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Year 2005 *Paper* 1451

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Michael A Matthias, M Mónica Díaz, Kalina J Campos, Maritza Calderon, Michael R Willig, Victor Pacheco, Eduardo Gotuzzo, Robert H Gilman, and Joseph M Vinetz, "Diversity of bat-associated Leptospira in the Peruvian Amazon inferred by Bayesian phylogenetic analysis of 16S ribosomal DNA sequences" (2005). American Journal of Tropical Medicine and Hygiene. 73 (5), pp. 964-974. Postprint available free at: http://repositories.cdlib.org/postprints/1451

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Abstract

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Am. J. Trop. Med. Hyg., 73(5), 2005, pp. 964–974
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DIVERSITY OF BAT-ASSOCIATED *LEPTOSPIRA* IN THE PERUVIAN AMAZON INFERRED BY BAYESIAN PHYLOGENETIC ANALYSIS OF 16S RIBOSOMAL DNA SEQUENCES

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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 phylogenic 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. kirschenri* 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. interrogans* 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.

Leptospiral diversity is reflected by the number of different wild and domestic animals that serve as reservoir hosts. More than 200 antigenic variants (serovars) have been described.[1] In the Peruvian Amazon, the most species-rich order of mammals is bats, with more than 150 species in 74 genera (Díaz MM, and Willig MR, unpublished data).[15] The high species richness of bats in the Peruvian Amazon suggests that these mammals might harbor an equally diverse group of leptospires.

Currently, the genus *Leptospira* is subdivided into 17 genomospecies, as shown by DNA-DNA hybridization studies.[16] Analysis of 16S rDNA gene sequences allows for the definitive delineation of *Leptospira* species. Previous studies have delineated three clades, but cannot differentiate serovars within a species.[17] The first comprises eight pathogenic species. A second intermediate group of unclear pathogenicity comprises *L. inadai* and *L. fainei*. The third contains only saprophytic serovars such as *L. biflexa*. Phylogenetic analysis of the leptospiral 16S rDNA gene is consistent with the classification of leptospires based on DNA-DNA hybridization.[18]

The purpose of this study was to determine the frequency of kidney infection in bats in Iquitos, to provide insight into the ecologic characteristics of the bats in relation to leptospiral colonization, and to describe the genetic diversity of the leptospiral strains carried by these animals using phylogenetic analyses of polymerase chain reaction (PCR)–amplified 16S rDNA genes. These data will provide insight into the diversity of leptospires in a hotspot of biodiversity,[15,19,20] and establish

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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°4′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 reason 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.[21]

Analysis of bat kidneys for leptospiral infection by PCR amplification. The DNA for the PCR was extracted using the Qiagen 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.[22] 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 (G^2) 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 $\alpha = 0.05$.

16S rRNA gene amplification and sequencing. Total genomic DNA was extracted from cultures of isolates MMD1493 and MMD1562, containing 2×10^8 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.[23] 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 $1{:}10^3$ 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′-TGT GTA CAA 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 (Qbiogene, Irvine, CA) according to manufacturer's directions, then cloned into the pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA). Clones were screened for leptospiral 16S rDNA inserts using a real time PCR procedure,[24] 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 TG-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 leptospiral 16S rRNA gene sequences (Table 1) available in GenBank using CLUSTAL W (version 1.83; http://www.ebi.ac.uk/clustal/w/) 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

	Species	Serovar	Accession no.
Pathogens	*L. alexanderi*	Manhoa3	AY631880
		Ballum	AY631884
	L. borgpetersenii	Hardjo/harjobovis	U12670
		Balcanica	U12669
	L. genomospecies 1	Sicuani	AY631881
	L. interrogans	Icterohaemorrhagiae	AY631894
		Canicola	X17547
	L. kirschneri	Cynopteri	AY631895
		Cynopteri	Z21628
		Fortbragg	U12677
	L. noguchii	Panama	AY631886
		Panama	Z21635
		Shermani	AY631883
	L. santarosai	Atlantae	U12672
		Shermani	Z21649
		Celledoni	AY631877
		Ecochallenge	AY034037
	L. weilii	Sarmin	U12673
		Worsfold	U12677
		Celledoni	Z21637
Intermediate	*L. fainei*	Hurstbridge	AY631885
		Hurstbridge	Y19243
		Aguarana	AY631891
	L. inadai	Kaup	AY631887
		Lyme	AY631896
		Lyme	Z21634
Saprophytes	*L. biflexa*	Andamana	AY631893
		Patoc	AY631876
	L. genomospecies 3	Holland	AY631897
	L. genomospecies 4	Hualin	AY631888
	L. genomospecies 5	Saopaolo	AY631882
	L. meyeri	Ranarum	AY631878
		Samarang	AY631892
		Semaranga	AF167353
		Ranarum	Z21648
		Gent	AY631890
	L. wolbachii	Codice	AY631679
		Codice	Z21638
	Leptonema illini	Illini	AY714984

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.[25]

Phylogenetic trees were constructed using the program MrBayes version 3.0b4[26] adopting the General Time Reversible Model with gamma distributed rates and invariant sites (GTR + I + G) of nucleotide substitution.[27] The Metropolis-Coupled Markov Chain Monte Carlo (MCMC) algorithm running four chains was used to estimate posterior probabilities of the phylogenetic model.[26] The analysis was adapted from that of Miller and others[28] 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 $\leq 3\%$ 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.[29] 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 Akaike 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 Peña Negra. Females (49.2%) were caught as often as males (50.8%), and most (77.6%) indvididuals 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*, *Mimon 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

TABLE 2
Summary of polymerase chain reaction (PCR) or culture positive bats by species, location, habitat, age, and sex*

ID no.	Species	Location	Habitat†	Season	Date of collection	Age	Sex	Culture	PCR	Accession no.
MMD1220	*Artibeus obscurus*	Varillal	B	Wet	3/31/03	Adult	F	–	+	AY995727
MMD1235	*Artibeus planirostris*	Varillal	B	Wet	4/1/03	Adult	F	–	+	AY995719
MMD1236	*Aribeus planirostris*	Varillal	B	Wet	4/1/03	Adult	F	–	+	AY995718
MMD1231	*Carollia perspicillata*	Varillal	B	Wet	4/1/03	Adult	M	–	+	AY995716
MMD0990	*Desmodus rotundus*	Moralilo	A	Wet	2/7/03	Adult	F	–	+	AY995715
CPB2568	*Glossophaga soricina*	La Habana	A	Dry	11/18/02	Adult	F	–	+	AY995721
MMD1388	*Glossophaga soricina*	Peñe Negra	B	Wet	5/14/03	Adult	M	–	+	AY995726
MMD1234	*Lonchophylla thomasi*	Varillal	B	Wet	4/1/03	Adult	F	–	+	AY995717
MMD1239	*Lanchophylla thomasi*	Varillal	B	Wet	4/1/03	Adult	M	–	+	AY995724
MMD1351	*Mimon crenulatum*	Peña Negra	B	Wet	5/12/03	Adult	M	–	+	AY995722
MMD2461	*Mimon crenulatum*	Peña Negra	A	Dry	10/12/03	Adult	F	+	+	
MMD1361	*Myotis riparius*	Peña Negra	B	Wet	5/12/03	Adult	M	–	+	AY995723
MMD1493	*Phyllostomus hastatus*	Peña Negra	C	Wet	5/26/03	Adult	M	+	+	AY995730
MMD1562	*Promops nasutus*	Iquitos	D	Dry	6/6/03	Adult	M	+	+	AY995729
MMD0965	*Rhinophylla pumilio*	Moralillo	A	Wet	2/6/03	Adult	M	–	+	AY995720
MMD1221	*Rhinophylla pumilio*	Varillal	B	Wet	3/31/03	Subadult	M	–	+	AY995728
MMD0955	*Sturnira lilium*	Moralillo	B	Wet	2/4/03	Adult	F	–	+	AY995713
MMD1233	*Sturnira tildae*	Varillal	B	Wet	4/1/03	Adult	F	–	+	AY995714
CPB2650	*Uroderma bilobatum*	La Habana	C	Dry	11/23/02	Adult	M	–	+	AY995725
MMD1100	*Uroderma bilobatum*	Varillal	A	Wet	3/14/03	Adult	M	–	+	AY995712

* All specimens were collected in Iquitos between December 2002 and February 2004.
† B = mature forest; A = second growth forest, C = agricultural land; D = urban.

icterohaemorrhagiae and the other was *L. kirschneri* serovar *grippotyphosa*.

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

Genus	No. collected
Artibeus	141
Artibeus (Dermanura)	37
Carollia	172
Chiroderma	1
Cormura	2
Dermanura	2
Desmodus	2
Glossophaga	22
Lonchophylla	11
Lophostoma	8
Mesophylla	6
Mimon	17
Molossus	1
Myotis	3
Phyllostomus	35
Platyrrhinus	13
Promops	1
Rhinophilla	31
Sturnira	50
Thyroptera	1
Tonatia	7
Trachops	1
Trinycteris	1
Uroderma	21
Vampyressa	3
Total	589

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

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

TABLE 5
Frequency of positive polymerase chain reactions for leptospires in bats by location, sex, age, habitat, year, and season

Variable	No. collected	No. (%) positive
Location		
Iquitos	1	1 (100)
La Habana	75	2 (3)
Moralillo	124	3 (2)
Peña Negra	142	5 (4)
Varillal	166	9 (5)
Zungarococha	81	0
Sex		
Male	299	11 (4)
Female	290	9 (3)
Age		
Adult	457	19 (4)
Offspring	1	0
Juvenile	14	0
Not recorded	17	0
Subadult	100	1 (1)
Habitat		
Agricultural land/new forest	404	7 (2)
Intermediate	3	0
Mature	181	12 (7)
Urban/sub-urban	1	1 (100)
Year		
2002	156	2 (1)
2003	433	18 (4)
Season		
Dry	157	3 (2)
Wet	432	17 (4)

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 ($\chi^2 = 3 \times 10^7$, 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 introduced 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 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 polytomies, 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), but 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. interrrogans*; subgroup 2 contained *L. alexanderi*, *L. borgpetersenii*, and *L. weilii*; 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 sequences 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 leptopsiral genomospecies 5, whereas subgroup 2 comprised leptospiral genomospecies 4, leptospiral geneomospecies 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.

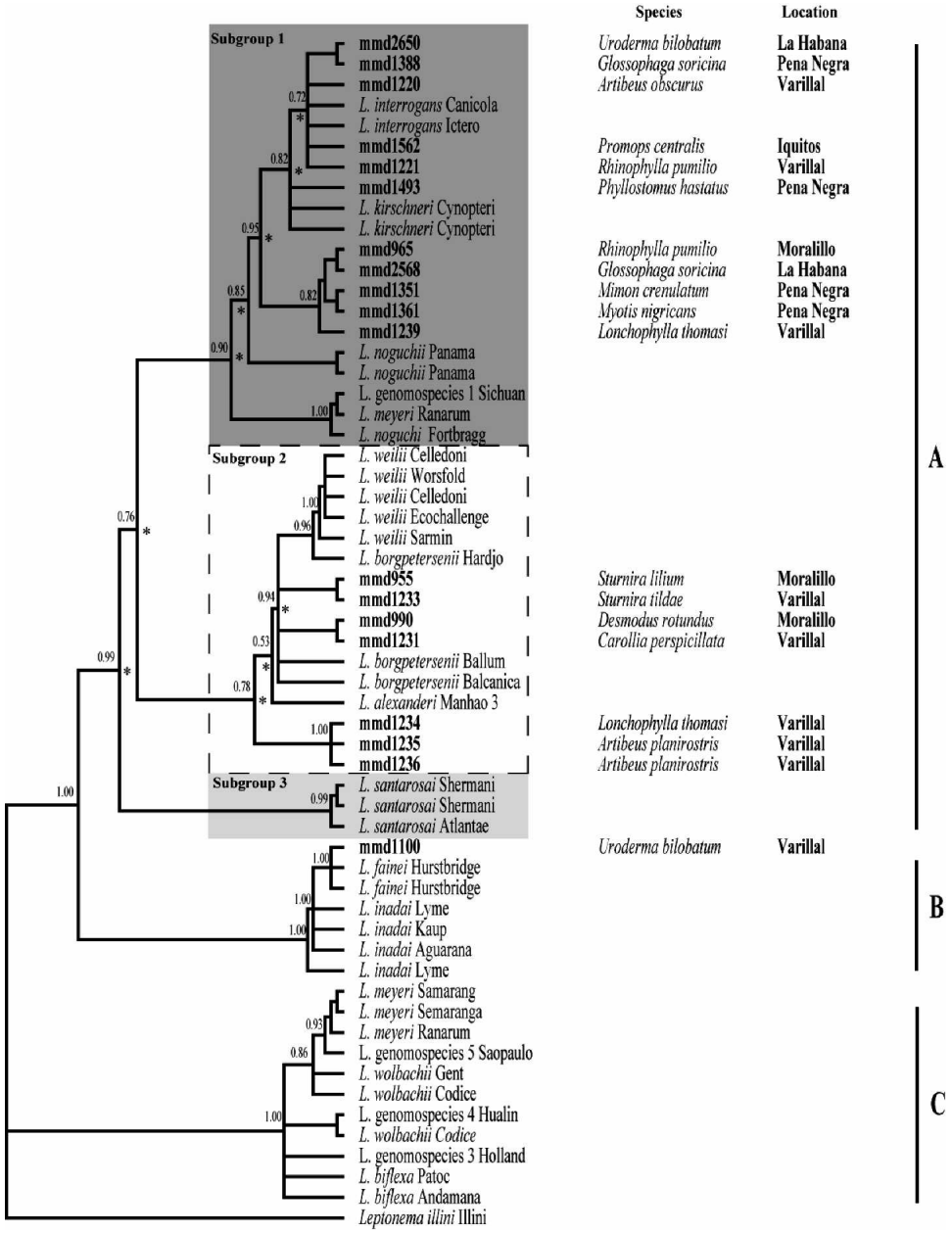

FIGURE 1. Phylogenetic tree based on 16S ribosomal DNA gene sequences using Bayesian analysis showing the 50% majority rule consensus of 29,000 trees from Markov Chain Monte Carlo sampling. Bayesian posterior probabilities are given above the node. Shaded boxes show individual subgroups: **A** = pathogenic strains; **B** = intermediate strains; and **C** = saprophytic strains. Nodes with less than 50% bootstrap support are indicated by an asterisk. Iquitos is the urbanized center of Iquitos. Peña Negra is a patch of mature forest surrounded by second-growth forest. Varillal is mature forest and second-growth forest. La Habana is second-growth forest and a cultivated area. Moralillo is mature and second-growth forest along the Iquitos-Nauta Highway. Highlighted (bold type) clones were detected in bats caught in Iquitos.

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.[30]

Although most of the Amazon remains intact,[31] the rate of deforestation in the neotropics is alarming[32] 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,[33] their spatial and temporal dynamics are particularly sensitive to anthropogenic activity.[34] The consequences of such altered spatial and temporal dynamics to the

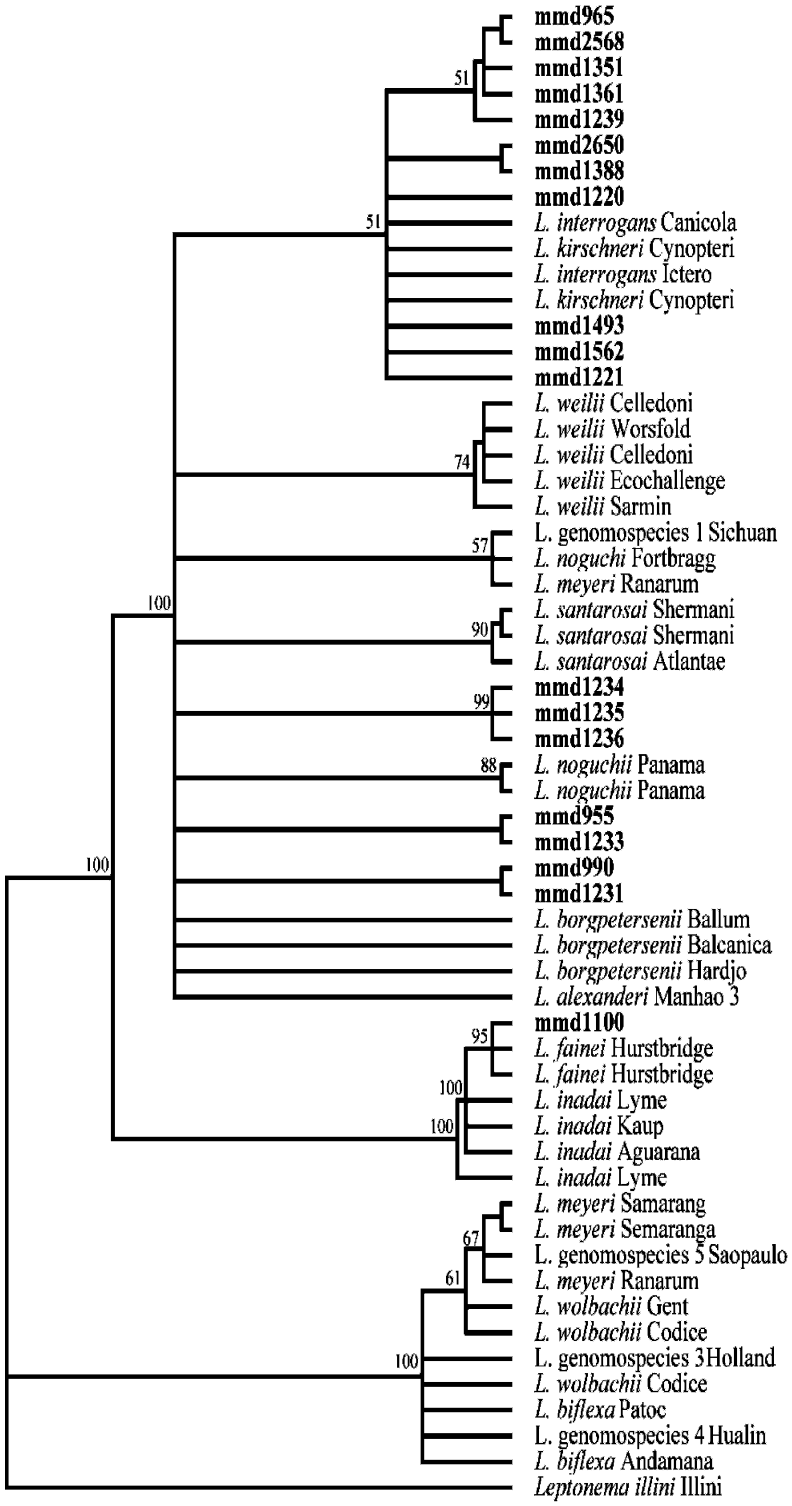

FIGURE 2. Bootstrap 50% majority rule consensus tree generated by weighted parsimony analysis of 1,000 pseudoreplicates. Bootstrap proportions appear above selected nodes. Hightlighted (bold type) clones were detected in bats caught in Iquitos.

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.[9] 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,[9] our data derive from animals prospec-

tively collected over an extended period of time at several sites in and around Iquitos. As such, our results likely represent a more accurate estimate of the rate of leptospiral infection of bats in proximity to human populations. In Australia, Smythe and others found that 28% of flying foxes had antibodies to leptospira.[13] However, these investigators did not isolate the organism or assess the frequency of renal carriage among these potential mammalian reservoirs.[13] 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 megachiropterans 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 possible 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,[9] which may fail to detect all leptospiral species.[22] Generally, our results should be interpreted as an underestimation of renal colonization 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.[35–37] 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.[36] High population densities facilitate transmission; non-gregarious species that infrequently come into contact with conspecifics should transmit leptospires between themselves infrequently. This is not true for bats in the genus *Carollia*, which have low infection rates yet are gregarious.[38,39]

Environmental factors also could contribute to observed leptospiral transmission and infection rates. In the present study, statistical analyses have shown that the variables habitat, location and species are all inter-related in affecting PCR positivity. It is therefore difficult to determine how each factor influences the rate of leptospiral positivity in bats of Iquitos. Nonetheless, bats collected from mature forests were significantly more likely to carry leptospires than were bats collected in areas associated with human activity. This association could be due in part to differences in species composition 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 leptospiral species was determined in an additional 17 bats. This report 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. Leptospires are fastidious organisms that are not easily grown in culture media, although some leptospires grow more easily than do others.[1,3] 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.[1] 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 serum. 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 structures, and bridges.[10] Although speculation, inhabitants of rural 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 marsupials 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. Molecular and ecologic approaches similar to those in the present study could assess this contention.

Traditional serologic (phenotypic) identification of leptospires is difficult and often does not reflect genetic relatedness.[1,3] Molecular approaches based on DNA-DNA hybridization have been described,[16,40,41] but are time-consuming and require the analysis of large numbers of bacteria in pure culture.[3] Since the late 1980s, rDNA genes universally have used to define phylogenetic relationships among bacteria.[42] 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 distinguished strains at the species level.[17,18] More recently, phylogenetic reconstructions also have been used to identify novel leptospiral strains.[43–45] The 16S rDNA analysis has the major advantage of not requiring an isolate to enable the analysis.

The application of Bayesian analyses to phylogenetic studies is relatively new, but has generated considerable excitement. Bayesian inferences of phylogeny produce a tree estimate with quantified support for each node. As do maximum likelihood analyses, Bayesian approaches fully capture phylogenetic relationships under a given nucleotide substitution model,[46] but require considerably less computation time, otherwise a serious constraint in phylogenetic reconstructions. Other methods, such as neighbor-joining (NJ) and maximum parsimony analysis, although faster, do not fully use all information in a particular dataset.[46] Bayesian phylogenetic analysis was used previously to describe the evolutionary relationships among known species of *Leptospira* using 16S rDNA *lipL32*, *lipL41*, and *ompL1* sequences.[47] 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,[18] indicating that the method could be applied to leptospiral phylogeny.[44,47]

Our phylogenetic inferences were based on leptospiral 16S rDNA sequences amplified directly from kidney. Tree topology was consistent with published reports.[18,47] Three large clusters consistent with groupings based on DNA-DNA hybridization 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 polytomous in-group within the *L. kirschneri* cluster. Although other investigators have reported similar branching patterns,[47] 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.[48] 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.[47] 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),[49] 3) stem-loop secondary structure (structural RNA genes),[49,50] or 4) mixed partitions including both DNA and protein sequences.[51]

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.[52] 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. interrrogans* serovar isolated in Peru is serovar Canicola, which is typically associated with dogs. *Leptospira interrrogans* 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[53] and humans in Europe.[43,44] In Australia, serologic evidence of *L. fainei* infection in humans has also been reported.[54] 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.

Received June 14, 2005. Accepted for publication July 22, 2005.

Acknowledgments: We thank the Instituto Nacional de Recursos Naturales and the Ministerio de Agricultura of Peru for permits to capture and collect specimens; the Universidad de la Amazonia Peruana for permission to conduct fieldwork on their property; Texas Tech University for additional support; Victor Linares, Sixto Mananita, Cesar Ahuanari, and students from the Museo de Historia Natural de San Marcos (Lima, Peru) for collaborations in the field; Fabiola Diaz and Henry Vega for laboratory assistance; and Christopher Bloch for bat kidney specimens.

Financial support: This work was supported by the United States Public Health Service, National Institutes of Health, Fogarty International Center (RO1 TW05860 and D43 TW007120). Ancillary support was provided by a research grant from the U.S. Public Health Service, National Institutes of Health, Fogarty International Center (RO1 AI49725).

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