age tested positive for H. pylori infection: 58 (71%) of 82 women and 39 (74%) of 53 men. Thirty (77%) of 39 participants >50 years of age tested positive for H. pylori compared to 67 (70%) of 96 participants <50 years of age.

Seventy-one (84%) of 85 participants residing in neighborhoods of low-socioeconomic status were infected versus 26 (52%) of 50 participants residing in neighborhoods of medium- or high-socioeconomic status (crude prevalence odds ratio 4.68). Twenty-three (85%) rural participants were infected compared to 74 (69%) of 108 urban participants (crude prevalence odds ratio 2.64).

Gastrointestinal symptoms were common, but did not correlate with active infection. One hundred five participants (78%) reported recurrent epigastric pain within the past year;

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and 120 participants (89%) reported a variety of gastrointestinal symptoms, including epigastric pain. Persons with active infection were no more likely to report epigastric pain (75 [78%] of 97) than persons without infections (30 [79%] of 38). One participant reported gastric cancer and was *H. pylori*–positive. Seven participants reported a family history of gastric cancer; all were *H. pylori*–positive. Five participants, of whom 3 were *H. pylori*–positive, reported a history of gastric surgery for peptic ulcer disease. More rural participants (23 of 27) reported a history of ruptured ulcers or a family member with gastric cancer than did urban participants (74 of 108). The frequency of reported gastrointestinal symptoms was similar between urban and rural participants.

To our knowledge, this is the first survey of *H. pylori* infection in Georgia. We found a high rate of infection with *H. pylori*. Participants also reported a very high rate of dyspeptic symptoms, although these were not correlated with infection. This small convenience sample survey has several limitations, however. First, our participants did not constitute a systematically selected population sample. Second, rural populations were underrepresented. Finally, we used neighborhood or village of residence as a marker for socioeconomic status without specific income information from the participants. Therefore, socioeconomic status misclassification possibly occurred and the association between infection and socioeconomic status may not be accurate.

Nevertheless, it is unlikely that we substantially over- or underrepresented infection prevalence in the general population. Despite the limitations of this study, our results clearly indicate that *H. pylori* is a serious public health problem in Georgia. There is a pressing need to educate medical professionals and the general public about *H. pylori* infection and gastrointestinal illness and to introduce diagnosis of this infection and appropriate treatment for it into standard medical practice. In addition, rigorous population surveys that include children are needed to identify high-risk groups of persons for targeted public health interventions (7).

**Acknowledgments**

We thank John Heinrich for facilitating the use of an accurate point-of-care test of active *H. pylori* infection in the field. We also thank Maiko Chokheli, Ekaterina Jhorjholiani, and Tamuna Zardashvili for assistance with data collecting.

This study was supported by a grant from the Biotechnology Engagement Program (BTEP) #13, Department of Health and Human Services. Dr. Gold is supported in part by a grant from the National Institutes of Health, NIDDK (DK-53708).

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**References**


enced diplopia or blurred vision and at least 1 of the following symptoms: dysphagia, dry mouth, dysarthria, upper/lower extremity weakness, dyspnea, and severe constipation. Those who met the probable case definition and had laboratory-confirmed botulism were considered definite case-patients.

We tested for botulinum neurotoxin in serum and spores in stool samples as described (1). Serum or stool specimens from 24 patients with ≥2 symptoms were sent to the Italian National Institute of Health for testing. Twenty-eight persons reported ≥2 symptoms (42% attack rate); 25 (89%) were considered probable cases and 3 (11%) were considered confirmed cases. Two members of the restaurant owner’s family and 1 employee were among the probable case-patients. Onset of symptoms occurred 4 hours to 6.5 days after eating at the restaurant (median 36 hours). Twenty persons (71%) had been seen in emergency rooms, 15 (53%) were admitted to a hospital, and 18% were admitted to intensive care. None required ventilatory support, and no deaths occurred.

The main symptoms reported by 28 probable and confirmed patients included dry mouth in 25 (89%), dysphagia in 25 (89%), severe constipation in 22 (79%), and blurred vision in 27 (96%). Three weeks after onset of symptoms, 15 (68%) reporting severe constipation, 11 (41%) reporting blurred vision, 10 (40%) reporting dry mouth, and 11 (44%) reporting dysphagia still had these symptoms. Of the 24 patients for whom rectal swabs were available, 3 were culture-positive for Clostridium botulinum type B. None of 5 serum samples tested positive.

Food-specific attack rates, relative risks (RRs), and 95% confidence intervals (CIs) were calculated. A Poisson model with robust error variance was used to estimate RR with adjustments for possible confounding and effect modification (2). Foods associated with illness with p-values <0.20 were considered in the model.

In a univariate analysis in which all 28 patients were considered, the RR of illness was higher among diners who ate home-preserved green olives in salt water (RR 5.2, 95% CI 1.4–19.8), ate cream pastries (RR 2.5, 95% CI 1.8–3.4), and drank homemade lemon liqueur (RR 2.1, 95% CI 1.3–3.4). After multivariate analysis, only the risk associated with eating green olives remained significant (RR 5.2, 95% CI 1.4–19.8).

None of the food items served on February 22 or 24 was available for sampling, and none of the other 13 food samples obtained from the restaurant was positive for C. botulinum. However, the pH of a jar of olives that had been prepared at the same time as those eaten on February 22 and 24 was 6.2, far above the level of 4.6 required to prevent growth of C. botulinum. No salinity testing was performed by the local laboratory, and inadequate storage during transit made it impossible to conduct salinity and water activity tests at the national reference laboratory.

Interviews with the restaurant proprietors indicated that the olives were prepared on site during the fall of 2003 from local olives. After soaking in salt water for 35 days, the olives had been decanted into jars, and salt water had been replaced with fresh water. Neither the amount of salt used in the salt water mixture nor the pH at any stage was standardized during preparation.

Both epidemiologic evidence and information obtained regarding preparation of the olives strongly suggest that they were the likely source of the outbreak. This outbreak highlights the previously documented risk associated with improperly prepared olives (3–5). In Italy and elsewhere in Europe, an increasing trend favors traditional foods and preparation methods over large-scale industrial products. This outbreak underlines the importance of providing training and periodic monitoring of those involved in small-scale preparation to ensure that disease risks from improperly prepared or stored foods are minimized.

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Medical Bacteriology: a Practical Approach

Peter Hawkey
and Diedre Lewis, editors

Oxford University Press
Oxford, United Kingdom
ISBN: 0-19-963778-4
Pages: 409, Price: US$59.50

Medical Bacteriology is a multi-contributor work with chapters provided by various expert medical microbiologists from the United Kingdom. The information is organized into 4 basic areas totaling 12 chapters; 6 covering the analysis of various types of patient specimens; 2 chapters on antimicrobial analysis, susceptibility testing, and direct assay in patient specimens; 2 chapters on laboratory management issues, including information technology, quality control, and quality assurance; and 2 chapters on the role of the laboratory in hospital infection control programs and in the support of epidemiologic investigations. Distributed throughout the text are 81 individual testing protocols, which can be found either by referring to a table at the front of the book or by searching the index. Also included are 4 appendixes.

Multiple options exist for presenting the fundamentals of medical bacteriology. Some texts have used a disease-based approach. Others have used an organism-based approach. Medical Bacteriology takes a specimen-based approach: bacterial diseases and their causative agents are addressed through the proper collection, processing, and analysis of clinical specimens. From a laboratory perspective, the specimen-based approach has substantial advantages. In this text, the critical importance of proper collection and transport of specimens is clearly communicated for each specimen type, and appropriate protocols are provided. In addition, an in-depth discussion is presented on the proper interpretation of cultures and other laboratory findings for each specimen type. For example, the chapter on urine bacteriology contains not only guidelines for interpreting various types of urine cultures, but also explains the relevance of other routine urinalysis findings to infections of the urinary tract. One disadvantage of the specimen-based approach is that organism identification protocols may appear in multiple chapters.

The chapters devoted to antimicrobial issues provide basic testing protocols and valuable insights on the selection of appropriate agents for routine testing and reporting. The relative advantages and disadvantages of diffusion versus dilution methods are clearly described, and readers from the United States may find some of the alternatives to the standard Kirby-Bauer procedure to be of interest.

The chapters devoted to laboratory management provide excellent insights on the rapidly evolving field of laboratory information technology. Laboratorians should find the comparison of stand-alone “legacy” systems with more integrated hospital-wide and health system–wide designs useful in making decisions on laboratory information management.

The chapters on infection control and epidemiology provide an overview of the technical and administrative issues encountered by the laboratory. The organization and function of hospital infection control committees are discussed, and guidance for performing basic cohort and case-control studies is presented.

Emphasis is placed on the processing and analysis of specimens with well-established protocols and materials. Bacterial identification and characterization protocols are based on techniques that have been in general use for many years. Readers interested in newer approaches and in analyses of some of the more exotic bacterial zoonoses are provided with appropriate references.

The book flows smoothly from chapter to chapter. Similar material appearing in various chapters is appropriately cross-referenced. The text is clearly written, with jargon and acronyms kept to a minimum. At 409 pages, including the appendixes and index, this book can be easily read in a few sittings, and readers, including students, technologists, laboratory supervisors, and senior scientists, should find it to be a useful reference.

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Cases in Human Parasitology

Judith S. Heelan

ASM Press, Washington, DC
Pages: 243, Price: US$59.95

This compact, glossy, paperbound text contains 62 cases in 5 sections: I) Intestinal Protozoa, II) Blood and Tissue Protozoa, III) Cestodes, Trematodes and Intestinal Nematodes, IV) Blood and Tissue Nematodes, and V) Challenging Cases. The intent was to emphasize the relationship between diagnosis and patient care. This goal is laudable; unfortunately, serious shortcomings limit the
book’s usefulness for students, professors, or laboratorians.

A major challenge is the format: the presented case is “textbook,” and the “answer” is given in the case presentation, leaving little need for the answer section. How many junior parasitologists or biologists with casual interest in parasites don’t know that bile-stained, barrel-shaped nematode eggs with prominent polar plugs represent *Trichuris* eggs? If the clinical history and illustrations are presented and the detailed description of the organism is left to the answer, the reader can look at the illustrations, decipher the morphologic features, consider the possibilities, and then differentiate by using existing features.

Some case presentations had no illustrations, which is a prerequisite. Most illustrations were adequate, but some were unacceptable. Figure 4.1 presumes to illustrate an *Entamoeba histolytica* cyst, but the diagnosis could not be made from the image. Figure 16.1 is listed as typical of *Babesia* infection, yet after close study, if *Babesia* organisms are present, they are not typical. The illustrations of microsporidia at low-power magnification were perplexing. Use of identical images to illustrate East African and West African trypanosomes is unacceptable. The illustration for case 52 (onchocerciasis) shows a Giemsa-stained microfilaria with a sheath. The morphologic features and the sheath stained with Giemsa indicate a *Brugia* microfilaria, not an *Onchocerca* microfilaria.

In case 52 (*Onchocerca*), surgical removal of regional lymph nodes is advised, in addition to removal of nodules containing adult worms. This is not standard medical advice. In case 48 (dracunculiasis), it is stated that cisterns in Iran and step wells in India are common sources of infection and that prevalence of this infection has been reduced in most areas, except India, Pakistan, and a few countries in Africa. Guinea worm has been absent from Iran since 1972, from Pakistan since 1993, and from India since 1996. In the same case study, it is stated that metronidazole is often used to complement or replace traditional removal of worms, and that niridazole, thiabendazole, and mebendazole are also useful. None of these drugs has any benefit in Guinea worm infection treatment. In case 3 (cyclosporiasis), it is stated that infections from ingestion of contaminated fruits, such as imported strawberries, have been reported. Not true; strawberries have never been implicated.

Given the multiple errors and lack of attention to detail (Colombia is misspelled; the width of Anisakis L3 is given as 1 cm), this book has little to offer, despite its reasonable price ($60). This is unfortunate because a well-done series of teaching cases could fill a much needed void.

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**Correction: Vol. 11, No. 4**

In "Methicillin-resistant *Staphylococcus aureus* Toxic Shock Syndrome" by Sophie Jamart et al., an error occurred. In a listing of laboratory results (third paragraph, fifth sentence), cyclic AMP receptor protein 43.7 ng/mL should be C-reactive protein 43.7 mg/dL.

The corrected article appears online at http://www.cdc.gov/ncidod/EID/vol11no04/04-0893.htm

We regret any confusion this error may have caused.
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During his long ocean passage to Australia in 1852, Eugene von Guérard reported that conditions were far from ideal, and “meals were late and bad” (1). He had boarded a sailing ship at Gravesend, England, to seek his fortune in Victoria. At the gold fields of Ballarat, he described mining as “arduous at first” and causing “much backache and blistering of hands” (1). His gold mining efforts in Ballarat and environs were unsuccessful, and after a year, he abandoned the venture. Even so, he had struck gold. His illustrated diaries chronicled the history of the region, the harsh life of the gold digger, and the scarring of landscape from gold mining. He opened a studio in Melbourne, soon to become the most important Australian artist of his day.

The son of an artist and court painter, von Guérard was born in Vienna, Austria. He toured Italy with his father and lived in Rome for a while, where he became familiar with the work of famed French landscapists Claude Lorraine and Nicholas Poussin. Later, he studied landscape painting at the Dusseldorf Academy, where he was influenced by German romanticism—a movement that also dwelled on the visual aspects of nature. During his 30 years in Australia, he became a renowned landscape painter, as well as teacher and honorary curator at the National Gallery in Victoria. He died in London, where he had settled near the end of his life (2).

The 1850s gold rush that lured von Guérard to Victoria coincided with a revived interest in landscape painting, particularly in Australia and the United States (the Hudson River School). In the midst of 19th-century urbanization grew longing to connect with nature. Travelers sought areas of untouched wilderness, and artists labored to bring exotic freshness to the homebound. From his studio in Melbourne, von Guérard traveled to and explored many regions, among them timbered Illawarra and Tasmania, seeking the picturesque and generating drawings for his monumental landscape paintings (3).

At Illawarra, von Guérard was able to capture, in minute detail, the character of local flora: cabbage palms, ferns, fig trees, and multiple varieties of vines within the dark green tones of the dense Australian forest. Meticulous geographic, geologic, and ecosystem markers lend his artistic work historical importance as backdrop to subsequent transformation of the landscape by widespread mining and population growth.

In a letter to Melbourne newspaper The Argus in 1870, von Guérard explained that he painted “with the greatest desire to imitate nature” and sought to capture not only her details but also her “poetical feelings” (4). Descriptive and emotional, his elaborate artistic observations imbued the physical world with inner life quite apart from human society. Like many of his contemporaries (e.g., American painter Frederic Church), von Guérard was influenced by prominent German naturalist Alexander von Humboldt (1769–1859), who advocated a “mutual reinforcement of art and science.” In this context, topographic detail was acceptable in paintings only if motivated and sustained by

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emotional connection and personal relevance. Landscape painting was a way to express love of nature. And nature was constantly changing, driven by forces that shaped it throughout the eons (5).

Ferntree Gully in the Dandenong Ranges on this month’s cover was hailed a masterpiece in its day. A lush mountain panorama, this painting excels in its faithful depiction of both the forest and the trees. Balanced and lyrical, the work is a romantic rendering of pure, unadulterated nature. The scene is carefully structured: background fully outlined, center well-lit, and foreground intentionally shaded to frame and enhance view of the cloistered center. Each leaf is described in detail.

A broad path leads inward for a better look at the botanical life nearby as well as the treed horizon afar. A couple of lyrebirds walk the brush, their ancient silhouettes outlined against the grounds sheltering their fare of insects, myriapods, and snails. Curving fern tops and tree branches create a circular feeling as the eye moves from dark to light, from mountaintop to forest floor, from live greenery to fallen tree limbs and skeletal trunks, recounting a natural cycle of death and regeneration (6).

Von Guérard provided a respectful glimpse at unspoiled wilderness. His artistic eye scanned the exotic flora and through the bucolic stillness saw the real Arcadia, a goldmine of natural elements in constant change.

The semitropical rainforest idyll witnessed by von Guérard in Illawarra repeats itself in tropical and subtropical regions around the globe. Under a canopy of green, away from direct light, rain, and wind, moisture seeps down or hangs in mid-air, creating a fertile environment for propagation and growth. Along with fern spores, wildlife and microbial life are beneficiaries of the gullies’ hothouse. Tiny creatures of the forest and blood-sucking insects, nature’s fine detail, populate the underbrush—among them, mosquitoes, which feed on wild animals and thrive in this habitat.

As human development encroaches on the forest and urbanization transforms the native environment, mosquitoes become able to travel to all global destinations. These adaptable insects, some of them vectors of dengue viruses, have become anthropophilic, domesticated, and dangerous to humans. Environmental change affects nature’s cycle, once more frustrating efforts to disrupt the persistent reemergence of tropical diseases like dengue (7).

References

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Upcoming Infectious Disease Activities

May 1, 2005
International Society of Travel Medicine (ISTM) offers certificate of knowledge in travel medicine exam (Given prior to the opening of 9th Conference of the ISTM)
Contact: exam@istm.org
http://www.ISTM.org/

May 1–5, 2005
9th Conference of the International Society of Travel Medicine
Lisbon, Portugal
Contact: 49-89-2180-3830
http://www.ISTM.org/

May 3–5, 2005
Controlling Infectious Agents and Other Contaminants in Healthcare Facilities through Planning and Design
Madison, WI, USA
Contact: 800-462-0876
http://epd.engr.wisc.edu/emaG041

May 9–11, 2005
The Eighth Annual Conference on Vaccine Research
Baltimore Marriott Inner Harbor Hotel
Baltimore, MD, USA
Contact: 301-656-0003 ext. 12 or vaccine@nfid.org
http://www.nfid.org

May 11–14, 2005
7th International Meeting on Microbial Epidemiologic Markers (IMMEM7)
Victoria, British Columbia, Canada
http://www.asm.org/Meetings/index.asp?bid=27725

May 23–27, 2005
Pennsylvania Department of Health Public Health Institute Spring 2005
Penn Stater Conference Center
State College, PA, USA
Contact: 570-826-2062 or cpolachek@state.pa.us
http://www.dsf.health.state.pa.us/health/cwp/view.asp?a=333&q=236330

Look in the June issue for the following topics:

- Integrating *Escherichia coli* Antimicrobial Susceptibility Data from Multiple Surveillance Programs
- Nonprescribed Antimicrobial Drugs in Latino Community, South Carolina
- Community-associated Methicillin-resistant *Staphylococcus aureus* in Hospital Nursery and Maternity Units
- Relative Fitness of Fluoroquinolone-resistant *Streptococcus pneumoniae*
- Global Spread of Vancomycin-resistant *Enterococcus faecium* from Distinct Nosocomial Genetic Complex
- Antimicrobial Resistance Determinants and Future Control
- Emergence and Spread of *Streptococcus pneumoniae* with *erm*(B) and *mef*(A) Resistance
- *pVir* and *Campylobacter jejuni* Enteritis
- Hidden Epidemic of Macrolide-resistant Pneumococci

Complete list of articles in the June issue at http://www.cdc.gov/ncidod/eid/upcoming.htm
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.
Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/eid/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

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), a one-sentence summary of the conclusions, and 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); tables (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no 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 epidemiologic 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. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only (effective January 2005). Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.