Multiplex-PCR for detection of natural *Leishmania* infection in *Lutzomyia* spp. captured in an endemic region for cutaneous leishmaniasis in state of Sucre, Venezuela

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We studied the natural infection of Lutzomyia (Lutzomyia) *sp. with* Leishmania *in endemic foci of cutaneous leishmaniasis in the Paria peninsula, state of Sucre, Venezuela. Sand flies were collected between March 2001 and June 2003, using Shannon light-traps and human bait. Of the 1291 insects captured, only two species of phlebotomines were identified:* L. ovallesi *(82.75%) and* L. gomezi *(17.42%). A sample of the collected sand flies (51 pools of 2-12 individuals) were analyzed by using a multiplex-PCR assay for simultaneous detection of New Word* Leishmania *and* Viannia *subgenera. The results showed a total of 8 pools (15.68%) infected; of these, 7 were* L. ovallesi *naturally infected with* L. braziliensis *(2 pools) and* L. mexicana *(5 pools) and 1 pool of* L. gomezi *infected by* L. braziliensis.

Key words: cutaneous leishmaniasis - sand flies vectors - natural infections - polymerase chain reaction - *Lutzomyia* spp. - Venezuela

Leishmaniasis in Venezuela has been endemic for more than a century. According to epidemiological reports from the Ministry of Health and Social Development, the Northeastern region (Anzoátegui and Sucre states) ranks as the fifth most important, with an annual average rate of prevalence of cutaneous leishmaniasis (CL) between 18 and 24 in 100,000 inhabitants (Bonfante-Garrido & Barroeta 2002). Identification of reservoirs and vectors and the determination of natural infection rates with *Leishmania* spp. parasites have been important for the definition of risk factors and epidemiological control of leishmaniasis in Venezuela (Añez et al. 1994, Alcais et al. 1997, Feliciangeli et al. 1999, Aransay 2000). However, the classical histopathological and dissection methods employed in these processes are time consuming and are not always sensitive enough for parasite species identification (Degrave et al. 1994, Davies et al. 1997, Dinesh et al. 2000, Rodríguez et al. 2002). In contrast, molecular biology techniques such as PCR, have resulted advantageous showing greater sensitivity, specificity, versatility and speed for the processing of large sample numbers (Noyes et al. 1998, Belli et al. 1998, Rodríguez et al. 1999, Zerpa 2000, Mendoza-León et al. 2001).

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Previous CL studies in endemic zones in eastern Venezuela have provided important data in relation to prevalence of the disease, epidemiological characterization of the affected populations and the identification of phlebotomine species with anthropophilic habits (Jorquera et al. 1989, González et al. 1999, 2002, Marchán et al. 2001). The present study provides relevant information about naturally infected phlebotomines captured from a highly endemic CL zone in Northeastern Venezuela (Marchán et al. 2001). For this purpose we employed a multiplex PCR system which allowed us, in a single-step assay, to detect phlebotomines infection by parasites of *Leishmania* and *Viannia* subgenera.

MATERIALS AND METHODS

Study area - We studied the area surrounding La Llanada de Cangua (10° 41'36" N and 62° 54'44" W., 199 m altitude) and La Viciosa (10° 42' 06" N and 62° 51'24" W, 32 m altitude) villages, both in the Arismendi municipality, in the Paria peninsula, state of Sucre (Fig. 1). The vegetation is made up of a wet thropophilic forest of marine influence, with annual average temperatures up to 24°C and annual rainfall between 600-1200 mm. The major economic activity of the region consists of the production of root vegetables and fruit, as well as fishing using traditional methods, typical of the coastal regions.

Sand flies collections and species identification - Sand flies were captured between March 2001 and June 2003, using Shannon light-traps and human bait. The traps were set from 18:00 to 20:00 in the forests around the dwellings where at least one member of the family was CL positive. Captured insects were transported in humid chambers to be processed in situ.

For dissection, phlebotomines were killed with excess CO_2 and immediately put on glass slides in a physiologi-

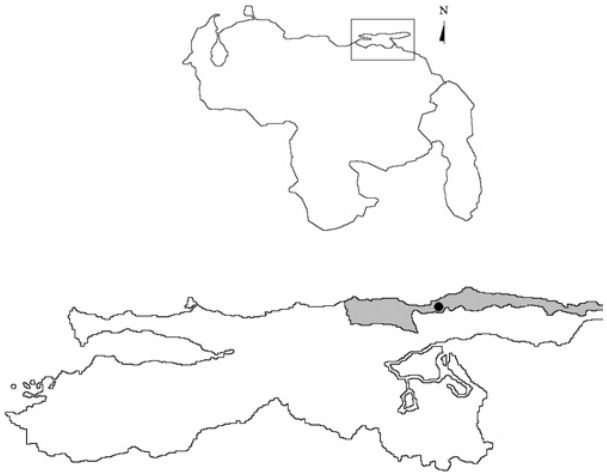

Fig. 1: map showing relative location of studied localities in the Paria peninsula, state of Sucre, Venezuela. Black circle show the area where sand flies were collected.

cal solution (NaCl, 0.85%). Taxonomic identification of each specimen was undertaken according to criteria defined by Young and Duncan (1994).

PCR assays - After dissection and specimen identification, we prepared DNA crude extracts from pools of 2-12 females of the same species. Briefly, the insects were placed directly in Eppendorf tubes containing 100 µl of Chelex-100 5% solution (Biorad), shaken vigorously for 5 min and heated in a water bath at 100°C for 10 min; after that, the samples were centrifuged at 13,600 g × 1 min. and the supernatant stored at –20°C until the PCR assay. The same procedure was applied for preparation of PCR controls, consisting of crude extracts from two non-infected female *L. longipalpis* or *L. youngi* (negative controls) or females experimentally infected with *L. amazonensis* (MHOM/BR/67/PH8) (positive control), proceeding from our laboratory colonies.

For the detection of *Leishmania* DNA in the samples, we used the procedure described by Harris et al. (1998). This consists of a one step multiplex assay for simultaneous detection of the genome of *Leishmania* (*L.*) and *Viannia (V.)* New Word parasites. Primers used were sequences of multicopy spliced leader RNA (mini-exon) gene able to cluster the New Word *Leishmania*, based in the gene size and sequence of the intergenic región, in three groups: *Viannia* species, dermotropic *Leishmania* species and *L.* (*L.*) *chagasi* (Degrave et al. 1994). The primers and their sequences are as follows: LU-5A, 5'-TTTATTGGTATGCGAAACTTC-3', which corresponds to a highly repetitive and conserved sequence in all *Leishmania* species and LM-3A, 5'-GCACCGCACCGG(A/G)CCAC-3'; LB-3C, 5'-CGT(C/G)CCGAACCCCGTGTC-3'; and LC-3L, 5'-GCCCGCG(C/T)GTCACCACCAT-3' which generate products of size range 218-240 bp for *L.* (*Leishmania*), 146-149 bp for *L. (Viannia)* and 351-397 bp for *L. chagasi* (Harris et al. 1998). Primers were synthesized by Operon Technologies Inc. (Alameda, CA) and kindly donated by Dr María Elena Peñaranda, Scientific Header of Sustainable Sciences Institute (San Francisco, CA). All the pools founded as positive for *Leishmania* were confirmed by a second determination.

For DNA amplification, 2.5 µl of each sample was added to 22.5 µl of reaction mixture containing KCl 50 mM, Tris buffer 10 mM (pH 8.3), 0.2 mM each deoxynucleotide triphosphate, $MgCl_2$ 1.5 mM, DMSO 10.5%, tetramethyl ammonium chloride 50 mM, betaine 0.6 M, dithiothreitol 1mM, LU-5A probe 0.4 µM, 0.2 µM each 3' primer (LM-3A, LB-3C and LC-3L) and, *Taq* DNA polymerase 0.04 U/µl (Sigma). Amplification was undertaken in a Thermolyne-Amplitron II thermal cycler, using an initial denaturation step of 95°C x 5 min, followed by 35 cycles of 95°C x 30 s, 54 °C x 45 s, and 72°C x 30 s with a final extension at 72°C x 5 min.

Finally, 8 µl of the amplification products were analyzed by electrophoresis on 1.5% agarose gel in TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.3) containing ethidium bromide 0.5 µg/ml. The amplification products were visualized under UV light and the gels documented by a Kodak DC 120 camera.

RESULTS

Sand fly species identification and abundance - Of the 1291 insects captured, only 2 species of phlebotomines were identified: *Lutzomyia ovallesi* and *L. gomezi*, with relative abundances of 82.75 and 17.42% respectively (Table I). The unique presence of these two species and the predominance of *L. ovallesi* were constant characteristics throughout the study.

PCR assays - For analyses of naturally infected insects, 461 *L. ovallesi* and 88 *L. gomezi* females (42.29% of the total number of insects captured) were grouped in 51 pools and PCR tested. In total, 8 pools (15.68%) were found infected by *Leishmania* (Table II), 7 of them correspond-

TABLE I

Number and species of *Lutzomyia* (♀) captured according to collection method and locality. State of Sucre, Venezuela, March 2001-June 2003

Species	Localities				Total	
	La Viciosa (32 m)		La Llanada de Cangua (199 m)			
	Light trap	Human bait	Light trap	Human bait	Light trap	Human bait
Lutzomyia ovallesi	196	17	719	134	915	151
L. gomezi	96	41	59	29	155	70
Total	292	58	778	163	1070	221

m: meters of altitude above see level

ing to *L. ovallesi* naturally infected with *L.* (*Viannia*) species (2 pools) and *L.* (*Leishmania*) species (5 pools). Additionally, *L.* (*Viannia*) parasites were detected in the one pool of *L. gomezi* infected (Fig. 2, line 10). It is important to point out that approximately one third of naturally infected pools (3 out of the 8) were captured using human bait, suggesting a high risk of leishmaniasis infection in this region of the country.

DISCUSSION

Considering that at least one specimen was infected in each positive pool, we estimate a minimal infection of 1.3% for the total sample evaluated (51 pools) and relative values of 1.5% and 0.64% for *L. ovallesi* and *L. gomezi* respectively. In general these values are high in comparison with reported infection values from different regions of Venezuela, calculated using similar methodologies (Feliciangeli & Rabinovich 1988, Feliciangeli et al.1999, Rodríguez et al. 2002). However, percentage infections of this magnitude would sustain the relatively high CL average annual rates of prevalence for state of Sucre (24 cases in 100,000 inhabitants) reported by Bonfante-Garrido and Barroeta (2002), and explain the findings of Marchán et al. (2001) who reported that between 1995 and 1998, half of the accumulated CL incidence in Venezuela occurred in the zone selected for the present study.

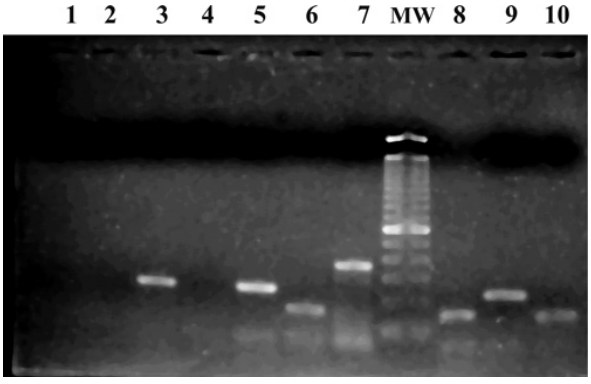

Fig. 2: multiplex-PCR products after amplification with primers LU-5A, LB-3C, LM-3A, and LC-3L for detection of *Leishmania* DNA within sand flies captured in La Llanada de Cangua and La Viciosa in Arismendi municipality, state of Sucre, Venezuela. Lines 1: negative control (water); 2: *Trypanosoma cruzi* (EP stock); 3: pool of 2 females of *Lutzomyia longipalpis* experimentally infected with *L. mexicana amazonensis* (L ma: M112,IFLA/RR67/PH8) (positive control); 4: pool of 2 non-infected females *L. longipalpis* (negative control); 5: positive control *L. mexicana* (Lm: MHOM/VE/80/NR); 6: positive control *L. braziliensis* (MHOM/BR//84/LTB300); 7: positive control *L. donovani chagasi* (Ldch: MHOM/BR/74/PP75); MW: 100 pb DNA ladder (BioLabs; lowest band shown, 100 pb); 8: pool of 10 females *L. ovallesi* captured in La Viciosa; 9: pool of 12 females *L. ovallesi* captured in La Llanada de Cangua; 10: pool of 12 females *L. gomezi* captured in La Viciosa.

According to original descriptions carried out by Harris et al. (1998) the multiplex assay employed in this study is highly sensitive and specific for the simultaneous detection of parasites belonging to *Viannia* subgenus (detecting as little as 0.01 parasites/sample) and *Leishmania* subgenus (detecting 10-100 parasites/sample). In addition, the simplicity of sample preparation allows us to consider the present PCR assay appropriate to be applied in field work conditions, due to the fact that crude extracts of whole insects or the digestive tract can be easily preserved and used without requiring previous DNA purification.

Furthermore, in our case, the validity and sensitivity of assay were assessed by including positive controls prepared from only two *L. longipalpis* or *L. youngi* individuals experimentally infected, that gave specific signals for the 218-240 pb fragment, corresponding to parasites from the *Leishmania* subgenus (Fig. 2, line 3).

The use of PCR for DNA detection in sandflies captured in the field is a useful procedure for detecting *Leishmania* infection in large numbers of specimens, identifying species suspected as vectors of leishmaniasis, without the common mistake of considering all motile flagellates in sandfly guts as indication of *Leishmania* infection (Perez et al. 1994, Rodríguez et al. 1999). By the other hand, despite the fact that the finding of infected insects among pools of captured females is no guarantee of transmission of leishmaniasis, it is a condition that should be considered in the definition of transmission risk for man and other hosts that could be reservoirs of the parasites in nature, contributing to understand the epidemiology of leishmaniasis in endemics areas.

Our results indicate the circulation of two phlebotomine species naturally infected with *Leishmania* parasites from both the *Leishmania* and *Viannia* subgenera, without wishing to underestimate the importance of *L. gomezi* as a Leishmaniasis vector, *L. ovallesi* was shown to be both abundant and capable of carrying parasites from both subgenera, and thus should be considered as one of the principal vectors of CL in this region, as for other endemic zones in our country (Bonfante-Garrido et al. 1991, Gómez et al. 1998, Feliciangeli & Rabinovich 1998).

At present we are analyzing samples of cutaneous

TABLE II

Number of pools[a] of *Lutzomyia ovallesi* and *L. gomezi* naturally infected by *Leishmania* sp.

Species	*L. ovallesi*		*L. gomezi*		Total	
Locality	Light trap	Human bait	Light trap	Human bait	Light trap	Human bait
La Viciosa	3/14	0/2	0/6	1/2	3/20	1/4
La Llanada de Cangua	2/17	2/5	0/2	0/3	2/19	2/8
Total	5/31	2/7	0/8	1/5	5/39	3/12

a: numbers of infected pools/total pools examined.

tissue from patients diagnosed with CL using the same PCR system, in order to establish the relationship between the natural infections found in the phlebotomines and the taxonomy of the infective parasites taken from the inhabitants of the region.

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