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# Natural infection of *Lutzomyia neivai* with *Leishmania* spp. in northwestern argentina

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**Abstract**

The natural infection of *Lutzomyia neivai* with *Leishmania* in the endemic area of American cutaneous leishmaniasis (ACL) in northwestern Argentina was analyzed by the polymerase chain reaction (PCR)-hybridization technique. Phlebotominae sand flies were captured in the provinces of Tucumán and Salta between 1999 and 2003. From a sample of 440 *Lu. neivai* females analysed for the detection of the *Leishmania* (*Viannia*) and *Leishmania* (*Leishmania*) subgenera, 9.1% of the samples resulted infected with a parasite of the subgenus *Viannia* and none with the *Leishmania*. This is the first report of naturally infected sand flies in Argentina besides the first report of infected *Lu. neivai* sensu strictu. Our results contributed to further incrimination of this specie as vector of leishmaniasis in the area and the identification of the main circulating parasite as belonging to the *Leishmania* (*Viannia*) subgenera.


*Keywords:* American cutaneous leishmaniasis; *Lutzomyia neivai*; *Leishmania* (*Viannia*) subgenera; PCR; Argentina

## 1. Introduction

Leishmaniases are vector-borne diseases caused by parasites of the genus *Leishmania* and transmitted in the Americas by the bite of sand flies of the genus *Lutzomyia* (Diptera: Psychodidae: Phlebotominae). American cutaneous leishmaniasis (ACL) has been reported in northern Argentina since 1916. An epidemic outbreak in 1984–1987 occurred in the northwestern province of Salta, followed by an increase of ACL incidence thorough the endemic region (Salomón et al., 1995). In the province of Tucumán a smaller magnitude epidemic outbreak occurred in 1986–1988. The Statistical Department of the Province Health Service (SI.PRO.SA) registered 203 new leishmaniasis cases between 1991 and 1997 in the province.

Parasites isolated and identified from patients in Argentina belongs to the *Leishmania braziliensis* complex (Cuba et al., 1996; Segura et al., 2000; Frank et al., 2003; Marco et al., 2005; Córdoba-Lanús et al., 2005) but it has also been detected *L.* (*Leishmania*) *amazonensis* (Frank et al., 2003) and *L.* (*Viannia*) *guyanensis*

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(Cupolillo et al., 1994; Marco et al., 2005), both by enzyme characterization. In Latin America *L.* (*Viannia*) and *L.* (*Leishmania*) subgenera are both causative agents of cutaneous and mucocutaneous leishmaniasis, differentiate between them has epidemiological and therapeutical implicances.

*Lutzomyia neivai* has been incriminated as the main vector of ACL in Argentina by its ecology and the epidemiology of the outbreaks studied (Salomón et al., 2004). However, there is no information about natural infection of sand flies by these parasites in the country. The rate of naturally infected sand flies and the identification of the circulating parasite are of prime importance in the design of vectorial and epidemiological surveillance studies.

Detection of *Leishmania* in several clinical samples of human, dogs and sandflies by polymerase chain reaction (PCR) have shown to be a highly sensitive and specific method. Phlebotominae analysis by the PCR technique is a very useful procedure to detect parasite infection in a great number of insects, also considering that the rate of natural infection of *Lutzomyia* spp. with *Leishmania* in endemic areas is very low.

The aim of the present study was to detect Phlebotominae natural infection with *Leishmania* spp. from an endemic area of ACL in northern Argentina and determine which subgenera the parasite belongs to by using the PCR-Hybridization technique. Moreover, these findings will confirm *Lu. neivai* as the vector of the disease in the area, already incriminated according to its spatial and seasonal abundance and epidemiological patterns in several foci in the country.

## 2. Material and methods

*Phlebotominae capture and identification*: A sample of sand flies collected in the framework of an ecological research of the Phlebotominae fauna in nothwestern Argentina was analysed for leishmanial infection in this study. Phlebotominae were captured in five localities in Tucumán and one in Salta provinces between November/1999 and January/2003 using a modified Shannon trap (Salomón et al., 1995) and CDC-like mini light traps, located in the primary and secondary vegetation, or in peridomestic habitats (animal pens, poultry yards, etc.). Sand flies were processed and identified according to Young and Duncan (1994) and Marcondes (1996).

*DNA extraction*: After morphological identification, 470 *Lu. neivai* females were pooled in groups of 10 individuals, immersed and homogenized in 300 μl lyses buffer (10 mM Tris–HCl, pH 8.2, 2 mM EDTA, 0.2% Tritón X-100), incubated with 3 μl of Proteinase K (20 mg/ml) at 55 °C for 2 h, placed at 95 °C for 5 min and cooled for 10 min, protocol modified from Jowett (1998). The DNA was recovered by the phenol:chloroform extraction method (Medina-Acosta and Cross, 1993) resuspended in 20 μl of bidestiled water and stored at 4 °C for its immediate use. Specific Phlebotominae primers: 5′ GTG GCC GAA CAT AAT GTT AG 3′and 5′ CCA CGA ACA AGT TCA ACA TC 3′ were used in a PCR as a DNA extraction confirmational test (Peixoto et al., 2001). This reaction amplified a sequence of 220 bp of the Phlebotominae *cacophony gene* homologue to the *Drosophila melanogaster* one.

*L.* (*V.*) *braziliensis* (MHOM/PE/95/LQ2), *L.* (*L.*) *tropica* (MON 58/LEM 2578) and *L.* (*L.*) *amazonensis* (MHOM/77/LTB0016) cultures were washed twice in 1× PBS and were then resuspended in extraction buffer TELT (Tris–HCl 50 mM, pH 8; EDTA 62.5 mM, pH 9; LiCl 2.5 M; Tritón X-100 4% (v/v)). A 100 g/ml of RNAse was added and then incubated at 60 °C for 1 h. The DNA was extracted as described above.

*PCR procedure*: The primers used to detect *Leishmania* spp. (**JP1** 5′ CCG CCC CTA TTT TAC ACC AAC CCC 3′ and **JP3** 5′ GGG GAG GGG CGT TCT GCG AA 3′), previously designed (Fernandes et al., 1994) amplified a 120 bp fragment of a minicircle sequence of the parasite kDNA. Briefly, the PCR reaction mixture (50 μl total volume) consisted of PCR buffer 1× (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (Perkin-Elmer), 2.5 U/μl of *Taq* DNA polymerase (Perkin-Elmer), 200 μM of each deoxynucleoside triphosphate (dNTPs) (Pharmacia), 15 pmol of each primer and 2 μl of the DNA sample. In this reaction, DNA amplification was performed for a first cycle at 94 °C for 5 min of incubation, 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s with a final extension cycle of 10 min at 72 °C.

For the specific amplification of the species of the *L.* (*Viannia*) and *L.* (*Leihmania*) subgenera were used the oligonucleotide primers **B1** 5′ GGG GTT GGT GTA ATA TAG TGG 3′ and **B2** 5′ CTA ATT GTG CAC GGG GAG G 3′ and **M1** 5′ CCA GTT TCG AGC CCC GGA G 3′ and **M2** 5′ GGT GTA AAA TAG GGG CGG ATG CTC TG 3′, respectively. These primers amplified a 760 bp minicircle sequence of the kDNA common to each subgenera species. Briefly, the reaction mixture consisted of 20 pmol of each oligonucleotide primer, 300 μM of each deoxynucleoside triphosphate (dNTPs) (Pharmacia), 2.5 U of *Taq* DNA polymerase (Perkin-Elmer) in PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (Perkin-Elmer) and 3 μl of DNA (10 ng/μl). DNA amplification was performed for 35 cycles (De Bruijn and Barker, 1992; Eresh et al., 1994).

No DNA, *Lutzomyia* spp. male DNA and *L.* (*L.*) *tropica* DNA (MON 58/LEM 2578) were used as negative controls. Positive controls consisted of *L.* (*V.*) *braziliensis* (MHOM/PE/95/LQ2) and *L.* (*L.*) *amazonensis* (MHOM/77/LTB0016) DNA. PCR products were analysed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. Extensive precautions for avoiding cross-contamination of samples were taken.

All PCR products were transferred to a nylon membrane by a Southern blot procedure according to standard conditions (Sambrook et al., 1989). Two PCR amplified minicircle kDNA fragments from *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* strains were used as probes in different reactions. The amplified products were isolated from an agarose gel and purified by the Quiaex II gel extraction Kit (Quiagen). Twenty-five nanograms of each purified fragment were labelled with digoxigenin coupled to dUTP (deoxyuracile triphosphate) using the DIG DNA Labeling Kit and following the manufacturer procedure (Boehringer Mannhein). The blot was prehibrydized at 68 °C for 2 h in 5× SSC (0.15 M sodium chloride, 0.015 M trisodium citrate), 0.02% sodium duodecyl sulphate (SDS), 0.02% *N*-lauroylsarcosine, 2% blocking reagent (ROCHE). Hybridization with the specific *L.* (*V.*) *braziliensis* or *L.* (*L.*) *amazonensis* (25 ng) labelled probe, depending on the reaction, was performed at 68 °C overnight. Washing conditions were 2 min × 5 min in 2× SSC/0.1% SDS at room temperature and 2 min × 15 min in 0.1× SSC/0.1% SDS at 68 °C. The hybridized products were immunodetected with anti-digoxigenin-AP, Fag fragments and then visualized with the colorimetric substrates NBT/BCIP using the DIG Nucleic Acid Detection Kit (ROCHE).

## 3. Results

A total number of 2749 insects were collected in Tucuman province. *Lu. neivai* was the prevalent species (96.5%) captured in the area followed by *Lu. migonei* (3.2%) and *Lu. shannoni* (0.3%). Isolated captures performed in Salta resulted in 656 sand flies, with *Lu. neivai* as the prevalent species (94%) again. A sample of 470 *Lu. neivai* females (310 insects from Tucuman and 100 from Salta) were analysed for leishmanial infection.

The PCR performed with specific Phlebotominae primers confirmed a good quality of the Phlebotominae DNA in 44 of 47 samples. These 44 samples were analyzed in two steps. Firstly, a PCR-hybridization assay with *Leishmania* gender specific primers. Secondly, in our attempt to identify which subgenera the parasite belonged to, we performed two PCR-hybridization assays specific for the *L.* (*Viannia*) and *L.* (*Leishmania*) subgenera, respectively.

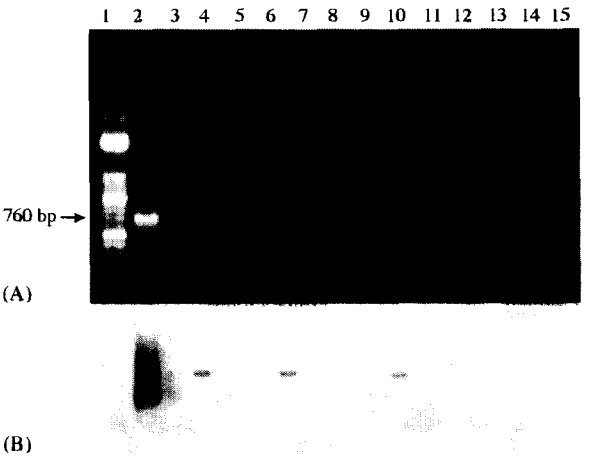


Fig. 1. (A) Polymerase chain reaction (PCR) amplification of *Leishmania* (*Viannia*) DNA in sand flies samples. Lane 1: molecular weigh marker XVI; lane 2: *L.* (*V.*) *braziliensis*, positive control; lanes 3–14: *Lutzomyia neivai* samples (lanes 4 and 6, positive samples from El Molino; lane 10, positive sample from Yánima); lane 15: *L.* (*L.*) *amzonensis*, negative control. (B) Hybridization with *Leishmania* (*Viannia*) *braziliensis* probe.

Four samples amplified the 120 bp fragment corresponding to a sequence of the kDNA of every *Leishmania* spp. (9.1%). The result was confirmed by Southern-blotting.

The second PCR assay revealed these four samples as positives for the subgenus *Viannia*. These results were confirmed by the Southern blot-hybridization of the PCR products with the specific *L.* (*V.*) *braziliensis* probe (Fig. 1). None sample amplified the kDNA sequence common to the species of the subgenus *Leishmania* in a third PCR assay. This result was confirmed with a Southern blot-hybridization with a specific probe for *L.* (*L.*) *amazonensis*. We exclude the possibility of PCR inhibition using these primers because the PCR specific primers for Phlebotominae detected the sand flies DNA in the 44 samples analysed and no false-positives findings were found.

Three *Lu. neivai* infected samples were collected in El Molino-Iltico, one in Yánima, Tucumán and one in El Oculto, Salta. Two of the infected samples from El Molino correspond to insects collected with a CDC trap placed in secondary forest (a tree trunk, posible Phebotominae shelter) and the other corresponds to a Shannon trap capture in the primary forest. The infected sample from Yánima was obtained with a CDC trap in the peridomestic habitat (pig pen) of a dwelling with human cases of the disease. The infected sample from

Table 1
Pools of *Lutzomyia neivai* females analysed for *Leishmania* infection organized by locality, habitat and trap method of captured in Tucumán and Salta provinces, Argentina

| Localities | Habitat | Trap type | Pools of *Lu. neivai* (Inf)[a] | Results of PCR-hybridization[b] | |
|---|---|---|---|---|---|
| | | | | Genus | Subgenus |
| Iltico | Primary forest | CDC | 3 | Neg | Neg |
| | | Shannon[c] | 3 | Neg | Neg |
| | Secondary forest | CDC | 4 | Neg | Neg |
| El Molino | Primary forest | Shannon | 1 (1) | *Leishmania* | *L. (Viannia)* |
| | Secondary forest | CDC | 3 (1) | *Leishmania* | *L. (Viannia)* |
| Yánima | Primary forest | Shannon | 2 | Neg | Neg |
| | Secondary forest | CDC | 2 | Neg | Neg |
| | Peridomestic habitat | CDC[d] | 7 (1) | *Leishmania* | *L. (Viannia)* |
| El Guayal | Secondary forest | CDC | 3 | Neg | Neg |
| El Cadillal | Primary forest | Shannon | 2 | Neg | Neg |
| El Oculto | Primary forest | CDC | 5 | Neg | Neg |
| | | Shannon | 3 | Neg | Neg |
| | Secondary forest | CDC | 9 (1) | *Leishmania* | *L. (Viannia)* |
| Total *Lu. neivai* pools | | | 47 | 4 | 4 |

[a] Pools of *Lu. neivai* analysed: each pool consists of 10 individuals females. (Inf): number of pools found infected by the molecular methods.
[b] Neg, negative pools of insects for *Leishmania* infection.
[c] Modified Shannon trap with protected human bait (Salomón et al., 1995).
[d] CDC light trap in relation to a pig pen in the peridomestic habitat of a dwelling with human related cases of leishmaniasis.

EL Oculto, Salta corresponded to a collection made with a CDC trap in secondary forest (Table 1).

## 4. Discussion

The oligonucleotides B1/B2 used in this assay are capable to detect 1 fg of purified DNA, or in the case of a pool of insects contain at least one parasite or 10 fg of kDNA (De Bruijn and Barker, 1992).

In our study, the PCR-hybridization for the *L. (Viannia)* subgenera was positive for 9.1% of the samples of *Lu. neivai* analyzed; and the proportion of infected sand flies was 0.9%. Further, no *Leishmania* parasites were found in any of 3341 *Lu. neivai* and 94 *Lu. migonei* females microscopically examined, that had been collected from the hyperendemic leishmaniasis area of Argentina (Salomón et al., 2004). The low proportion of females infected suggested by previous data and current results are consist with the sporadic nature of the outbreaks and the scarcity of cases during the inter-epidemic periods, even where populations of species with proven vectorial capacity and competence are abundant in peridomestic habitats. In this scenario, the infection of the vector became the "rare event" that can be related with the parasite offer from an unknown non domestic reservoir.

Pérez et al. (1994) in a study in Huarochiri, Perú, found a positivity of 1.1% by PCR for the *L. braziliensis* complex, subgenus *Viannia*, with a proportion of infected flies of 0.14%. Miranda et al. (2002), using a PCR with primers specific for *Leishmania* spp. found 8.9% of positive samples, with 0.4% of infected phlebotomines. Santos da Silva and Grunewald (1999) in a study in Rio Grande do Sul, Brazil demonstrated *Leishmania (Viannia)* infections using PCR in 0.6% for *Lu. pessoai* and 0.8% for *Lu. misionensis*.

This is the first report of naturally infected sand flies in Argentina. Further, up to our knowledge, this is also the first report of naturally infected *Lu. neivai* sensu strictu (Andrade-Filho et al., 2004) certainly identified since this species was revalidated from the *Lu. intermedia* complex (Marcondes, 1996). The infected insects found in this study corresponded to captures made just before the humid season which is in concordance with the higher density of phlebotomines in the environment in Tucumán province (Córdoba-Lanús et al., unpublished data; Córdoba-Lanús and Salomón, 2002). Consistently, Pérez et al. (1994) in a study in Perú, found *Lu. peruensis* naturally infected by *Leishmania* spp. in collections performed in the months before the warm-humid period. The infected phlebotomines belong to primary and secondary forest, and peridomestic habitat captures. These

sand flies collections proceed mainly from the southern localities of the province, where the highest incidence of the disease was reported. Miranda et al. (2002) proposed that determining the micro-regions where this pathology occurs in high frequency is fundamental for the comprehension of ecological and geographical factors which may influence the transmission of the disease.

The PCR-hybridization technique allowed us to identify the circulating parasite and probable causative agent of leishmaniasis in northwestern Argentina as belonging to the *Leishmania* (*Viannia*) subgenera, and contributed to its vector species incrimination in the area. Further studies are needed in order to characterize the parasite specie.

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