Natural *Leishmania* Infection of *Lutzomyia auraensis* in Madre de Dios, Peru, Detected by a Fluorescence Resonance Energy Transfer–Based Real-Time Polymerase Chain Reaction

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Abstract. *Leishmania* species of the *Viannia* subgenus are responsible for most cases of New World tegumentary leishmaniasis. However, little is known about the vectors involved in disease transmission in the Amazon regions of Peru. We used a novel real-time polymerase chain reaction (PCR) to assess *Leishmania* infections in phlebotomines collected in rural areas of Madre de Dios, Peru. A total of 1,299 non-blood fed female sand flies from 33 species were captured by using miniature CDC light traps. *Lutzomyia auraensis* was the most abundant species (63%) in this area. Seven of 164 pools were positive by PCR for *Leishmania* by kinetoplast DNA. The real-time PCR identified four *Lu. auraensis* pools as positive for *L. (Viannia) lainsoni* and *L. (V.) braziliensis*. The minimum infection prevalence for *Lu. auraensis* was estimated to be 0.6% (95% confidence interval = 0.20–1.42%). Further studies are needed to assess the importance of *Lu. auraensis* in the transmission of New World tegumentary leishmaniasis in hyperendemic areas of Peru.

INTRODUCTION

Leishmaniasis is a complex of vector-borne diseases caused by protozoan parasites of the genus *Leishmania*. It occurs in as many as 70 countries worldwide, with more than 2 million new cases a year.¹ In South America, New World tegumentary leishmaniasis (NWTL) is mainly caused by species of the *Viannia* subgenus, including *L. (V.) braziliensis*, *Leishmania (V.) peruviana*, *Leishmania (V.) guyanensis*, and *Leishmania (V.) panamensis*.^{2,3} In Peru, NWTL is endemic in 74% of the country,^{3,4} and there were 6,761 reported cases in 2010.⁵

The transmission cycle of *Leishmania* begins with the bite of an infected phlebotomine sand fly. Of the 500 known sand fly species in South America, only approximately 30 are known vectors for NWTL.^{4,6,7} In Peru, four species have been incriminated as vectors of *L. (V.) peruviana*, and all are in the Andean Mountain regions.^{8–11} However, little is known regarding vectors of NWTL in the Peruvian Amazon Basin despite the large number of human cases reported.^{8,9,11}

Vector surveillance studies are often focused on studying the distribution of potential vectors and the prevalence of *Leishmania* infections in field-captured sand flies. These studies are critical to better understand the dynamics of disease transmission and to identify new parasite-vector associations.¹²

The detection of *Leishmania* in sand fly specimens has traditionally been based on the finding of flagellate forms in sand fly midgut dissections. However, this method requires advanced expertise¹³ and is cumbersome and time-consuming. Consequently, its use is limited under field conditions or in laboratories in remote-endemic areas where technical resources may be scarce. Improved tools to identify *Leishmania* species in sand fly specimens are greatly needed to enhance entomologic surveillance studies.

Tsukayama and others reported *Leishmania* species-specific DNA polymorphisms in the genes encoding mannose phosphate isomerase (*MPI*) and 6-phosphogluconate dehydroge-

nase (*6PGD*).¹⁴ On the basis of these polymorphisms, we have designed a fluorescence resonance energy transfer (FRET)–based real-time PCR that can identify up to five *Leishmania (V.)* species (Nuñez JH and others, unpublished data). In the present study, we applied this assay to identify *Leishmania* parasites in several species of *Lutzomyia* sand flies collected from a rural community in Madre de Dios, Peru, a region where NWTL is hyperendemic.

MATERIALS AND METHODS

Study area. The study was conducted in the community of Flor de Acre in the District of Iberia, Province of Tahuamanu, Department of Madre de Dios, Peru. This community is located along the recently inaugurated Peru–Brazil inter-oceanic highway on the Iberia–Pacahuara route. The site is located at approximately 11°23'09"S, 69°30'55" (Figure 1) at 300 meters above sea level and has humid sub-tropical climate. Annual temperature in this region ranges from 22°C to 34°C, and annual precipitation ranges from 4,000 mm to 8,000 mm. In 2010, the Department of Madre de Dios reported 415 cases of cutaneous leishmaniasis and 47 cases of mucocutaneous leishmaniasis.⁵ Previous reports show that *L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) lainsoni*, and *Leishmania (V.) shawi* are *Leishmania* species identified in human samples from the study area.^{3,15–17}

Capture of sand flies and identification. Peridomestic sand fly specimens were captured in September, November, and December of 2009 (before and during the rainy season) in the vicinity of 10 arbitrarily selected houses during 10 consecutive days each month (Figure 1). This period was chosen on the basis of the seasonal increase in sand fly density regularly observed in the region at this time. Miniature CDC light traps equipped with a CO₂ generator were placed outside each house for 12 hours per night. No humans or animals were used for collection of specimens, and no collection of information on humans was obtained as part of this study. Phlebotomines were stored in 70% ethanol and transported at room temperature to the central laboratory of the US Naval Medical Research Unit No. 6 in Lima.

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FIGURE 1. Flor de Acre, Iberia in Madre de Dios, Peru. **Inset** shows satellite images of 10 houses around where sand flies were collected. Source: "Flor de Acre" S11° 23' 09" and W69° 30' 55" Google Earth.

Species determination was based on keys developed by Young and Duncan.⁷ To preserve DNA integrity for PCRs, sand fly species identification was carried out by using a modified procedure that uses lactophenol only on specific parts of the sand fly body, thus preserving the remainder of the specimen for PCR analysis. Sand flies were sexed and non-blood-fed females were pooled in groups of 1–10 specimens according to species and capture location and stored again in 70% ethanol until total DNA was isolated. Male sand flies were used as negative controls because they are not infected by *Leishmania*.

DNA extraction. DNA isolation was performed by using the Chelex-100 method according to reported techniques.¹⁸ In brief, pooled sand flies were homogenized in 0.85% NaCl. Homogenized pools were treated with 5% (w/v) Chelex-100 for 5 minutes, followed by incubation at 100°C for 10 minutes. Samples were then centrifuged at 16,000 × *g* for 10 minutes. Supernatants were carefully separated from the pellet and then mixed with 200 µL of 100% ethanol and centrifuged at 16,000 × *g* for 15 minutes. The supernatants were mixed with 100 µL of 70% ethanol and pellets were dried by using a SpeedVac (Savant, Irvine, CA) and resuspended in 50 µL of nuclease-free distilled water.

Polymerase chain reaction for detection of sand fly DNA. For the detection of *Lutzomyia* DNA, a published PCR protocol was used¹⁹ with primers T1B 5'-AAA CTA GGA TTA GAT ACC CT-3' and T2A 5'-AAT GAG AGC GAC GGG CGA TGT-3' specific for 12S ribosomal DNA. The reactions were carried out in a volume of 25 µL containing 1X *Taq* polymerase buffer (Invitrogen, Carlsbad, CA), 1.5 mM MgCl₂, 125 µM dNTPs (Invitrogen), 0.5 µM of each primer, 1 unit of *Taq* DNA polymerase (Invitrogen), and 5 µL of DNA sample. The PCR was run on a thermocycler (GeneAmp PCR system 9700; Applied Biosystems, Foster City, CA)

for 5 minutes at 94°C; followed by 35 cycles for 20 seconds at 94°C, 30 seconds at 56°C, and 25 seconds at 72°C; followed by a final extension step for 5 minutes at 72°C. A PCR product of approximately 400 basepairs confirms the presence of sand fly DNA in the extracted samples and the absence of potential PCR inhibitors in the DNA preparations.

Polymerase chain reaction identification of *Leishmania Viannia* subgenus. A modified PCR protocol²⁰ was used to detect *Leishmania* (*Viannia*) subgenus from sand fly pools. A conserved region of the kinetoplast DNA minicircle was amplified by using primers MP1-L 5'-TAC TCC CCG ACA TGC CTC TG-3' and MP3-H 5'-GAA CGG GGT TTC TGT ATG C-3'.²⁰ The reactions were carried out in a volume of 20 µL containing 1X *Taq* polymerase buffer (Invitrogen), 1.5 mM MgCl₂, 125 µM dNTPs (Invitrogen), 0.5 µM of each primer, 1 unit of *Taq* DNA polymerase (Invitrogen), and 5 µL of DNA sample. The PCR was run on the aforementioned thermocycler for 5 minutes at 94°C; followed by 35 cycles for 45 seconds at 94°C, 45 seconds at 58°C, and 1 minute at 72°C; followed by a final extension step for 5 minutes at 72°C. This reaction generates a 70-basepair DNA fragment that is specific for the *Viannia* subgenus.

Fluorescence resonance energy transfer-based real-time PCR for speciation of *Leishmania*. This PCR approach is based on known mutations in the *6PGD* and *MPI* genes that yield distinct melting peaks, which are used to distinguish between five *Leishmania* species.¹⁴ The validation of this real-time assay will be published separately. For the *6PGD* gene, we used a 50-µL reaction containing 1X *Taq* polymerase buffer (Invitrogen), 1.5 mM MgCl₂, 200 µM dNTPs (Invitrogen), 0.8 µM of each primer, 1.5 units of *Taq* DNA polymerase (Invitrogen), and 5 µL of DNA sample. For the *MPI* gene, reactions were performed in a volume of 50 µL containing 1X *Taq* polymerase buffer (Invitrogen), 1.5 mM MgCl₂, 200 µM dNTPs (Invitrogen), 1 µM of each primer, 1.5 units of *Taq* DNA polymerase (Invitrogen), and 5 µL of DNA sample. The PCR was run on a thermocycler (GeneAmp PCR system 9700; Applied Biosystems).

TABLE 1

Species of *Lutzomyia* sand flies captured in Flor de Acre, Iberia, Madre de Dios, Peru

Species	No. sand flies	%
<i>Lu. auraensis</i>	821	62.9
<i>Lu. davi</i>	107	8.2
<i>Lu. choti</i>	86	6.6
<i>Lu. llanosmartinsi</i>	68	5.2
<i>Lu. hirsuta</i>	67	5.1
<i>Lu. carrerai</i>	33	2.5
<i>Lutzomyia</i> spp.*	28	2.2
<i>Lu. yucumensis</i>	20	1.5
<i>Lu. aragaoi</i>	16	1.2
<i>Lu. shawi</i>	8	0.6
<i>Lu. richardwardi</i>	7	0.5
<i>Lu. umbratilis</i>	4	0.3
<i>Lu. falcata</i>	3	0.2
<i>Lu. trinidadensis</i>	3	0.2
Other <i>Lutzomyia</i> †	28	2.8
Total	1,299	100

* The species of these sand flies could not be determined. They were only identified as belonging to the genus *Lutzomyia*.

† Other *Lutzomyia* species: *Lu. barrettoii*, *Lu. migonei*, *Lu. nevesi*, *Lu. quechua*, *Lu. saulensis*, *Lu. serrana*, *Lu. sherlocki*, *Lu. Nyssomyia whitmani*, *Lu. Psychodopygus amazonensis*, *Lu. antunesi*, *Lu. clausenae*, *Lu. cortelezzi*, *Lu. evangelistai*, *Lu. furcata*, *Lu. guyanensis*, *Lu. nematoducta*, *Lu. punctigeniculata*, *Lu. nuneztovari*, *Lu. preclara*, *Lu. walkeri*.

FIGURE 2. Detection of *Lutzomyia* 12S ribosomal DNA and of *Leishmania* kinetoplast DNA by polymerase chain reaction (PCR) of pools of field-collected *Lutzomyia* sand flies. **A**, Agarose gel electrophoresis of 12S ribosomal DNA PCR products in a representative subset of pools. Lane 1, 100-basepair (bp) ladder; lanes 2–8, DNA from *Lutzomyia* pools; lane 9, positive control of male *Lutzomyia* spp.; lane 10, blank. **B**, Agarose gel electrophoresis of kinetoplast DNA PCR products. Lane 1, 100-bp ladder; lanes 2–8, positive *Lutzomyia* pools; lane 9, positive control of *L. (V.) braziliensis* strain MHOM/BR/84/LTB300; lane 10, negative control of male *Lutzomyia* spp.; lane 11, blank.

After the first round of amplification reactions, a FRET based real-time assay was performed. Both gene reactions were conducted in a volume of 20 μ L containing 1X LightCycler® 480 Genotyping Master (Roche, Indianapolis, IN), 1.25 μ M of forward primer, 0.25 μ M of reverse primer, 0.75 μ M of anchor and sensor probes, and 5 μ L of PCR product from the first reaction. The real-time PCR was run on a LightCycler 480 (Roche) (Nuñez JH and others, unpublished data). To estimate the analytical sensitivity of the real-time PCR, known amounts of parasite DNA were spiked into parasite-free male sand fly DNA preparations. The limit of detection was the lowest parasite DNA concentration that yielded an amplification product visualized on the FRET real-time PCR.

RESULTS

We collected 1,299 female specimens belonging to 33 sand fly species. As shown in Table 1, the most prevalent species were *Lutzomyia auraensis* (62.9%), followed by *Lu. davis* (8.2%), *Lu. choti* (6.6%), *Lu. llanosmartinsi* (5.2%), and *Lu. hirsuta* (5.1%). The PCR analysis of 12S ribosomal DNA confirmed the identity of these sand flies as belonging to the genus *Lutzomyia*. In addition, the positive results indicated good DNA quality and the absence of PCR inhibi-

tors and served as an internal control to rule out false-negative results (Figure 2A).

Sand fly specimens were grouped into 164 pools on the basis of sand fly species and collection site. Seven of 164 sand fly pools were positive for parasites of the *Leishmania* (*Viannia*) subgenus by kinetoplast DNA PCR (pools I–VII; Table 2 and Figure 2B). Four of the seven pools were composed of 10 specimens each of *Lu. auraensis*, and the remaining three comprised 1, 3, and 10 specimens of *Lu. punctigeniculata*, *Lu. trinidadensis*, and *Lu. davis*, respectively. DNA samples from male sand flies, which are known not to be infected by *Leishmania*, did not yield any amplification product.

Our FRET-based real-time PCR was used to identify the species of *Leishmania* in kinetoplast DNA-positive pools. This assay detects down to 60 fg of parasite DNA, which is equivalent to less than five parasites per reaction.²¹ We identified the species of *Leishmania* from five of the seven kinetoplast DNA-positive pools (III–VII). The real-time PCR identified three positive pools as *L. (V.) lainsoni* (V–VII), and two positive pools (III and IV) as *L. (V.) braziliensis* (Table 2 and Figure 3). All but one of these pools were *Lu. auraensis*. Pool VII did not yield a positive result for the *MPI* locus, possibly because of the larger amplicon size for *MPI* (1,464 basepairs) than for kinetoplast DNA (70 basepairs) and *6PGD* (628 basepairs). Two additional kinetoplast DNA-positive, real-time PCR-negative pools did not yield any amplification product for *6PGD* or *MPI*. Therefore, we could not identify the *Leishmania* species.

Five of the seven kinetoplast DNA-positive pools were collected around house no. 9. Another positive pool was collected near house no. 1, which was approximately 12 km from house no. 9 (Figure 1). The minimal infection prevalence for *Lu. auraensis* and *Lu. davis* was 0.60% (5 of 821) (95% confidence interval = 0.20–1.42%) and 0.90% (1 of 107), (95% confidence interval = 0.02–5.10%), respectively. We were unable to estimate the minimal infection prevalence for *Lu. punctigeniculata* and *Lu. trinidadensis* because there were only one and three insects captured of each species, respectively.

DISCUSSION

We report infection of *Lu. auraensis* with New World species of *Leishmania*. Using a newly developed real-time PCR that can discriminate among five *Leishmania* species, we identified that *Lu. auraensis* is a natural carrier of *L. (V.) lainsoni* and *L. (V.) braziliensis* in the study area. Positivity among non-blood-fed females supports the likelihood of infection as opposed to only parasite carriage. There are no previous reports of natural *Leishmania* infection in this sand fly species,

TABLE 2

Identification of *Leishmania* species by real-time polymerase chain reaction in seven kinetoplast DNA PCR-positive sand fly pools*

<i>Lutzomyia</i> species	Pool no.	House†	Kinetoplast DNA PCR	Real-time PCR	No. insects in pool	Month of capture
<i>Lu. punctigeniculata</i>	I	9	+	–	1	September
<i>Lu. trinidadensis</i>	II	3/9	+	–	3	September
<i>Lu. davis</i>	III	9	+	<i>L. (Viannia) braziliensis</i>	10	September
<i>Lu. auraensis</i>	IV	1	+	<i>L. (V.) braziliensis</i>	10	November
<i>Lu. auraensis</i>	V	9	+	<i>L. (V.) lainsoni</i>	10	December
<i>Lu. auraensis</i>	VI	9	+	<i>L. (V.) lainsoni</i>	10	December
<i>Lu. auraensis</i>	VII	9	+	<i>L. (V.) lainsoni</i>	10	December

*PCR = polymerase chain reaction.

†House number from which sand fly pools were collected.

FIGURE 3. Melting curve analysis of real-time polymerase chain reaction of 6-phosphogluconate dehydrogenase (6PGD) and mannose phosphate isomerase (MPI). **A**, Melting curves corresponding to 6PGD-based amplification reactions of seven sand fly pools (I–VII) were run and compared with a battery of six standard strains of the New World *Leishmania*. **B**, Melting curves of MPI-based real-time amplification reactions of seven sand fly pools (I–VII) were run and compared with a battery of six standard strains of New World *Leishmania*. Species were identified on the basis of melting curves that had the same or similar peaks. pan = *panamensis*; guy = *guyanensis*; lain = *lainsoni*; bra = *braziliensis*; per = *peruviana*; ama = *amazonensis*.

despite being highly prevalent in hyperendemic settings for leishmaniasis in Brazil and Peru.^{22,23}

This study also confirmed the large predominance of *Lu. auraensis*, which comprised 63% of all collected sand flies in the study area. The abundance of this species in the

southeastern Peruvian rainforest has been reported,²² and reached approximately 80% of all collected sand flies in Alto Tambopata, Peru. *Lu. auraensis* has been reported in the State of Acre, Brazil, and comprised 19% of all collected sand flies.²³ According to Young and Duncan, there are also

reports of *Lu. auraensis* in Venezuela, Suriname, Bolivia, and Colombia.⁷ Moreover, *Lu. auraensis* may have anthrophilic behavior on the basis of studies in Puno, Peru,²² although existing evidence lacks confirmation.²⁴ These findings underscore the urgency to determine the importance of this species in the transmission of NWTL in this region.

To determine the vectorial role of *Lu. auraensis*, future studies will need to assess if this sand fly species is capable of sustaining the parasite and transmitting it to the vertebrate host.²⁵ The possibility that *Lu. auraensis* transmits *L. (V.) braziliensis* is a particularly important consequence of our findings, given that this parasite species is the major etiologic agent of the disfiguring mucosal presentation of the disease. Overall, our results highlight the limited knowledge we have regarding the vectors implicated in the transmission of leishmaniasis in many parts of South America.

The PCR-based techniques that detect low amounts of parasite DNA are highly desirable because of their increased sensitivity and specificity in detecting *Leishmania* parasites^{12,13,26,27} compared with midgut dissection techniques.⁶ In this study, the real-time PCR could detect and discriminate *Leishmania* species in field-collected sand fly preparations with high reliability. This method is ideal for field conditions because entomologic specimens can be collected and preserved in alcohol at room temperature until transported to the laboratory for processing.¹³ The real-time assay was evaluated in 192 clinical samples from patients with suspected leishmaniasis throughout Peru and showed a sensitivity of 91% and a specificity of 77%, when compared to kinetoplastid DNA PCR for leishmania diagnosis (Nunez JH, unpublished data). In addition, the real-time PCR assay can identify species within the *Leishmania Viannia* complex with 100% of concordance compared to multilocus sequencing typing (tested in 72 positive clinical samples). Furthermore, unlike reported PCRs,^{20,28,29} no post-PCR processing such as electrophoresis, restriction fragment length polymorphism, or sequencing was required.

Most (5 of 7) positive sand fly pools were collected near a single house. Two other houses with positive pools were located 5 and 12 km away from this house and the pools from seven other houses were negative. These findings are consistent with the clustering and heterogeneous spatial distribution observed in a small area of *Leishmania*-infected sand flies in Rio Branco, Brazil, where the location with the most positive phlebotomines also had the highest number of new cases of cutaneous leishmaniasis.³⁰ Future studies in the Amazon basin need to assess larger number of more dispersed collection points to better describe if transmission risk variability remains over broader geographic ranges.

Lutzomyia davis represented the second most frequent sand fly species (8.2%), and one pool of this species was positive for *L. (V.) braziliensis*. *Lutzomyia davis* is a vector for NWTL and is commonly found in Brazil, Colombia, and French Guiana.^{31,32} Although *Lu. davis* has been reported in the Peruvian rainforest,²² *Leishmania* infections have not been reported in this species in Peru. The presence of natural infection by *L. (V.) braziliensis* confirmed in this study indicates that this sand fly species may also act as vector for leishmaniasis transmission in the study area, although experimental confirmation is lacking.

Less common sand fly species such as *Lu. trinidadensis* and *Lu. punctigeniculata* were positive by PCR for kineto-

plast DNA, which indicated infection with New World *Leishmania* species. However, the real-time PCR showed negative results for these samples and consistent results after three repetitions, leaving us unable to identify the infecting *Leishmania* species. We found that the detection limits of the kinetoplast DNA and real-time PCRs were equivalent when using the same parasite DNA preparation. Therefore, it is unlikely that this discordance could reflect lower sensitivity of the real-time PCR. The kinetoplast DNA PCR may have amplified other *Leishmania (Viannia)* species that are not within the five species that the real-time PCR detects.

The specificity of the real-time PCR was 100% and the sensitivity was also high but could be improved. Therefore, the two kinetoplast DNA-positive, real-time PCR-negative pools could be infected with other *Leishmania* species less commonly reported in the study region but not evaluated in our assays. For example, *L. (V.) shawi* was reported in the same region of Peru where our study was conducted.¹⁷ Also, a study in Venezuela detected *Leishmania (L.) venezuelensis* in *Lu. trinidadensis*, the same sand fly species found to be *Leishmania* positive in our study but without an identifiable species.³³ However, it is unlikely that the *Leishmania* species found in *Lu. auraensis* could be a misclassification of other species. For example, *L. (V.) braziliensis* and *L. (V.) shawi* have different 6PGD multilocus enzyme electrophoresis patterns.³⁴ Also, the MPI real-time PCR can identify *Leishmania (L.) amazonensis/mexicana* (Nuñez JH, unpublished data) and probably other *Leishmania* subgenus species as a complex, differentiating them from *L. (V.) braziliensis* and *L. (V.) lainsoni*. Therefore, none of these potential limitations affect our main conclusion, which is that *Lu. auraensis* is a common and important carrier of multiple *Leishmania* species in the southern Amazon basin. Expanding the list of species detected by our assay and more extensive vector studies will further enhance our knowledge of the distribution of *Leishmania* vectors in this little-described disease-endemic region.

In summary, we identified natural *Leishmania* infection in *Lu. auraensis*, a widespread sand fly species in Peru and Brazil. Its role as a new vector for leishmaniasis remains to be determined to better understand the risk of leishmaniasis transmission in the southern Amazon Basin. Our real-time PCR proved to be a viable and efficient tool for identification of *Leishmania* species in field-collected sand fly specimens, compared with traditional dissection and microscopic examination techniques. The use of this assay in field conditions could be useful in identifying *Leishmania*-carrying sand fly species and enhancing research and prevention efforts.

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