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# *LEISHMANIA AMAZONENSIS* INFECTIONS IN *ORYZOMYS ACRITUS* AND *ORYZOMYS NITIDUS* FROM BOLIVIA

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*Abstract.* Three of thirteen *Oryzomys acritus*, Emmons and Patton 2005 (Rodentia: Muridae: Sigmodontinae) and 3 of 17 *Oryzomys nitidus*, Thomas 1884, collected from Noël Kempff National Park, Bolivia, from 2002 to 2005, tested positive for *Leishmania (Leishmania) amazonensis* or *L. (L.) mexicana* and negative for *Leishmania (Viannia)* spp. using the polymerase chain reaction (PCR). Based on previous records of *L. (L.) amazonensis* in humans, rodents, and sand flies from Bolivia, and the geographic distributions of *L. (L.) amazonensis* and *L. (L.) mexicana*, it was concluded that the *Oryzomys* were infected with *L. (L.) amazonensis*. These results identify two additional species of *Oryzomys* as hosts of *L. (L.) amazonensis*, and identify an ecological region of Bolivia where *L. (L.) amazonensis* is enzootic.

## INTRODUCTION

Cutaneous and mucocutaneous leishmaniases are endemic in both the Andean highland and the Amazon basin of Bolivia, but visceral leishmaniasis is rare.[1] Although most human infections have been attributed to *L. (Viannia)* spp., *L. (Leishmania) amazonensis* also has been reported.[2–5] The rice rat *Oryzomys capito* Olfers (1818) was reported to be a host in Cochabamba, Bolivia.[6] The taxonomy has been revised, and the currently accepted epithet for Western Bolivian specimens of this taxon is *Oryzomys perenensis* J.A. Allen, 1901.[7] *Leishmania (L.) amazonensis* also was detected in *Akodon* sp. Meyen, 1833 (Rodentia: Muridae: Sigmodontinae) and *Oligoryzomys* sp. Bangs, 1900 (Rodentia: Muridae: Sigmodontinae) and the sand fly *Lutzomyia nuneztovari anglesi* (Le Pont & Desjeux, 1984).[8,9] The purpose of our investigation was to test the hypothesis that *Oryzomys* sp. are reservoirs of *L. (L.) amazonensis* in Bolivia.[10,11]

## MATERIALS AND METHODS

**Field methods.** Rodents were captured at two localities in Bolivia, Santa Cruz, Parque Nacional Noël Kempff Mercado: El Refugio Huanchaca (UTM 20L 0712187; 8368228, WGS 84, 220 m elevation) each September from 2002–2005, and Los Fierros (UTM 20 L 0724182; 8387597, 240 m elevation) in October 2002 and September 2003. The vegetation and ecology of the region has been described in detail.[12] Small mammals were captured in live traps (H.B. Sherman, Tallahasee, FL) as part of a project by Emmons to study the faunal communities within the park. Procedures have been approved by Dr. Emmons' institutional animal care and use protocol. Animals were transferred from the trap to a cloth bag. One person who was wearing leather gloves restrained the animal by gripping it firmly behind the head while another performed other procedures. The area to be biopsied was sprayed with benzocaine first-aid spray; with this procedure the subjects showed no signs of distress. Because long-term monitoring is being continued, most captured animals were marked and released, although a few were preserved as vouchers. Series of up to 20 voucher specimens per species, with tissue samples, have been collected for the localities, and are preserved in the Museo de Historia Natural Noël Kempff Mercado, Santa Cruz, with duplicates in the United States National Museum.

In 2002, animals with scars on the basal, dorsal surface of the tail were chosen for biopsy (Figure 1). A 2-mm punch biopsy was taken from the edge of the scarred area and from the rim of each ear, placed in 1.5-mL conical bottom tubes with screw tops (PGC Scientifics, Frederick, MD), containing 95% EtOH. Pooled samples of skin from each ear and the base of the tail were screened by the polymerase chain reaction (PCR) as described below.

During September 11–13, 2003, rodents were captured at El Refugio Huanchaca and biopsies were taken only from the base of the tails from 25 rodents with or without scars or lesions, except that a biopsy from one ear was taken in the case of three *Proechimys longicaudatus* that were missing tails. These were screened by both PCR and culture. In 2004 biopsies were taken only from the tails of rodents with scars; in 2005 they were taken from all rodents captured in forests. These biopsies were only screened by PCR.

**Culture methods.** Unsuccessful attempts were made to culture *Leishmania* promastigotes from the tissue samples collected in September 11–13, 2003. They were placed in 1X medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (GIBCO), 1M Hepes buffer (pH 7.4) (Sigma Chemical Co., St. Louis, MO), 100X penicillin/streptomycin (GIBCO), 10 mM hypoxanthin (SIGMA), 0.25% (v/v) bovine hemin (Sigma), and 5 mL human urine cleared at 600 g for 20 minutes in a Centra-CL2 benchtop centrifuge. The cultured medium was sterilized by passage through a 0.2-μm filter and adjusted to pH 7.4 by 1M Sodium Hydroxide (Fisher Scientific Company, Fair Lawn, NJ). The cultures were kept at 25°C and monitored at least three times a week for the presence of promastigotes of *Leishmania*.

**Primers and probe.** DNA was amplified with the universal *Leishmania* primers 13A and 13B.[13] To identify kDNA sequences that were uniquely conserved among *L. (L.) amazonensis* and *L. (L.) mexicana* parasites, minicircle DNA sequences from *L. (Viannia)* spp., *L. (L.) amazonensis*, and *L. (L.) mexicana* that had been deposited in GenBank were aligned using the CLUSTAL software. To detect DNA from *L. (L.) amazonensis* or *L. (L.) mexicana* the forward primer

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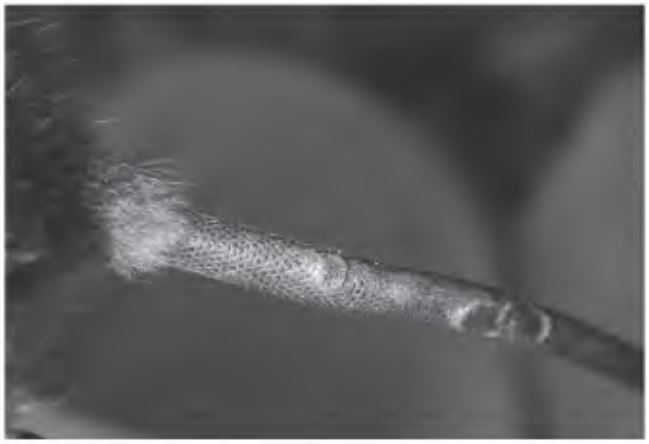

FIGURE 1. Tail of *Oryzomys acritus* from Noel Kempff National Park, Bolivia, showing lesions that were positive for *Leishmania*.

was the genus specific primer 13A (5′-GTGGGGGAGGGGCGTTCT-3′), and the reverse primer (M1.1; 5′-CCAGTTTCGACCGCCGGAGC-3′) targeted a sequence that was conserved in the *L. (L.) amazonensis* and *L. (L.) mexicana* sequences but differed from the *L. (Viannia)* spp. sequences. To specifically amplify *L. (Viannia)* spp. the forward primer was specific to *L. (Viannia)* spp. (B4; 5′-TCGTACTCCCCGACATGCCTC-3′) and the reverse primer was the genus specific primer 13B (5′-ATTTTACACCAACCCCCAGTT-3′).

**Parasites and parasite DNA.** Sixty-three *Leishmania* isolates from both the Old and New Worlds were used to define the amplification specificity of the M1.1/13A PCR primers. The specificity of the B4/13B (*Viannia* subgenus) will be described in another report (Guarin N, Melby PC, unpublished). Reference isolates were generously provided by Dr. Nancy Saravia, Centro Internacional de Entrenamiento e Investigaciones Medicas-CIDEIM, Cali, Colombia (*L. (Viannia)* spp.); Dr. Fernando Andrade-Narvaez, Universidad Autonoma de Yucatan, Merida, Mexico (*L. (L.) mexicana*); and Dr. Hechmi Louzir, Institut Pasteur, Tunis, Tunisia (*L. major*). Identification of the reference isolates to the subgenus or species level had been determined previously by analysis of isoenzyme profile and/or reactivity to specific monoclonal antibodies.

*Leishmania* spp. promastigotes were cultured in M199 medium supplemented with 15% heat inactivated fetal calf serum (HIFCS), 0.1 mM adenine, 5 μg/ml hemin, 1 μg/ml biotin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.[14] For isolation of parasite DNA the cultured promastigotes were washed in PBS and $1 \times 10^3$ were pelleted in a microcentrifuge tube. The parasite DNA was isolated by adding 20 μL of 10 mM Tris, 10 mM EDTA and placing the sample in boiling water for 5 minutes. The sample was then diluted in nuclease-free water and an aliquot used for the PCR reaction.

**Amplification of *Leishmania* DNA from cultured parasites.** Primers were re-suspended in nuclease-free water to a stock concentration of 100 pmol/μl and stored at −70°C. The DNA was amplified by PCR in a master mix containing 1.5U Taq DNA polymerase, 200 μM dNTPs, 0.4 μM of each primer, and 1.5 mM of $MgCl_2$. The PCR reaction conditions were: 94°C × 5 minutes, followed by 94°C × 1 minute, 65°C × 1 minute, 72°C × 1.5 minute for 30 cycles, followed by 72°C × 7 minutes for 1 cycle. DNA equivalent to 20 parasites was added to each reaction mix.

**Detection of amplified *Leishmania* DNA.** The amplification products were detected by 1.8% (w/v) agarose gel electrophoresis with ethidium bromide staining under UV light. The 13A/M1.1 primers gave a ~105 bp amplification product from *L. (L.) amazonensis* and *L. (L.) mexicana* DNA, the B4/13B primers gave a ~135 bp amplicon from DNA from *Viannia* subgenus parasites, and the universal primers 13A/13B gave a ~ 120 bp amplicon for all *Leishmania* strains used to test the specificity of the primers (Table 1).

**Polymerase chain reaction screening of Bolivian rodents.** The rodent tissue was lysed and the conserved region of kDNA amplified with the primers described previously in this article. PCR products amplified from two of the animals whose test results were positive were cloned into the plasmid vector pCR®2.1-TOPO® (TOPO TA Cloning® kit, Invitrogen Corporation, Carlsbad, CA). The plasmids were purified by QIAGEN® Plasmid Maxi Purification kit (QIAGEN, Valencia, CA), and sequenced at the Advanced Nucleic Acid Core Facility, Department of Microbiology at University of Texas Health Science Center at San Antonio (UTHSCSA). Sequences were compared and aligned using the National Center for Biotechnology Information, U.S. National Library of Medicine, Basic Local Alignment Search Tool (BLAST).[15]

## RESULTS

Using a large number of Old World and New World *Leishmania* isolates the M1.1/13A primers were determined to spe-

TABLE 1
PCR detection of *Leishmania* DNA from cultured promastigotes*

| Species/WHO International Code | 13A/13B | 13A/M1.2 |
|---|---|---|
| *L. (V.) braziliensis* (MHOM/CO/87/1277) | + | − |
| *L. (V.) braziliensis* (MHOM/CO/88/1407 | + | − |
| *L. (V.) braziliensis* (MHOM/BR/75/M2903) | + | − |
| *L. (V.) guyanensis* (MHOM/BR/75/M4147) | + | − |
| *L. (V.) guyanensis* (MHOM/CO/88/1390) | + | − |
| *L. (V.) guyanensis* (MHOM/BR/75/M4147) | + | − |
| *L. (V.) panamensis* (MHOM/CO/86/1166) | + | − |
| *L. (V.) panamensis* (MHOM/PA/71/LS94) | + | − |
| *L. (V.) panamensis* (MHOM/CO/84/2122) | + | − |
| *L. (L.) mexicana* (MHOM/MX/91/390) | + | + |
| *L. (L.) mexicana* (MHOM/MX/87/67) | + | + |
| *L. (L.) mexicana* (MNEO/US/90/WR972) | + | + |
| *L. (L.) mexicana* (MOTO/MX/97/0p5) | + | + |
| *L. (L.) mexicana* (MHOM/MX/98/847) | + | + |
| *L. (L.) mexicana* (MHOM/MX/94/663) | + | + |
| *L. (L.) mexicana* (MHOM/MX/98/840) | + | + |
| *L. (L.) mexicana* (MHOM/MX/98/830) | + | + |
| *L. (L.) mexicana* (MHOM/MX/94/681) | + | + |
| *L. (L.) mexicana* (MHOM/MX/98/838) | + | + |
| *L. (L.) mexicana* (MHET/MX/97/Hd18) | + | + |
| *L. (L.) mexicana* (MHOM/MX/94/758) | + | + |
| *L. (L.) mexicana* (MPER/MX/97/Py4) | + | + |
| *L. (L.) mexicana* (MHOM/MX/97/820) | + | + |
| *L. (L.) mexicana* (MHOM/MX/97/823) | + | + |
| *L. (L.) amazonensis* (IFLA/BR/67/PH8) | + | + |
| *L. (L.) amazonensis* (MHOM/BR/73/M2269) | + | + |
| *L. (L.) major* (MHOM/TN/88/TN435) | + | − |
| *L. (L.) major* (MHOM/IL/80/Friedlin) | + | − |
| *L. (L.) major* (MHOM/SY/94/Abdou) | + | − |
| *L. (L.) donovani* (MHOM/SD/00/1S-2D) | + | − |
| Negative control | − | − |

* DNA equivalent to 20 parasites was used in each PCR reaction. Amplification products were detected by agarose gel electrophoresis and ethidium bromide staining.

TABLE 2
Number of individual mammals positive for *Leishmania* spp./Number screened by PCR using primers 13A/13B[12]

| Locality | Species | Sept–Oct 2002 | Sept 2003 | Sept 2004 | Sept 2005 | Total |
|---|---|---|---|---|---|---|
| El Refugio Huanchaca | *Akodon dayi* Day's Grass Mouse | | 0/3 | | | 0/3 |
| | *Didelphis marsupialis* Southern Opossum | 0/1 | | | | 0/1 |
| | *Marmosops ocellatus* Bolivian Slender Mouse Opossum | | 0/3 | | | 0/3 |
| | *Mesomys hispidus* Spiny Tree Rat | 0/1 | | | | 0/1 |
| | *Oecomys bicolor* Bicolored Arboreal Rice Rat | | 0/1 | | 0/1 | 0/2 |
| | *Oligoryzomys microtis* Small-eared Pygmy Rice Rat | 0/1 | 0/1 | | | 0/2 |
| | *Oryzomys acritus* Rio Itenez Rice Rat | 1/1 | 1/5 | 1/1 | 0/3 | 3/10 |
| | *Oryzomys nitidus* Elegant Rice Rat | | 0/4 | 1/2 | 1/9 | 2/15 |
| | *Proechimys longicaudatus* Long-tailed Spiny Ray | 0/5 | 0/10 | 0/1 | 0/1 | 0/17 |
| | Total | 1/9 | 2/17 | 2/4 | 1/14 | 5/54 |
| Los Fierros | *Juscelinomys huanchacae* Huanchaca Juci | 0/1 | | | | 0/1 |
| | *Kunsia tomentosus* Wooly Giant Rat | 0/3 | | | | 0/3 |
| | *Necromys lenguarum* Bay Mouse | | 0/1 | | | 0/1 |
| | *Oryzomys acritus* Rio Itenez Rice Rat | 0/3 | | | | 0/3 |
| | *Oryzomys nitidus* Elegant Rice Rat | 1/2 | | | | 1/2 |
| | Total | 1/9 | 0/1 | | | 1/10 |
| Both | Total | 2/18 | 1/28 | 2/4 | 1/14 | 6/64 |

cifically amplify DNA from members of the *L. (L.) amazonensis* or *L.* (*L.*) *mexicana* (Table 1). Most notably, under the standardized reaction conditions, DNA from members of the *Viannia* subgenus was not amplified by these primers. Furthermore, the *Viannia* subgenus-specific B4/M13 primers did not amplify DNA from *L. (L.) amazonensis* or *L.* (*L.*) *mexicana.*

Ultimately 64 individual mammals, 10 species of rodent and two species of opossum, were screened for *Leishmania* (Table 2). Three of thirteen *O. acritus* (Figure 2) and 2 of 17 *O. nitidus* (Figure 3) from El Refugio Huanchaca, and 1 of 2 *O. nitidus* from Los Fierros tested positive for *Leishmania* spp. and negative for *L.* (*Viannia*) spp., using PCR.[16] Efforts to culture the parasite were unsuccessful. No positive for *Leishmania* was detected in any other mammal species. Both traplines where *Leishmania* positive rodents were collected were in semideciduous tall forest formations.

The sequence of the DNA amplified from *O. acritus* (field no. 444) and *O. nitidus* (field no. 575) compared with a reference kDNA sequence [(*L. (L.) amazonensis*, MHOM/BR/00/Raimundo (=MHOM/BR/1983/M1132), NCBI Accession number M21326)] is shown below. The primer (13A and M1.1) sequences are underlined and a vertical line indicates identity between the cloned and reference sequences.

Each of the sequenced kDNA fragments was 102 nucleotides in length, including the primer sequences. The sequence of amplified DNA (excluding the primer sequences) from *O. acritus* (field no. 444) and *O. nitidus* (field no. 575) showed 93% and 78% identity to the reference *L. (L.) amazonensis* kDNA sequence, respectively. A similar level of identity was observed with other *L. (L.) amazonensis* and *L.* (*L.*) *mexicana* sequences. Notably, the BLAST search did not identify homology to kDNA sequences of parasites of the *Viannia* subgenus, even though more than 40 such sequences of the conserved region of the minicircle DNA are present in the NCBI database.

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444   GTGGGGGAGGGGCGTTCTGCGGAATCCTCAAAAATGAGTGCAGAAACCCCGTTCATATTTTGGGGGAATTTTG
      |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||| |||||
La    GTGGGGGAGGGGCGTTCTGCGGAAACCTCAAAAATGAGTGCAGAAACCCCGTTCATATTTTGGGGGATTTTT
      |||||||||||||||||||||||||  |  ||||||||||||||||||||||||||||||| ||||||||| ||
575   GTGGGGGAGGGGCGTTCTGCGGGGAAGCCAAAAATGAGTGCAGAAACCCCGTTCATAATTTGGGGGAATTCC

444   GGGAATTCCGGCTCCGGCGGTCGAAACTGG
      |||||||| ||| || | |||| ||||||||
La    GGGAATTTCGGTTCGGACGGTGGAAACTGG
        || | ||| || | |||| ||||||||
575   TCAAAATCCGGCTCCGGCGGTCGAAACTGG
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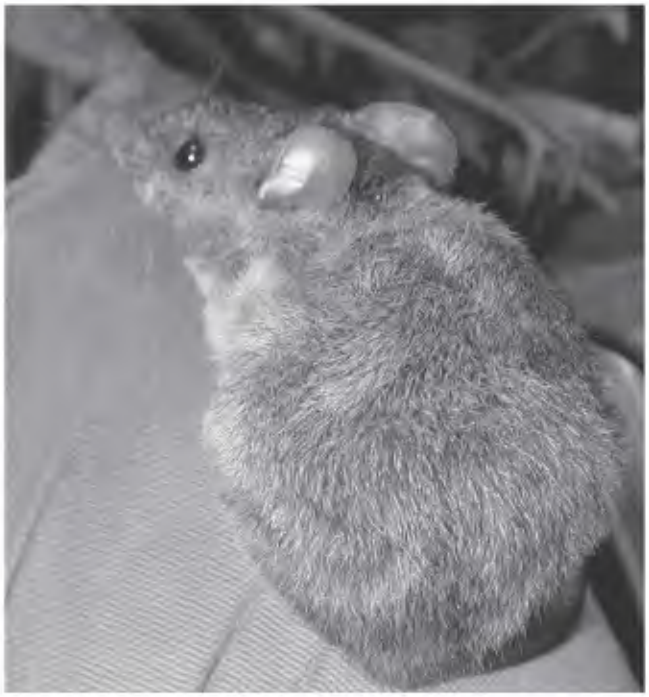

FIGURE 2. Three of thirteen *Oryzomys acritus* collected from Noël Kempff National Park, Bolivia, from 2002–2005, tested positive for *Leishmania*.

## DISCUSSION

Results indicate that both *O. acritus* and *O. nitidus* were infected with either *L. (L.) amazonensis* or *L.* (*L.*) *mexicana*. It is noteworthy that both PCR products that were sequenced showed high homology with DNA from parasites collected from humans in Brazil. Previously, eight human stocks from the sub-Andean region of LaPaz were identified as *L.* (*L.*) *amazonensis* based on isoenzyme electrophoresis and comparison with reference strains.[4] *Leishmania* isolates from one *Akodon* and two *Oligoryzomys* collected at this focus were identified as *L.* (*L.*) *amazonensis* based on the similarity of kDNA-PCR profiles to that of the human isolates.[8] Isoenzyme profiles of three isolates from the sand fly *Lu.*(*n.*) *anglesi* collected at this focus also indicated *L.* (*L.*) *amazonensis*.[9] PCR using SSU rRNA was used to compare a large number of *L.* (*L.*) *amazonensis* strains from broadly distributed geographical areas.[5] Results indicated that of these two species, *L.* (*L.*) *amazonensis* is the main species occurring in South America, whereas *L.* (*L.*) *mexicana* is primarily confined to North and Central America. Therefore, based on the previous identification of *L.* (*L.*) *amazonensis* from humans, rodents, and sand flies from Bolivia, and the geographic distributions of *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana*, we concluded that the rice rats were infected with *L.* (*L.*) *amazonensis*.

FIGURE 3. Three of seventeen *Oryzomys nitidus* collected from Noël Kempff National Park, Bolivia, from 2002–2005, tested positive for *Leishmania*.

These results identify two additional species of *Oryzomys* as hosts of *L.* (*L.*) *amazonensis*, and identify Noël Kempff National Park as an ecological region of Bolivia where *L. (L.) amazonensis* is enzootic. The fact that infections were detected in either *O. acritus* or *O. nitidus* each year from 2002–2005, and the lack of infections in other potential reservoirs such as *Proechimys longicaudatus*, suggest that *O. acritus* and *O. nitidus* are reservoirs of *L. (L.) amazonensis* in this area. Seemingly, *Oryzomys* is a host, and possible reservoir, of *L. (L.) amazonensis* or *L.* (*L.*) *mexicana* over a broad geographic range, including Mexico, Central America, and South America.[4,17–22] Since *Oryzomys* spp. also occur in the eastern United States where sand flies (*Lutzomyia* sp.) are abundant, it may also be a reservoir there.[23] According to Eisenberg (1989), oryzomyines are close relatives of the neotomine-peromyscine group of North America, and the tylomyine and nyctomyine of Central America.[24] Based on the information that reservoirs of *Leishmania* spp. are known from all of these closely related groups, Kerr (2000) suggested that oryzomyine rodents might be reservoirs of *Leishmania* in South America.[11] Our results support this hypothesis.

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