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 in these processes, typical of the coastal regions.

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 phlebotomines 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 therophophilic 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 CO₂ and immediately put on glass slides in a physiologi-
PCR for natural infection of Lutzomyia • Alicia Jorquera et al.

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 World parasites. Primers used were sequences of multicopy spliced leader RNA (mini-exon) gene able to cluster the New World Leishmania, based in the gene size and sequence of the intergenic region, 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'-TTATGGTAGTAGCGAATTTC-3', which corresponds to a highly repetitive and conserved sequence in all Leishmania species and LM-3A, 5'-GCCGCCAGGACCG(A/G)CCAC-3'; LB-3C, 5'-CGT(G/C)CGAAGGCGTGTC-3'; and LC-3L, 5'-GCCGCCAGG(T/G)GTCAACCACAT-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, MgCl2 1.5 mM, DMSO 10.5%, tetramethyl ammonium chloride 50 mM, betaine 0.6 M, diithiothreitol 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 |

<table>
<thead>
<tr>
<th>Species</th>
<th>La Viciosa (32 m)</th>
<th>La Llanada de Cangua (199 m)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light trap</td>
<td>Human bait</td>
<td>Light trap</td>
</tr>
<tr>
<td>Lutzomyia ovallesi</td>
<td>196</td>
<td>17</td>
<td>719</td>
</tr>
<tr>
<td>L. gomezi</td>
<td>96</td>
<td>41</td>
<td>59</td>
</tr>
<tr>
<td>Total</td>
<td>292</td>
<td>58</td>
<td>778</td>
</tr>
</tbody>
</table>

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.

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 sandflies guts as indication of Leishmania infection (Perez et al. 1994, Rodriguez 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>L. ovallesi</th>
<th>L. gomezi</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locality</td>
<td>Light trap</td>
<td>Human bait</td>
<td>Light trap</td>
</tr>
<tr>
<td>La Viciosa</td>
<td>3/14</td>
<td>0/2</td>
<td>0/6</td>
</tr>
<tr>
<td>La Llanada de Cangua</td>
<td>2/17</td>
<td>2/5</td>
<td>0/2</td>
</tr>
<tr>
<td>Total</td>
<td>5/31</td>
<td>2/7</td>
<td>0/8</td>
</tr>
</tbody>
</table>

\(^a\): 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.

ACKNOWLEDGEMENTS

To Leomery Romero for technical assistance in PCR assays, to Prof. Leonardo De Sousa for his comments and suggestions and to Dr Frances Osborn for English language revision of the manuscript.

REFERENCES


