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Authors
Matthias, Michael A
Díaz, M Mónica
Campos, Kalina J
et al.

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DIVERSITY OF BAT-ASSOCIATED LEPTOSPIRA IN THE PERUVIAN AMAZON INFERRRED BY BAYESIAN PHYLOGENETIC ANALYSIS OF 16S RIBOSOMAL DNA SEQUENCES

MICHAEL A. MATTHIAS, M. MÓNICA DÍAZ, KALINA J. CAMPOS, MARITZA CALDERON, MICHAEL R. WILLIG,* VICTOR PACHECO, EDUARDO GÔTUZZO, ROBERT H. GILMAN, AND JOSEPH M. VINETZ*

Division of Infectious Diseases, Department of Medicine, University of California, San Diego School of Medicine, La Jolla, California: Ecology Program, Department of Biological Science and Museum, Texas Tech University, Lubbock, Texas; Laboratory of Infectious Diseases and Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru; Museo de Historia Natural, Lima, Peru; Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

Abstract. The role of bats as potential sources of transmission to humans or as maintenance hosts of leptospires is poorly understood. We quantified the prevalence of leptospiral colonization in bats in the Peruvian Amazon in the vicinity of Iquitos, an area of high biologic diversity. Of 589 analyzed bats, culture (3 of 589) and molecular evidence (20 of 589) of leptospiral colonization was found in the kidneys, yielding an overall colonization rate of 3.4%. Infection rates differed with habitat and location, and among different bat species. Bayesian analysis was used to infer phylogenetic relationships of leptospiral 16S ribosomal DNA sequences. Tree topologies were consistent with groupings based on DNA-DNA hybridization studies. A diverse group of leptospires was found in peri-Iquitos bat populations including Leptospira interrogans (5 clones), L. kirschneri (1), L. borgpetersenii (4), L. fainei (1), and two previously undescribed leptospiral species (8). Although L. kirschneri and L. interrogans have been previously isolated from bats, this report is the first to describe L. borgpetersenii and L. fainei infection of bats. A wild animal reservoir of L. fainei has not been previously described. The detection in bats of the L. interrogs serovar Icterohemorrhagiae, a leptospire typically maintained by peridomestic rats, suggests a rodent-bat infection cycle. Bats in Iquitos maintain a genetically diverse group of leptospires. These results provide a solid basis for pursuing molecular epidemiologic studies of bat-associated Leptospira, a potentially new epidemiologic reservoir of transmission of leptospirosis to humans.

INTRODUCTION

Leptospirosis is a globally important zoonotic disease caused by spirochetes of the genus Leptospira, which includes pathogenic and saprophytic strains.1 These bacteria colonize the kidneys of reservoir animals and are excreted with urine into the environment.2 Transmission to humans occurs either directly from exposure to contaminated urine or infected tissues, or indirectly via contact with contaminated soil or water.3 Most studies, especially those in developing countries such as Peru, have focused on domestic animals as reservoir hosts because of their economic importance and close association with humans.4–8 However, because of their size, abundance, spatial distribution, and association with humans and domestic animals, wild mammals such as bats may be epidemiologically significant sources of leptospires. Scant data either support or refute the hypothesis that bats are involved in transmitting leptospirosis to humans,9 despite their oftentimes close association with human domiciles.10 Recent studies in Iquitos, Peru indicated that bats carry leptospires in their kidneys.9,11 Elsewhere, isolates have been obtained from 19 species of Microchiroptera.12 Moreover, circulating antibodies to Leptospira have been detected in fruit bats in Australia.13 In Sudan, serologic evidence of infection was found in megachiropteran and microchiropteran species.14 To definitively establish that bat colonization by leptospires leads to human infection requires rigorous evidence such as that provided by molecular epidemiologic methods (e.g., 16S ribosomal DNA [rDNA] gene sequencing). If the molecular identities of leptospires colonizing bats match those causing human infection, the role of bats in transmission of leptospirosis would be unequivocal.

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LEPTOSPIRAL DIVERSITY IN PERUVIAN AMAZON BATS

the basis for conducting molecular epidemiology studies to determine the role of bats in transmitting leptospirosis to humans.

MATERIALS AND METHODS

Study site. The study site included the city of Iquitos and surrounding areas in northeastern Peru (03°34'S, 73°15'N). Iquitos is on the bank of the Amazon River between the outlets of the Itaya River and the Nanay River. It is the third largest and most important city in the Amazon basin, with approximately 350,000 inhabitants in the city and surrounding local villages. The climate is hot (average temperature = 27.5°C), humid (mean annual humidity = 85%), and rainy (mean annual precipitation = 2,700 mm). Although temperatures are relatively constant, June to December is the hottest period, and a rainy season extends from January through June.

Field methods. Bats were trapped by mist netting from December 2002 to June 2004 at 18 sites in the vicinity of Iquitos that represented four habitats: urban-suburban, undisturbed tropical humid forest, secondary growth tropical humid forest, and cultivated areas. Six to eight mist nets (12 meters) were set at each site for two nights from dusk (~6:00 pm) to midnight. In urban areas, some specimens were obtained by netting or searching roosts.

Collected specimens were anesthetized with chloroform. In the laboratory, animals were killed by excess chloroform anesthesia. Thereafter, urine and kidneys were removed for leptospiral culture and PCR. Skins and skeletons were preserved as standard museum specimens. Specimens will be deposited at the Museo de Historia Natural de San Marcos in Lima, Peru. The specimens collected have not yet been accessioned institutionally, so they are here identified with the initials of the collectors (M. Mónica Díaz [MMD] and Christopher P. Bloch [CPB]). Animal work in this study was approved by the Institutional Animal Care and Use Committees of Texas Tech University and the University of California San Diego, and approved by the Interior Ministry of Peru.

Culture conditions. Kidneys were processed under aseptic conditions. Two small homogenized portions of kidney were inoculated into two tubes containing 5 mL of semisolid PLM-5 (Serologicals Corp., Norcross, GA) medium supplemented with the antibiotics neomycin (2 µg/mL) and 5-fluorouracil (200 µg/mL). Cultures were incubated at 28-30°C and examined bi-weekly by dark-field microscopy. The remaining kidney specimens and urine were stored at -20°C for PCR-based studies. Positive cultures were sub-cultured into liquid PLM-5 medium without antibiotics. Isolates were identified by a modified pulsed-field gel electrophoresis (PFGE) protocol (Matthias MA and others, unpublished data) and microscopic agglutination testing (MAT) with reference antisera.

Analysis of bat kidneys for leptospiral infection by PCR amplification. The DNA for the PCR was extracted using the Qiaqen DNeasy® tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s directions. Five microliters of purified DNA was amplified using the G1/G2 primer pair and conditions as described by Gravekamp and others. The cycling protocol included an initial denaturation step at 94°C for 5 minutes, followed by 34 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes. The PCR products were visualized after electrophoresis on ethidium bromide–stained 2% agarose gels.

Statistical analyses. Differences in the frequency of renal carriage were investigated using generalized log-linear analysis as implemented in SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL). Goodness-of-fit (G²) and chi-square tests in multi-dimensional contingency tables were used to determine complete and partial independence among the variables, PCR result, location, habitat, sex, age, and genus. Complete independence of variables was assessed using a saturated model, which included all four-, three-, and two-way associations. Due to computational limitations, only five-way associations (PCR result and all permutations of four of the other five variables) were evaluated. Subsequent analyses were designed to fit the most parsimonious model to the data, i.e., the model with the fewest number of variables (and their interactions) that could explain the distribution of the data. All four-way associations were evaluated. Models that were significantly different from the saturated model were rejected. Significance of all statistical tests was determined at α = 0.05.

16S rRNA gene amplification and sequencing. Total genomic DNA was extracted from cultures of isolates MMD1493 and MMD1562, containing 2 × 10⁶ leptospires/mL using the QIAamp DNA extraction kit according to manufacturer’s directions (Qiagen). The PCR amplification was performed as described previously, using the 16S rDNA primers fD1/rD1. In addition, 16S rDNA was amplified from kidney extracts initially positive by G1/G2 PCR using a multiplex PCR procedure. After an initial round of amplification using primers rD1 and fD1, PCR products were diluted 1:10³ with sterile double-distilled water and subjected to a second round of amplification using the nested primers lepto16S11f (5’-GGC GGC GCG TCT TAA ACA TGC-3’), and lepto16S1338r (5’-TGG GTA CAA ACA GGT CCG GGA AC-3’). A reaction volume of 25 µL was used for all amplifications and consisted of 12.5 µL of HotStarTaq® mastermix (Qiagen), 5 µL of Q solution (Qiagen), 0.2 µM of each primer, and 0.1 units of Pfu DNA polymerase (Qiagen). The first cycle consisted of incubation at 95°C for 15 minutes for enzyme activation and was followed by 34 cycles at 94°C for 10 seconds, 64°C for 1 minute, and at 68°C for 2 minutes. The PCR products were purified after electrophoresis from 1.0% agarose gels in Tris-acetate-EDTA buffer using the GENECLEAN® II gel extraction kit (Oxigene, Irvine, CA) according to manufacturer’s directions, then cloned into the pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA). Clones were screened for leptosporal 16S rDNA inserts using a real time PCR procedure, then cycle-sequenced. Sequencing was performed on an ABI 3100 automated sequencer (Perkin Elmer, Wellesley, MA) using three forward primers: lepto16S11f, lepto16S505f (5’-TCA TTG GGC GTA AAG GGT G-3’), and lepto16S1006f (5’-TCA GCT CGT GTC GTG AGA-3’), and the reverse primer lepto16S1338r. Real-time PCR conditions were according to manufacturer’s (Qiagen) directions.

Phylogenetic analyses. The 16S rRNA gene sequences were aligned with those of published leptosporal 16S rRNA gene sequences (Table 1) available in GenBank using CLUSTAL W (version 1.83; http://www.ebi.ac.uk/clustalw/) with default parameters. Alignments were optimized manually using a multiple sequence alignment editor BioEdit version 7 (Tom Hall, http://www.mbio.ncsu.edu/BioEdit/)
TABLE 1
GenBank accession numbers of leptospiral 16S ribosomal RNA gene sequences used in this study

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<th>Species</th>
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<th>Accession no.</th>
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</thead>
<tbody>
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<td>Pathogens</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Ballum</td>
</tr>
<tr>
<td></td>
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<td>Hardjo/harbovii</td>
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<td></td>
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<td>L. interrogans</td>
<td>Interohaemorrhagiae</td>
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<td></td>
<td></td>
<td>Canico</td>
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<tr>
<td></td>
<td>L. kirschneri</td>
<td>Cynopteri</td>
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<tr>
<td></td>
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<td>Cynopteri</td>
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<td>Codice</td>
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<tr>
<td></td>
<td>Leptonema illini</td>
<td>Illini</td>
</tr>
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</table>

Bioedit.html): gaps were treated as missing data. Homogeneity of base frequencies across taxa was tested using algorithms implemented in PAUP* version 4.0b10 (Swofford DL, 2003. PAUP*, Phylogenetic Analysis Using Parsimony and other Methods. Version 4. Sinauer Associates, Sunderland, MA). The suitability of 56 nucleotide substitution models was evaluated using PAUP* in conjunction with Modeltest version 3.6, as described previously.

Phylogenetic trees were constructed using the program MrBayes version 3.0b4 adopting the General Time Reversible Model with gamma distributed rates and invariant sites (GTR + I + G) of nucleotide substitution. The Metropolis-Coupled Markov Chain Monte Carlo (MCMC) algorithm running four chains was used to estimate posterior probabilities of the phylogenetic model. The analysis was adapted from that of Miller and others and used uniform prior distributions ranging from 0 to 1 for the shape parameter of the gamma (γ) distribution, and from 0 to 0.8 for the proportion of invariable sites (I). Branch lengths were inferred from a uniform exponential prior distribution. A flat prior was used for the topology and a Dirichlet distribution was used for base frequencies (0.5, 0.5, 0.5, 0.5) and rate matrix (0.5, 0.5, 0.5, 0.5, 0.5, 0.5). Unique random starting trees were used. Trees were sampled from the MCMC analysis every 100 generations to increase independence of samples. The first 100,000 generations were discarded as burn-in to ensure sampling of the chain at stationarity. Convergence of the Markov Chains was verified by comparing variation in the posterior probabilities of each clade among 5 replicates of 3,000,000 generations and another 5 replicates of 5,000,000 generations. Convergence was indicated when this variation was ≤ 3% at each node. The 16S rRNA sequence of Leptonema illini (GenBank/ European Molecular Biology Laboratory/DNA Data Bank of Japan accession no. AY714984) was used as an outgroup to establish the root of the tree. Bayesian inference of phylogeny is still developmental, and certain aspects of these analyses are under investigation.

Consequently, weighted parsimony analysis was used to corroborate tree reconstructions inferred by the Bayesian phylogenetic approach. Maximum parsimony analysis was performed using PAUP*, and the nucleotide substitution rates were estimated by the Bayesian approach. Heuristic searches with tree bisection-reconnection swapping, and MULTREES options were conducted using 10 random-addition replicates; five trees were held per replicate. Branch support was estimated using 1,000 bootstrapped pseudoreplicates.

**Model selection.** Modeltest version 3.6 was used to evaluate the suitability of 56 nucleotide substitution models using corrected Akaikes information criteria. Briefly, a batch process was passed to PAUP* to generate a matrix of likelihoods for each of 56 nucleotide substitution models that was analyzed subsequently in Modeltest. The general time reversible model with gamma distribution and invariable sites (GTR + I + G) was the suggested model and was adopted for all phylogenetic reconstructions.

**GenBank accession numbers.** Previously unpublished sequences have been submitted to GenBank and assigned accession numbers AY995712 to AY995730 (Table 2).

**RESULTS**

**Animal data.** A total of 3,510 bats (60 species, 33 genera, 6 families) were captured; 2,237 were analyzed (2,077 kidneys cultured) and 1,273 were released in accordance with permit restrictions. Of these, culture and PCR data were available for 589 bats. Two frugivorous genera, Artibeus (141, 23.9%) and Carollia (172, 29.2%) were captured most often (Table 3) and were ubiquitous with respect to habitat associations. In general, bats were obtained more frequently in forested (mature or secondary growth) habitats than elsewhere, and were most abundant near the peri-Iquitos villages of Moralillo and Pena Negra. Females (49.2%) were caught as often as males (50.8%), and most (77.6%) individuals were sexually mature.

**Renal carriage.** Evidence of leptospiral infection (by PCR or culture) was found in the kidneys of 20 (3.4%) bats representing 12 genera (Table 2). Except for a single vespertilionid (Myotis riparius) and a single molosid (Promops nasutus), all species with leptospiral infection were from the family Phyllostomidae. Isolates were recovered from kidneys of only three species: Phyllostomus hastatus, Miniom crenulatum, and Promops nasutus; each was caught at a different location. All other bat kidney specimens were positive for leptospiral infection only by PCR (Table 2). One isolate (MMD2461) was lost upon subculture; of the 2 remaining isolates, typing by MAT and PFGE showed that one was L. interrogans serovar
The rate of renal infection differed among bat genera. Renal carriage was most common in species of three genera Promops, Desmodus, and Myotis, of which 100% (1 of 1), 50% (1 of 2), and 33% (1 of 3), respectively, were positive by PCR (Table 4). None of the 14 collected juvenile specimens were PCR positive. Renal carriage in adults was four times more frequent than in non-sexually mature specimens (Table 5). Leptospiral positivity differed with location and habitat; however, small sample sizes precluded more powerful statistical analysis (Table 5). At sites where more than one specimen was captured, renal infection was most common (9 of 166; 5.4%) in the more rural area of Varillal and lowest (0 of 81) in Zungarococha, where more forest destruction and human development are present (Table 5). Twelve (7%) of 181

TABLE 3
Generic distribution of 589 bats caught and analyzed by polymerase chain reaction or culture methods

<table>
<thead>
<tr>
<th>Genus</th>
<th>No. collected</th>
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<td>Artibeus (Dermanura)</td>
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</tr>
<tr>
<td>Vampyressa</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>589</td>
</tr>
</tbody>
</table>

however, small sample sizes precluded more powerful statistical analysis (Table 5). At sites where more than one specimen was captured, renal infection was most common (9 of 166; 5.4%) in the more rural area of Varillal and lowest (0 of 81) in Zungarococha, where more forest destruction and human development are present (Table 5). Twelve (7%) of 181

TABLE 4
Frequency of positive polymerase chain reactions by bat species (does not include data from genera for which no specimens were positive)

<table>
<thead>
<tr>
<th>Species</th>
<th>No. collected</th>
<th>No. (%) positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artibeus jamaicensis</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Artibeus lituratus</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Artibeus obscurus</td>
<td>39</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Artibeus planirostris</td>
<td>70</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Carollia brevicauda</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>Carollia castanea</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Carollia perspicillata</td>
<td>99</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Carollia sp.</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Desmodus rotundus</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Glossophaga soricina</td>
<td>21</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Glossophaga sp.</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lonchophylla thomasi</td>
<td>11</td>
<td>2 (18)</td>
</tr>
<tr>
<td>Minon crenulatum</td>
<td>17</td>
<td>2 (12)</td>
</tr>
<tr>
<td>Myotis nigricans</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Myotis riparius</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Phyllostomus discolor</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Phyllostomus elongatus</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Phyllostomus hastatus</td>
<td>29</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Promops nasutus</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Rhinophylla fischerae</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Rhinophylla pumilio</td>
<td>27</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Sturnira lilium</td>
<td>38</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Sturnira magnia</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sturnira tildae</td>
<td>10</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Sturnira sp.</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Uroderma bilobatum</td>
<td>11</td>
<td>2 (18)</td>
</tr>
<tr>
<td>Uroderma magniostrum</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

* All specimens were collected in Iquitos between December 2002 and February 2004.
† B = mature forest, A = second growth forest, C = agricultural land, D = urban.
Two five-sample replicates were used, and for 3,000,000 generations the estimate of tree topology was consistent for all replicates. In addition, variation in the posterior probability at each node was < 3%. Similarly, replicates run for 5,000,000 generations converged on a single tree topology, and variation at all nodes was < 3%. Taken together, these results indicate that 3,000,000 generations were sufficient for convergence.

Posterior clade probabilities were estimated from the 50% majority rule consensus of 29,000 trees. The overall tree topology was supported strongly because most nodes had posterior probabilities >70% (Figure 1). More than 30% of the clades received 100% support, and approximately half of the clades were found in at least 95% of the sampled trees.

Maximum parsimony analysis. Of the 344 variable sites, 185 were parsimony informative. Uninformative sites (1,075) were excluded from analysis. Weighted parsimony analysis recovered 180 equally parsimonious trees of length 359 (consistency index = 0.719, retention index = 0.948). The strict consensus of the 180 most parsimonious trees (Figure 2) resulted in a combination of both well and poorly resolved clades.

Branch support was evaluated using 1,000 bootstrap replicates. Most clades were well supported with > 80% bootstrap probabilities (Figure 2). Maximum parsimony analysis resulted in five polytomy, including two large polytomies within the pathogenic strains (Figure 2).

Phylogenetic relationships. The Bayesian approach (Figure 1) produced a tree similar to that based on parsimony analysis (Figure 2), and was able to resolve a more structured tree with fewer polytomies. Discrepancies between the two reconstructions were few (Figure 2).

On the basis of phylogenetic analyses, the leptospiral 16S sequences occur in three monophyletic groups that represent pathogenic (cluster A), intermediate (B), and saprophytic (C) strains (Figure 1). All sequences from this study clustered within the pathogenic group, except for MMD110, which clustered with intermediate strains. Sequences were distributed evenly among several subgroup lineages (L. interrogans, L. kirschneri, and L. borgpetersenii), reflecting heterogeneity among clones (Figure 1).

Cluster A could be resolved into three subgroups (Figure 1). Subgroup 1 contained sequences derived from leptospiral genomospecies 1: L. noguchii, L. kirschneri, L. meyeri, and L. interrogans; subgroup 2 contained L. alexanderi, L. borgpetersenii, and L. weillii; and subgroup 3 contained only sequences derived from strains belonging to L. santarosai.

Eleven and seven of the unknown sequences clustered within subgroup 2 and 3, respectively. Clones MMD1234, 1235, and 1236 (99.6% sequence similarity), and MMD965, 1239, 1351, 1361, and 2568 (99.4% sequence similarity) formed separate monophyletic groups that may represent novel species. All others clustered closely with sequences from known leptospiral species. The largest clade included five clones identified in the present study and published sequences derived from L. interrogans. Four subgroups formed a poorly resolved monophyletic group with L. borgpetersenii. Cluster C also contained two subgroups. In subgroup 1, L. meyeri, clustered with sequences from L. wolbachii and leptospiral genomospecies 5, whereas subgroup 2 comprised leptospiral genomospecies 4, leptospiral genomospecies 3, L. biflexa, and L. wolbachii.

Unknown sequences did not cluster with any particular bat species (Figure 1). Moreover, leptospiral 16S ribosomal sequences were distributed among the sampled locations except samples MMD1234, MMD1235, and MMD1236, which were obtained near the rural, peri-Iquitos village of Varillal.

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**Table 5**

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. collected</th>
<th>No. (%) positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iquitos</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td>La Habana</td>
<td>75</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Moralillo</td>
<td>124</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Peña Negra</td>
<td>142</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Varilal</td>
<td>166</td>
<td>9 (5)</td>
</tr>
<tr>
<td>Zagarrococha</td>
<td>81</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Location of collection for the leptospiral 16S rDNA gene sequences.*

Bats in mature forests were PCR positive compared with 7 (2%) of 404 from agricultural land or secondary growth forests combined (Table 5) ($\chi^2 = 8.53$, degrees of freedom $[df] = 1, P = 0.004$).

General loglinear analysis for complete independence showed that the variables were not independent ($\chi^2 = 3 \times 10^2$, $df = 23,012, P < 0.0001$). Further statistical analyses showed that the most parsimonious model included the four-way association of the variables PCR result, habitat, location, and species ($G^2 = 2,261.61, df = 22,745, P = 1.000$ and $\chi^2 = 11,055.26, df = 22,745, P = 1.000$).

Phylogeny of the leptospiral 16S rRNA gene sequences. Of the 20 specimens positive by G1/G2 PCR, 19 were analyzed further (kidney from specimen MMD2461 was not available). Nested 16S rDNA gene PCR products of approximately 1,252 basepairs were sequenced. These sequences were aligned with 16S rDNA sequences from 38 leptospiral reference strains retrieved from GenBank. Sequence length ranged from 1,238 to 1,253 basepairs (average = 1,249.0), with an average GC content of 52.1% (range = 50.8–52.9%) and an average pairwise sequence similarity of 95.5%. Five hundred sixty-two gaps were inserted to align the 58 sequences, producing a matrix of 1260 characters. Bayesian analyses used the complete alignment whereas maximum parsimony analysis was based only on phylogenetically informative sites.

Bayesian inference of phylogeny. Two five-sample replicates, the first running for 3,000,000 generations and the second for 5,000,000 generations, were used to assess convergence. For replicates run for 3,000,000 generations, the estimate of tree topology was consistent for all replicates. In addition, variation in the posterior probability at each node was < 3%. Similarly, replicates run for 5,000,000 generations...
DISCUSSION

We report a variety of pathogenic and intermediate *Leptospira*, including two putative new species, found associated with chronic renal colonization of bats in the Peruvian Amazon. Bat-associated leptospires are genetically diverse based on phylogenetic analysis of 16S rDNA sequences. These data are of substantial epidemiologic significance, providing the basis for detailed molecular epidemiologic investigations of bat-associated leptospires as the source of human infection by pathogenic *Leptospira* in the Iquitos region, where leptospirosis is highly endemic. Although most of the Amazon remains intact, the rate of deforestation in the neotropics is alarming and results in reducing forested areas, increasing habitat fragmentation, and subjecting natural assemblages to intrusion by humans along with their commensal species (i.e., horses, dogs, cattle, rats). Such ecologic changes promote the emergence of infectious diseases by placing humans into contact with novel reservoirs or infectious agents. Because bats respond to habitat modification, loss, and fragmentation at the level of populations and communities, their spatial and temporal dynamics are particularly sensitive to anthropogenic activity. The consequences of such altered spatial and temporal dynamics to the...
ecology of leptospirosis and the likelihood of disease transmission to humans is known poorly. However, anthropogenic activity in previously undisturbed areas could directly or indirectly increase the risk of infection by new serovars.

The role of bats in leptospiral transmission was not assessed here for humans. Evidence of renal infection was found in 20 (3.4%) of 589 bats, a rate lower than that in previous reports from the same area. In the previous study, 35% (7 of 20) of the bats were PCR positive as assessed by G1/G2 PCR. However, since only six bat species were evaluated, rates of renal infection might have been biased and artificially high. Unlike a previous report, our data derive from animals prospec-

**Figure 2.** Bootstrap 50% majority rule consensus tree generated by weighted parsimony analysis of 1,000 pseudoreplicates. Bootstrap proportions appear above selected nodes. Highlighted (bold type) clones were detected in bats caught in Iquitos.
tively collected over an extended period of time at several sites in and around Iquitos. As such, our results likely repre-
sent a more accurate estimate of the rate of leptospiral infec-
tion of bats in proximity to human populations. In Australia, Smythe and others found that 28% of flying foxes had anti-
bodies to leptospira. However, these investigators did not iso-
late the organism or assess the frequency of renal carriage
among these potential mammalian reservoirs. Serologic
analysis indicates only previous exposure to Leptospira, but
not the presence of renal infection. Antibodies to Leptospira
cannot precisely identify infecting leptospires (particularly a
novel leptospire). Therefore, this previous serologic study of
flying foxes does not indicate whether these megachiropter-
ans could have been reservoirs for leptospiral transmissions.
Because of the limitations of serologic analysis, we did not
determine the presence of antibodies to Leptospira in the
present study. It is well known that many leptospiral strains
are fastidious in culture or difficult to culture. It is also pos-
sible that PCR analysis may miss some infections both for
technical reasons (complexity of a DNA extract from kidney)
or because we used only the G1/G2 primer set, which may
fail to detect all leptospiral species. Generally, our results
should be interpreted as an underestimation of renal coloni-
zation of bats in the Peruvian Amazon.

The frequency of renal infection differed among bat species
(even among congeners), suggesting that some species may be
better suited to carry leptospires than are others. Variation in
the frequency of renal carriage among sister taxa has been
found in other mammals. In Hawaii, for instance, Rattus
norvegicus is a more significant carrier and disseminator of
leptospires than is either R. rattus or R. exulans. In that study,
more than 61% of the R. norvegicus yielded isolates, whereas
only 17.8% of R. exulans were culture positive. In that study,
renal carriage was associated strongly with rat population
density. High population densities facilitate transmission;
non-gregarious species that infrequently come into contact
with conspecifics should transmit leptospires between them-
selves infrequently. This is not true for bats in the genus
Carollia, which have low infection rates yet are gregari-
ous.

Environmental factors also could contribute to observed
leptospiral transmission and infection rates. In the present
study, statistical analyses have shown that the variables habi-
tat, location and species are all inter-related in affecting PCR
positivity. It is therefore difficult to determine how each fac-
tor influences the rate of leptospiral positivity in bats of Iq-
uitos. Nonetheless, bats collected from mature forests were
significantly more likely to carry leptospires than were bats
collected in areas associated with human activity. This asso-
ciation could be due in part to differences in species compo-
sition with respect to habitat or location.

In Iquitos, we isolated leptospires from the kidneys of three
bat species: P. hastatus, M. crenulatum, and P. nasutus. The
PCR evidence of infection with identification of the leptospiri-
ral species was determined in an additional 17 bats. This re-
port is the first to describe the isolation and identification of
leptospires from these bat species. Considering the number of
positive PCR reactions, the isolation rate was low. Lepto-
spires are fastidious organisms that are not easily grown in
culture media, although some leptospires grow more easily
than do others. Many formulations containing serum have
been described that appear to improve growth in primary
culture, and standard recommendations are to use more than
one type of medium for primary isolation of leptospires from
clinical specimens or tissues. However, because of logistic
concerns, we could only use one type of leptospiral culture
medium, and chose to use Ellinghausen-McCullough-
Johnson-Harris (EMJH) medium, which does not contain se-
rum. Medium supplemented with serum might have yielded a
higher and more accurate rate of isolation.

Despite the low infection rates in bat populations, bats may
be an important link in the transmission cycle of leptospirosis
in Iquitos. Bats forage in fruit orchards and forest clearings
created by human activities, and roost in buildings (under tiles or in attics), water cisterns, culverts, abandoned struc-
tures, and bridges. Although speculation, inhabitants of ru-
rnal villages may contact soil or surface waters contaminated
with bat urine (e.g., when harvesting wood in forest to make
charcoal). In contributing to a sylvatic cycle of leptospiral
transmission, ground-dwelling species such as rodents or mar-
supials that reside or forage under bat roosts could encounter
Leptospira-contaminated urine. Such a consideration leads to
the testable hypothesis that leptospires could be maintained
by a bat-rodent or bat-marsupial transmission cycle. Molecu-
lar and ecologic approaches similar to those in the present
study could assess this contention.

Traditional serologic (phenotypic) identification of lepto-
spires is difficult and often does not reflect genetic related-
ness. Molecular approaches based on DNA-DNA hybrid-
ization have been described but are time-consuming and
require the analysis of large numbers of bacteria in pure
culture. Since the late 1980s, rDNA genes universally have
used to define phylogenetic relationships among bacteria. The
application of phylogenetic analysis to the classification of
Leptospira showed that 16S rDNA analysis was consistent
with results obtained by DNA-DNA hybridization and dis-
tinguished strains at the species level. More recently, phy-
genetic reconstructions also have been used to identify
novel leptospiral strains. The 16S rDNA analysis has the
major advantage of not requiring an isolate to enable the
analysis.

The application of Bayesian analyses to phylogenetic stud-
ies is relatively new, but has generated considerable excite-
ment. Bayesian inferences of phylogeny produce a tree esti-
mate with quantified support for each node. As do maximum
likelihood analyses, Bayesian approaches fully capture phy-
genetic relationships under a given nucleotide substitution
model, but require considerably less computation time, oth-
erwise a serious constraint in phylogenetic reconstructions.
Other methods, such as neighbor-joining (NJ) and maximum
parsimony analysis, although faster, do not fully use all infor-
mation in a particular dataset. Bayesian phylogenetic anal-
ysis was used previously to describe the evolutionary relation-
ships among known species of Leptospira using 16S rDNA
sequences, lipL32, lipL41, and ompL1 sequences. Inferred tree topologies
were well resolved and consistent with trees generated
using NJ and unweighted pair group method with arithmetic
mean (UPGMA) tree-generating algorithms, indicating that
the method could be applied to leptospiral phylogeny.

Our phylogenetic inferences were based on leptospiral 16S
rDNA sequences amplified directly from kidney. Tree topol-
ogy was consistent with published reports. Three large
clusters consistent with groupings based on DNA-DNA hy-
bridization studies were generated. Because of the high de-
gree of similarity between sequences, some terminal taxa were not well resolved, particularly the *L. kirschneri* and *L. interrogans* strains. *Leptospira interrogans* formed a polytamous in-group within the *L. kirschneri* cluster. Although other investigators have reported similar branching patterns, they were able to resolve all terminal branches (no polytomies). The differences with our results are unclear. In our analyses, the overall sequence similarity of closely related (clustered) terminal taxa may have been higher than in previous studies. Phylogenetic analysis of leptospiral 16S rDNA genes often fails to resolve sequences of more than 97% similarity. Because of less resolving power of 16S rDNA sequence analysis at this level of similarity, Stackebrandt and Goebel suggested confirming conclusions based on 16S rDNA sequence analysis with DNA-DNA hybridization studies. Haake and others also concluded that none of the four genes that they evaluated adequately resolved leptospiral species, and suggested that simultaneous analysis of multiple genes would improve resolution. Indeed, current strategies explore the possibility of using partitioned datasets: with DNA sequences from 1) more than one gene, 2) a single gene partitioned by codon position (protein coding genes), 3) stemloop secondary structure (structural RNA genes), or 4) mixed partitions including both DNA and protein sequences.

We used two approaches to investigate the phylogenetic relationship of leptospiral 16S rDNA sequences: weighted parsimony and Bayesian inference. Overall tree topology was similar; however some clades, notably the *L. interrogans* and *L. kirschneri* strains, were less resolved by parsimony analysis. The disparity of results between the two methods highlights the unique approach that each uses when inferring phylogeny. Maximum parsimony does not adequately account for pleisiomorphy (convergent evolution), but rather assumes that common states in terminal taxa were inherited directly from a common ancestor. Bayesian analyses can implement complex nucleotide substitution models that better account for these invisible changes. It is currently being debated which approach better approximates the true tree. Maximum parsimony may perform better than either maximum likelihood or Bayesian analyses when branch lengths are between 0.15 and 0.35, but the latter generate better results when branch lengths are longer. However, with our dataset, Bayesian analysis proved superior: a higher proportion of poorly resolved clades were apparent when using weighted parsimony analysis as compared with Bayesian analysis.

Based on phylogenetic analysis of 16S rDNA sequences, bats in Iquitos maintain a genetically diverse group of leptospires. This is not surprising considering the number of different bat species in the region. Most strains belong to the *L. kirschneri* (1) or *L. interrogans* (5) lineage. Both *L. kirschneri* (serovar Cynopteri) and *L. interrogans* (serovar Schueffneri) previously have been isolated from bats (Serovar Database; National Veterinary Science Laboratory, Ames, IA). *Leptospira interrogans* serovar Icterohaemorrhagiae typically is maintained by *Rattus* spp., which was confirmed by our observations in the urban slum and market area of Belen, Iquitos, where we found that peridomestic rats (*R. norvegicus* and *R. rattus*) frequently carry serovar Icterohaemorrhagiae (between 40% and 50%, unpublished data). Of the five *L. interrogans* clones, one (serovar Icterohaemorrhagiae) was isolated from a bat caught in urban surroundings and was typed as serovar Icterohaemorrhagiae. In addition, four strains showed strong 16S rDNA sequence similarity to *L. borgpetersenii*, which has been isolated primarily from humans and peridomestic rodents. These results suggest that the transmission of leptospires from peridomestic rodents to bats could occur in Iquitos. The only other *L. interrogans* serovar isolated in Peru is serovar Canicola, which is typically associated with dogs. *Leptospira interrogans* serovars cause most severe human infections in Iquitos (Cunningham C, unpublished data); thus, bats may contribute to leptospiral transmission to man. To our knowledge, *L. borgpetersenii* has not been previously detected in bats.

Our data also indicate the possible existence of two new leptospiral species maintained by bats in Iquitos. The first included three clones in bats collected in Varillal, a village near Iquitos, which clustered most closely with *L. alexanderi*, a strain isolated from humans in China. The second consisted of five clones that clustered closely with *L. interrogans* and *L. kirschneri*, but formed a separate monophyletic group. A maintenance host of *L. alexanderi* has yet to be identified.

The remaining sequence clustered with the intermediate strains, and was almost identical to *L. fainei*. *Leptospira fainei* has been isolated from pigs in Australia and humans in Europe. In Australia, serologic evidence of *L. fainei* infection in humans has also been reported. The present report is the first demonstration that a wild, as opposed to a domestic, animal has a kidney infection with *L. fainei*. An rDNA sequence identical to MMD1100 was detected repeatedly in environmental water samples collected in and around Iquitos (Qanoza CA, unpublished data). Consequently, human exposure to and infection by a leptospiral strain similar to MMD1100 is quite likely.

We identified a number of potential bat reservoirs of leptospires in Iquitos, and highlighted the genetic diversity of bat-associated leptospires, including two undescribed leptospiral species. To understand more fully the role of bats in the maintenance and transmission of leptospires in Iquitos, the relationship of bats with their abiotic environment and with other mammal reservoirs with which they may come into contact needs to be clarified so that mechanisms of transmission and persistence of renal infection can be determined in an ecologic context. Studies to more precisely delineate the transmission of bat-associated leptospires to humans and to peridomestic and domestic animals are needed; this study forms an important starting point for that initiative.

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Authors’ addresses: Michael A. Matthias and Joseph M. Vinetz, Division of Infectious Diseases, Department of Medicine, University of...
California, San Diego School of Medicine, 9500 Gilman Drive, Mail Code 0640, La Jolla, CA, 92039-0640, Telephone: 858-534-6000, Fax: 858-534-6020, E-mails: mmmathia@ucsd.edu and jnvnetz@ucsd.edu. M. Mónica Díaz, Laboratory of Emerging Infectious Diseases, Morona 448, Iquitos, Peru. Telephone: 51-65-242-226, E-mail: mmonicadiaz@arent.net.ar. Michael R. Willig, Ecology Program, Department of Biological Science and Museum, Texas Tech University, Lubbock, TX 79409-3131, Telephone: 703-292-7197, Fax: 703-292-9064, E-mail: michael.willig@ttu.edu. Kaila J. Campos, Mariza Calderon, and Eduardo Gotuzzo, Universidad Peruana Cayetano Heredia, Correo Apartado 4314, Lima 300, Peru, Telephone: 51-1-482-3903, E-mail: kaila_cg@hotmail.com, mmcalderons@yahoo.es, and egh@upch.pe. Victor Pacheco, Museo de Historia Natural, Apartado 140434, Lima 14, Peru, E-mail: vpachecot@ummsm.edu.pe. Robert H. Gilman, Department of International Health, Johns Hop-kins Bloomberg School of Public Health, 615 North Wolfe Street, Room W5515, Baltimore, MD 21205, Telephone: 410-955-4195, E-mail: gilmanbob@yahoo.com.

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