DETECTION OF PATHOGENIC *LEPTOSPIRA* SPP. INFECTIONS AMONG MAMMALS CAPTURED IN THE PERUVIAN AMAZON BASIN REGION

JOSEPH E. BUNNELL, CHRISTINE L. HICE, DOUGLAS M. WATTS, VICTOR MONTRUEIL, ROBERT B. TESH, AND JOSEPH M. VINETZ

Department of Pathology, World Health Organization Collaborating Center for Tropical Diseases, University of Texas Medical Branch, Galveston, Texas; United States Naval Medical Research Center Detachment, Lima, Peru

Abstract. To identify potential zoonotic reservoirs of pathogenic leptospires in the Peruvian Amazon basin, wild mammals were trapped from July 1997 to December 1998 near the city of Iquitos. After extraction of nucleic acids from animal kidneys, DNA of pathogenic leptospires was identified by polymerase chain reaction (PCR) assays using one of two primer sets, one amplifying a region of the 23S rRNA gene, and the other amplifying a gene fragment specific for *Leptospira* spp (G1/G2 primers). Overall, 29% (40 of 136) of the mammals tested showed evidence of renal infection by *Leptospira* spp., including 20% (13 of 64) of the rodents, 39% (20 of 51) of the marsupials, and 35% (7 of 20) of the chiropterans (bats). Marsupials and chiropterans were implicated as more significant reservoir hosts of leptospires pathogenic to humans than previously recognized.

INTRODUCTION

Leptospirosis is a zoonosis with cosmopolitan distribution. The disease presents with protean clinical manifestations. It has recently been classified as a re-emerging disease, largely because of increased recognition and the recent rediscovery that it can present as a severe hemorrhagic illness, easily confused with some viral hemorrhagic fevers. The etiologic agents, *spirochetes* belonging to the genus *Leptospira*, are extracellular gram-negative bacteria whose natural life cycle depends on colonization of renal tubules of their vertebrate hosts and excretion via urine into the environment. The taxonomy of the genus *Leptospira* is confusing. Historically, antigenic characteristics based on serological test results were used to classify disease-causing leptospires. Later, when newer methods of classification based on nucleic acid technology and phylogenetic analysis became available, results of these methods were superimposed on the antigenic classification. Consequently, different species may be included in the same serovar, and different serovars may be recognized within a single species. Leptospires have a broad host range, and have been found in almost all mammals and in a variety of cold-blooded vertebrates. Human infection usually results from direct contact with an infected reservoir’s urine or water contaminated by it; the microorganism gains entrance to the human host via abrasions, wounds, or conjunctivae. Maintenance of leptospires in nature may require social contact among wild animals, since environmental contamination alone appears to be insufficient, at least for some arboreal mammals.

Leptospirosis has been recognized in Peru since 1917, and the seroprevalence of antibodies to *Leptospira* spp. in the Peruvian Amazon basin was reported in one study to be 58% (n = 426). However, leptospiral infection rates based on serology may underestimate the true prevalence because some animals are infected with multiple serovars and some hosts excrete spirochetes in their urine even after becoming seronegative.

It is clear that pathogenic *Leptospira* have a wide distribution among both feral and domesticated animals. A common misperception about leptospirosis is that the primary reservoir of the spirochete is rats. Such mistaken notions can have serious repercussions for the control of leptospirosis outbreaks, as occurred in the 1995 leptospirosis outbreak in Nicaragua. Despite suggestions that multiple serovars of leptospirosis were likely involved in the flood-related outbreak (including serovars not classically associated with *Rattus norvegicus* or *Rattus rattus*), control measures for that outbreak primarily aimed at killing domestic rats. The purpose of this study was to determine whether small mammals around Iquitos could be important reservoirs for introducing pathogenic leptospires into the environment.

MATERIALS AND METHODS

Mammal collections were made at the Allpahuayo Biological Station (S3°58’; W73°25’), a 3,000-hectare field station operated by the Instituto de Investigaciones de la Amazonia Peruana, located 25 km southwest of Iquitos in the Department of Loreto, in northeastern Peru (Figure 1). Iquitos, a city of approximately 300,000 inhabitants, is located in the lowlands (± 106 m elevation) of northeastern Peru along the Amazon River (Figure 1), has a mean annual temperature of 25°C, is surrounded by a vast expanse of humid tropical rainforest, and has an annual rainfall of about 3,000 mm.

As part of a study of biodiversity, small mammal communities were surveyed at the Allpahuayo Biological Station from July 1997 to December 1998, using a variety of ground and arboreal traps. After capture, the animals were killed with methoxyflurane and blood samples and tissues were collected aseptically and stored at ~70°C until DNA extraction. The skins and skulls of all animals were prepared for taxonomic studies and deposited in the Museum of Texas Tech University and at the Museum of Natural History at San Marcos University in Lima. Nucleic acids were extracted from kidneys under sterile conditions, using a commercially available kit (Qiagen, Valencia, CA) in a laboratory separate from the lab in which polymerase chain reaction (PCR) amplification was conducted and amplicons were strictly handled in order to minimize the risk of contamination. Positive-control DNA was prepared from mankarso and grippotyphosa/Moskva V serovars grown in PLM-5 medium (Intergen, Purchase, NY). *Leptospira* genus-specific primers targeting a portion of the 23S rRNA gene that amplify a 481 base-pair (bp) product, designated L737 and
were used to screen samples for the presence of leptospiral DNA. Positive samples, as well as randomly-selected negative samples, were also analyzed with primers G1/G2. Both of these PCR assays are specific for pathogenic members of the genus *Leptospira*, and do not amplify DNA from non-pathogenic, saprophytic leptospires or from any other known bacteria. Samples that were PCR-negative in both leptospiral assays were tested in a separate PCR for the presence of amplifiable DNA using primers designed to amplify the tumor suppressor gene, p53, to rule out false-negatives. These primers, originally designed to detect mouse p53, give a signature "footprint" for all mammal species on which they have been tested, including human, horse, shrew, vole, chipmunk, and the mice *Mus musculus* and *Peromyscus leucopus*, but do not amplify any product from invertebrates or purified spirochetes (data not shown). The products of the PCR assays were visualized in 1.5% agarose gels with ethidium-bromide staining with an Eagle Eye II (Stratagene, La Jolla, CA) instrument under UV transillumination. Direct DNA sequencing was performed on selected PCR products using an ABI 373 XL automated sequencer (PE Biosystem, Foster City, CA).

RESULTS

Among the nucleic acid preparations from kidney tissue of 148 mammals, amplifiable DNA was successfully extracted from 136 (92%) of the animals (Table 1). Individual samples are not represented in more than one cell in the table; e.g., if *Leptospira* spp. DNA using primers for the 23S rRNA gene was amplified from a sample, it was not included in the G1/G2 column. Thus, the overall estimate of mammals with evidence of active infection with a pathogenic leptospiro at the time of capture was 40 of 136 (29%). Kidney samples from three marsupials—one common grey four-eyed opossum (*Philander opossum*), one Bishop's slender mouse opossum (*Marmosops bishopi*), and one white-bellied slender mouse opossum (*Marmosops noctivagus*)—were found to have *Leptospira* spp. DNA amplifiable by G1/G2 primers but not by 23S rRNA gene-specific primers. This observation suggests that these three animals may have been excreting a subset (i.e., *Leptospira kirschneri*) species into the environment at the time of capture. Sequencing of the PCR products from these samples confirmed that they were from pathogenic *Leptospira* spp. (data not shown). The 40 mammals with evidence of *Leptospira* spp. infection included 13 of 64 (20%) of rodents, 20 of 51 (39%) of marsupials, and 7 of 20 (35%) of bats. One carnivore (*Potos flavus*; common name, kinkajou) tested negative by PCR.

DISCUSSION

This study demonstrates that diverse small mammals found around Iquitos, Peru in the Peruvian Amazon basin region are frequently infected with pathogenic leptospires. These carrier hosts are likely to be an environmental source for infecting humans as well as other feral and domesticated animals. It is widely thought that leptospirosis is far more common that is currently diagnosed or recognized. We have obtained evidence from a prospective febrile-illness study in Iquitos that least 5% of patients presenting to village clinics have greater than 4-fold rising titers (as determined by microagglutination assay), suggesting that acute leptospirosis is common (Vinetz JM and others, unpublished data). Only one report has attempted to estimate the large-scale burden of leptospirosis, and even this survey was
<table>
<thead>
<tr>
<th>Order</th>
<th>Genus</th>
<th>Species* (common name)</th>
<th>No. collected</th>
<th>No. of samples with amplifiable DNA (p53 PCR)</th>
<th>No. (%) PCR-positive using 23S RNA gene</th>
<th>No. (%) PCR-positive using G1/G2 primers</th>
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<tr>
<td>Rodentia</td>
<td>Proechimys</td>
<td>(spiny rat)</td>
<td>72</td>
<td>64</td>
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<td>13/64 (20)</td>
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<td>Oryzomys</td>
<td>yunganus (rice rat)</td>
<td>46</td>
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<td>megacephalas</td>
<td>5</td>
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<td>0</td>
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<td>11</td>
<td>10</td>
<td>0</td>
<td>8/10 (80)</td>
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<td>1</td>
<td>0</td>
<td>1/1 (100)</td>
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<td>0</td>
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<td>falanginosa</td>
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<td>andersoni (grey four-eyed opossum)</td>
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<td>51</td>
<td>3/51 (6)</td>
<td>20/51 (39)</td>
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<td>Philander</td>
<td>opossum</td>
<td>19</td>
<td>15</td>
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<td>5/15 (33)</td>
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<td>bishopi (slender mouse opossum)</td>
<td>3</td>
<td>3</td>
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<td>0/3 (0)</td>
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<td>Chiroptera</td>
<td>Platyrrhinus</td>
<td>helleri (white-lined bat)</td>
<td>20</td>
<td>20</td>
<td>0</td>
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<td>Artibeus</td>
<td>gnomus (fruit-eating bat)</td>
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<td>7</td>
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<td>Sturnura</td>
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<td>2</td>
<td>0</td>
<td>1/2 (50)</td>
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<td>Carolina</td>
<td>brevicauda (leaf-nosed bat)</td>
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<td>4</td>
<td>0</td>
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<td>Carolina</td>
<td>perspicillata</td>
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<td>2</td>
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<td>2/2 (100)</td>
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<td>Carnivora</td>
<td>Potos</td>
<td>flavus (kinkajou)</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Total</td>
<td></td>
<td></td>
<td>148</td>
<td>136</td>
<td>3/136 (2)</td>
<td>40/136 (29)</td>
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</table>

* Not all specimens were identified to the species level.

incomplete because of systematic deficiencies of diagnosis and reporting. Except for urban areas where domestic rats and dogs are likely to be predominant sources of pathogenic leptospires, and agricultural areas in New Zealand and Australia where cattle and pigs have been demonstrated to be sources of pathogenic *Leptospira*, the source of infection in many cases of leptospirosis is often unclear.

Of the three marsupials PCR-positive for *Leptospira* spp. using the G1/G2 primer pair, two had the expected product size (285 bp using primers G1/G2). However, DNA from the *Marmosops noctivagus* kidney amplified a slightly larger PCR product than expected, ~300 bp. Sequence analysis confirmed the identity of this amplicon as that of a pathogenic *Leptospira* species. This finding underscores the power of sequencing PCR products, not only for confirmatory purposes, but for clarifying the results of products similar to, but not exactly the expected size. Interestingly, in addition to the 40 mammals that amplified the expected 481 bp product in the pathogenic leptospire PCR using primers L737/ L1218, a number of other individuals amplified distinct products of unexpected sizes. Future studies will examine these anomalous results, and address the possibility that at least some of them could represent previously unrecognized pathogenic *Leptospira* serovars, or species variants. Another
possibility to address in the future is that some of these un-
expected results may be due to coinfection of the mamma-
lian host with more than one *Leptospira* species.

The finding that one-third of mammals tested were pre-
sumably excreting pathogenic leptospires in their urine is
notable. Such a high prevalence of excretion in a population
of feral animals in proximity to an urban area raises the
possibility that people living in Iquitos or its environs are at
risk for contracting leptospirosis. The possibility of cross-
over infection from feral zoonotic reservoir hosts to domes-
tic animals also seems possible. Of substantial technical re-
levance, leptospires are often difficult to isolate from tissue
and body fluid specimens. Therefore, previous studies rely-
ing solely on culture are likely to have underestimated the
prevalence of the disease leptospirosis and the carrier state.
A specific PCR assay, such as the one employed here, also
has substantial advantages in terms of sensitivity for epide-
miological surveys over the use of leptospiral culture and
serology alone. However, the PCR assay employed here does
not differentiate among pathogenic leptospiral serovars.
It is unknown whether certain leptospiral serovars are re-
stricted to specific mammalian hosts in the Peruvian Amazon.
Due to the intermittently arboreal habits of some of the ani-
mals tested, where carrier animals actually excrete leptospires
may be anywhere from the canopy to the forest floor. Because
of their abundance and diversity, marsupials may represent a
substantial reservoir for pathogenic leptospires in the Pe-
ruvian Amazon. Similarly, bats merit further investigation as
to their potential role as leptospiral reservoirs in this region
since 35% of bat kidneys tested contained *Leptospira* spp.
DNA. The present study identifies potential zoonotic reser-
vours of pathogenic leptospires in the Peruvian Amazon basin
region, where a variety of feral mammals are infected at a
relatively high prevalence. Future investigations are needed
to: 1) elucidate the transmission dynamics of pathogenic lep-
tospiros in this tropical forested area, 2) determine if humans
become infected with the same leptospires found in the feral
mammals, and 3) clarify the present confusion in the serovar
versus species-classification schemes.

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Authors’ addresses: Joseph E. Bunnell, Department of Marine Sciences,
Texas A&M University at Galveston, P.O. Box 1675, Galveston, TX
77553; Robert B. Tesh and Joseph M. Vinetz, Center for Tropical Dis-
eeases, Department of Pathology, University of Texas Medical Branch,
301 University Blvd., Galveston, TX 77555-0609; Christine L. Hice,
Department of Biological Sciences, Texas Tech University, Lubbock,
TX 79409; Victor Montrucchi, Instituto de Investigaciones de la Ama-
zonia Peruana, Iquitos, Peru; and Douglas M. Watts, U.S. Naval Med-
ical Research Center Detachment, Lima, Peru, APO AA 34031-3800.

Reprint requests: Joseph M. Vinetz, Center for Tropical Diseases,
Department of Pathology, University of Texas Medical Branch, 301
University Blvd., Galveston, TX 77555-0609, (409) 747-2962,
email: jovinetz@utmb.edu

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