ISOLATION OF *LEISHMANIA (VIANNIA) BRAZILIENSIS* FROM *LUTZOMYIA SPINICRASSA* (SPECIES GROUP *VERRUCARUM*) MORALES OSORNO MESA, OSORNO & HOYOS 1969, IN THE VENEZUELAN ANDEAN REGION

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Summary:

Natural infection with *Leishmania* spp. in phlebotomine sandflies was searched for during a longitudinal study carried out from July 1997 to July 1998 in the village Catarnica, Municipality Independencia, Táchira State. This hamlet is an old endemic focus of cutaneous leishmaniasis in the Venezuelan Andean region, which lies close to the Colombian border at 1,300 m a.s.l., in an agricultural area mainly used for cultivating coffee. Phlebotomine sandflies were collected using Shannon traps placed in the peridomestic habitat from 19:00 to 21:00 hs. Males were stored in alcohol 70 % while females were kept in Nunc vials with 10 % DMSO and cryopreserved in liquid nitrogen for subsequent dissection and identification. The most abundant anthropophilic species was *Lutzomyia spinicrassa* with 3,032 males and 4,290 females (85.4 %). Among 1,633 (38 %) females of *Lu. spinicrassa* dissected, 26 (1.6 %) were infected with promastigotes, while no natural infection was found in 209 females of other species. The flagellates were identified as *Leishmania braziliensis braziliensis* using PCR with species specific primers derived from nuclear DNA and hybridization using species specific probe labelled with digoxigenin. This parasite had been previously isolated from patients with cutaneous leishmaniasis from the same area. These results show *Lu. spinicrassa* as a new proven vector of *Leishmania braziliensis* in the Andean region of Venezuela.

KEY WORDS : *Lutzomyia spinicrassa*, vector, *Leishmania braziliensis*, Venezuela.

Résumé : ISOLEMENT DE *LEISHMANIA (VIANNIA) BRAZILIENSIS* CHEZ *LUTZOMYIA SPINICRASSA* (GROUPE *VERRUCARUM*) MORALES OSORNO MESA, OSORNO & HOYOS 1969, DANS LA RÉGION ANDINE DU VENEZUELA

L'infestation naturelle des phlébotomes par Leishmania *spp. a été l'objet d'une étude longitudinale menée de juillet 1997 à juillet 1998 dans le village de Catarnica, municipalité d'Independencia, État de Táchira. Ce hameau est connu de longue date pour être un foyer de leishmaniose cutanée dans cette région andine du Venezuela à la frontière avec la Colombie, à une altitude moyenne de 1 300 m, et où l'on cultive principalement le café. Les phlébotomes ont été capturés avec des pièges de Shannon placés en zone péridomestique de 19 à 21 heures. Les mâles ont été conservés dans de l'alcool à 70 %, tandis que les femelles ont été placées dans des ampoules de Nunc avec 10 % de DMSO et conservées dans de l'azote liquide pour les dissections et identifications ultérieures. L'espèce anthropophile la plus souvent capturée a été* Lutzomyia spinicrassa *: 3 032 mâles ; 4 290 femelles (85,4 %). Sur les 1 633 femelles (38 %) de* Lu. spinicrassa *disséquées, 26 (1,6 %) étaient porteuses de promastigotes, tandis qu'aucune infestation naturelle n'était observée chez les 209 femelles d'autres espèces. Par PCR, ces flagellés ont été identifiés comme étant* Leishmania braziliensis braziliensis. *Ce parasite avait été isolé précédemment chez des personnes atteintes de leishmaniose cutanée dans la même région. Les résultats prouvent que* Lu. spinicrassa *est un nouveau vecteur de* Leishmania braziliensis *dans la région andine du Venezuela.*

MOTS CLÉS : Lutzomyia spinicrassa, *vecteur,* Leishmania braziliensis, *Venezuela.*

INTRODUCTION

Cutaneous leishmaniasis (CL) in Venezuela is endemic and focal, but distributed almost all over a country where the population is about 25,000,000. The number of cases reported by the Ministry of Health during the period 1970-2003 was 54,624 with an incidence of 11 in 100,000 inhabitants in the last five years; 98.5 % were diagnosed as localized cutaneous leishmaniasis (LCL), 0.8 % as muco-cutaneous leishmaniasis (MCL) and 0.16 % as diffuse leishmaniasis (DL) (from Files of the Departmento de Informática, Instituto de Biomedicina, Caracas).

About 100 species of phlebotomine sandflies are known in Venezuela, 30 of which are anthropophilic and eight are recognized as suspected or proven vectors of CL (Feliciangeli, 1991).

In Southern Venezuela, the Amazonian region, no studies on the natural infection in phlebotomine sandflies have been carried out and *Lutzomyia umbratilis* Ward & Fraiha 1978, *Lutzomyia anduzei* (Rozeboom, 1942), *Lutzomyia flaviscutellata* (Mangabeira, 1942) and species in the subgenus *Psychodopygus* are suspected to be responsible for the transmission of CL on the basis of their anthropophily and because elsewhere they are recognized as proven vectors.

In Northern Venezuela, three species have been found infected with parasites that have been identified either by molecular methods or enzyme profiles: *Lutzomyia*

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ovallesi (Ortiz, 1952), *Lutzomyia panamensis* (Shannon, 1926) and *Lutzomyia gomezi* (Nitzulezcu, 1931). *Lutzomyia ovallesi* is the most widespread vector of CL below 800 m a.s.l. in Venezuela, and has been found infected in several foci: El Ingenio, Miranda State (north-central Venezuela) with *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) mexicana* (Feliciangeli, 1991; Feliciangeli *et al.*, 1994; Barrios *et al.*, 1994); in Duaca, Lara State (west-central Venezuela) it was found harboring a hybrid *Le. braziliensis-Le. guyanensis* (Bonfante-Garrido *et al.*, 1991) and recently in Eastern Sucre State (La Viciosa and La Llanada de Cangua) where with multiplex-PCR it was proven to harbor *Le. braziliensis* (Jorquera *et al.*, 2005). Parasites isolated from *Lu. panamensis* caught in Urama, Carabobo State, have also been identified as *Le. braziliensis* by PCR and species specific hybridization (Rodriguez *et al.*, 2002), confirming its role in the transmission of CL as proposed by pioneer researchers (Pifano *et al.*, 1959). *Lutzomyia gomezi* has also been found naturally infected with *Le. braziliensis* in El Ingenio (Feliciangeli *et al.*, 1994) and in La Viciosa (Jorquera *et al.*, 2005). However, because of poor man-vector contact (Gomez *et al.*, 1998) and its scant density, this species is considered a secondary vector (Feliciangeli & Rabinovich, 1998).

In the Andean region, the high anthropophilic *Lutzomyia youngi* Feliciangeli & Murillo 1975 [cited as *Lutzomyia townsendi* (Ortiz)] is considered to be the vector of CL because it was found infected with perypiloric promastigotes (Scorza *et al.*, 1984) and the experimental transmission to hamsters was achieved (Scorza & Añez, 1984).

In this paper, we give evidence, for the first time, on the role of *Lutzomyia spinicrassa* Morales Osorno Mesa, Osorno & Hoyos, 1969, as responsible for the transmission of *Le. braziliensis* in the Venezuelan Andean State of Táchira.

MATERIALS AND METHODS

Study area

A study on the natural sandfly infection with *Leishmania* spp. was carried out in the village of Catárnica, Municipality Independencia, Táchira State (72° 20' W-7° 53' N; 1,300 m a.s.l.), Venezuela (Fig. 1), during a long-term entomological study (July 1997-July 1998). Weekly sandfly collections were made using Shannon traps from 19:00 to 21:00 h (Perruolo, 2004). This village is an endemic focus of CL, whose inhabitants (n = 361 in 72 houses) mainly till the land, especially for coffee. This locality, lying in a pre-montane dry forest (Ewel & Madriz, 1968), has an average temperature of 18°-24° C and a variable precipitation that, during the year of study, was less than 200 mm (Perruolo, 2004). Between 1996-2004, 16 cases of cutaneous leishmaniasis were registered in Catarnica, which means an incidence of 0.55 cases per 100 inhabitants per year.

Fig. 1. – Map showing the relative location of the village Catárnica in Táchira State and in Venezuela.

Sandfly identification and search for *Leishmania* spp.

Males were kept in glass vials with 70 % ethylic alcohol, and later clarified in Nesbitt' s solution and mounted in Berlese medium. Batches of 20-25 females were stored in Nunc vials with 10 % dimethylsulfoxide (DMSO) and cryopreserved in liquid nitrogen (Young *et al.*, 1987) for posterior search for *Leishmania* parasites. Female dissection was performed in sterile PBS, pH = 7.2, using sterile slides, cover slips and needles. When flagellates were seen, the gut was disrupted and the solution was transferred to Eppendorf tubes containing 25 µl of lysis buffer (10 mM Tris–HCl pH 8, 10 mM EDTA, 0.1 % SDS, 10 mg/ml proteinase K). The slides were then stained with Giemsa in order to confirm the presence of promastigotes.

Heads, male genitalia, cibaria, spermathecae and other diagnostic morphological characters of the phlebotomine sandflies were examined with a phase contrast microscope (× 400) and the sandfly species identification was achieved using dichotomic keys by Young & Duncan (1994).

Identification of *Leishmania* spp.

Leishmania identification was carried out according to Rodriguez *et al.* (1999). The samples were heated for one hour at 56° C, and the DNA was extracted with

phenol:chloroform (1:1). The DNA was precipitated from the aqueous phase with 10 µl of 4 M LiCl and 100 % ethanol. PCR was performed with 2 µl of DNA sample (100 ng) resuspended in 25 µl of a reaction mix containing 200 ng of each primer 3J1 = 5'-TAC CTG ATG ACT CCA C-3' and 3J2 = 5'-CCT CAT CAT ACC GTT GAT C-3' for *Le. (V.) braziliensis* identification. The DNA was amplified in a thermocycler (PT-100 MJ Research, USA) during 35 cycles, each cycle consisting of one min at 94°C, one min at 60°C for annealing and one min at 72°C for extension; and a final extension at 72°C for 10 minutes more was permitted. PCR of negative controls such as other *Leishmania (Viannia)*, *Leishmania (Leishmania)* subgenera, *Trypanosoma (Schizotrypanum) cruzi*, *T. (Schizotrypanum) evansi* and non-infected sandfly DNAs was also performed.

Species specific identification was assessed by dot blot hybridization of the PCR product with LbJ38 probe for *Le. (V.) braziliensis*. The DNA probe was labelled using the digoxigenin-anti digoxigenin kit (Boehringer-Mannhein, Germany) according to the instructions of the manufacturer. Labelling and detection of the hybridized product was carried out as previously described (Rodriguez *et al*, 1999).

RESULTS

Table I shows species composition, number of males and females caught (sex ratio 1:1.28), number and proportion of females dissected for searching *Leishmania* spp. in Catárnica, Táchira State, Venezuela, from July 1997 to July 1998. A total of 5,127 females were collected and 1,842 females were dissected (35.9 %). The most abundant anthropophilic species was *Lu spinicrassa* with 3,032 males and 4,290 females (85.4 %). Among the 1,633 (38 %) females of *Lu. spinicrassa* dissected, 26 (1.6 %) were found infected with promastigotes while no natural infection was found in 209 females of other species. The parasites where seen attached to the epithelium of the pylorus, the abdominal and thoracic midgut, some of them close to the stomodeal valve. A few free flagellates were also scattered in the ileum.

Figure 2 shows the monthly fluctuations of males and females of *Lu. spinicrassa* and the months in which sandflies were found infected by *Leishmania* parasites. The sandfly population abundance was negatively asso-

Species	Males		Females		Dissected	
	No.	%	No.	%	No.	%
L. spinicrassa	3,032	(75.4)	4,290	(83.67)	1,633	(38.1)
Lu. gomezi	820	(20.39)	639	(12.46)	158	(24.7)
Lu. nuneztovari	70	(1.7)	134	(2.61)	47	(35.1)
Lu. ovallesi	72	(1.79)	51	(0.99)	2	(3.92)
Lu. shannoni	25	(0.62)	13	(0.25)	2	(15.4)
B. beaupertuyi	1	(0.02)	0	(0)	–	–
Lutzomyia sp.	1	(0.02)	0	(0)	–	–
Total	4,021	100	5,127	100	1,842	(35.9)

Table I. – Sandfly species composition, abundance and search for *Leishmania* spp. in Catarnica, Táchira State, Venezuela.

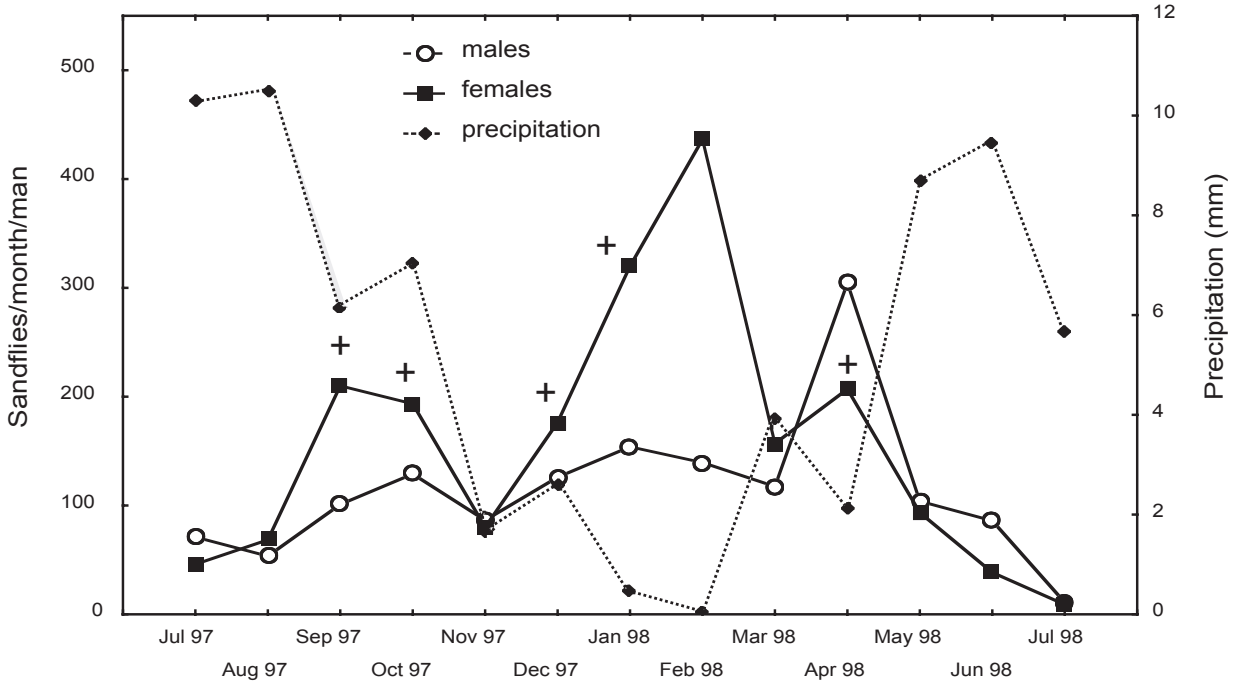

Fig. 2. – Monthly fluctuations of *Lutzomyia spinicrassa* in Catárnica, Táchira State, Venezuela and occurrence of infected females (+).

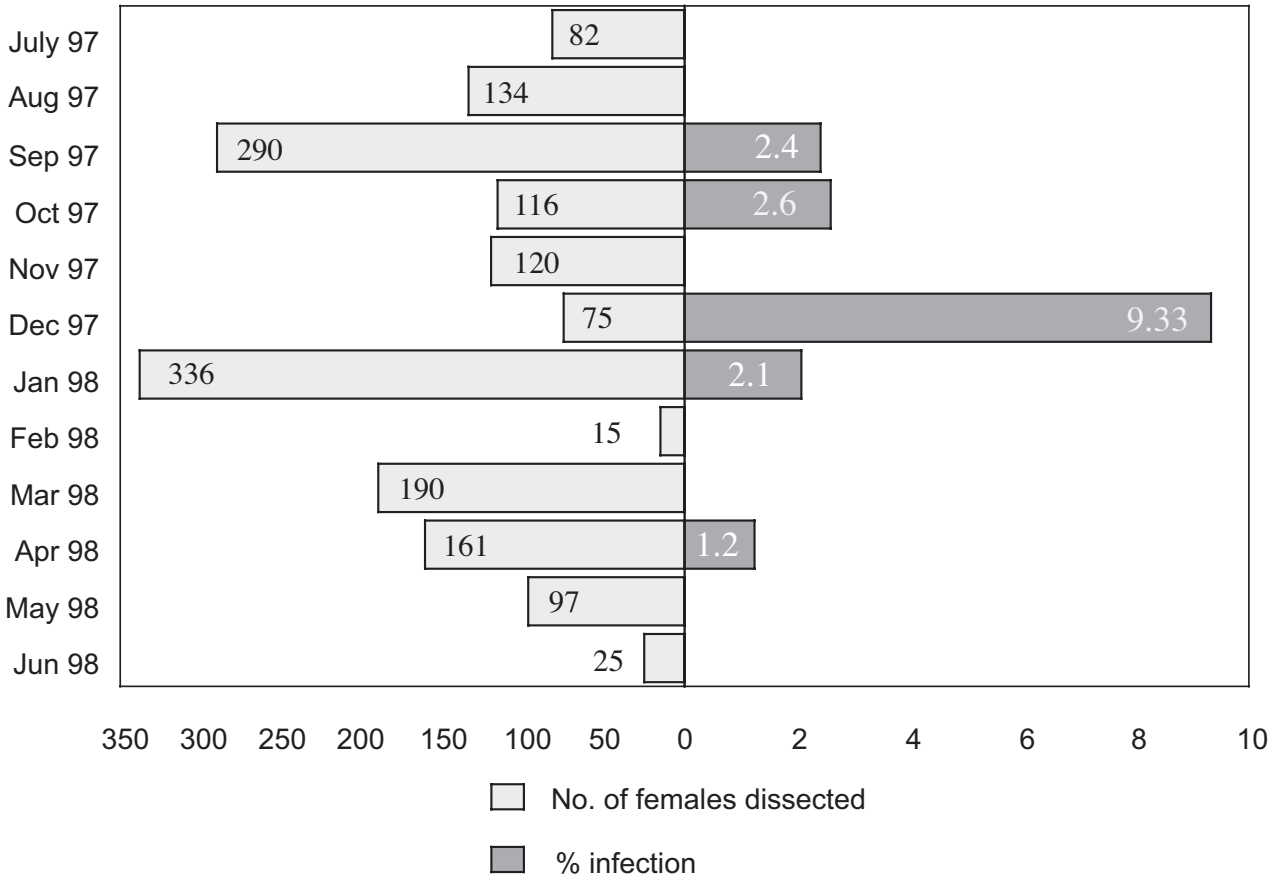

Fig. 3. – Monthly rates, July 1997-June 1998, of naturally infected *Lutzomyia spinicrassa* in Catárnica, Táchira State, Venezuela.

ciated with precipitation, with two peaks being observed, in July-August, 1997 and May-June, 1998 when rains were very scanty. Figure 3 gives the number of *Lu. spinicrassa* dissected and the monthly rates of infection.

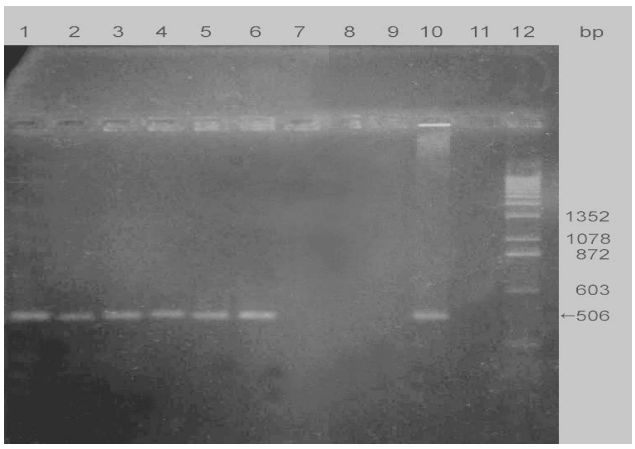

Fig. 4. – Polymerase chain reaction (PCR) products obtained after 35 amplification cycles with total DNA of *Lutzomyia* spp and controls: Lanes 1-6: *L. spinicrassa*; Lanes 7-9: negative samples; Lane 10: *Le. braziliensis* LTB300; Lane 11: negative control; Lane 12: molecular size markers.

The highest proportion of infection (9.3 %) was detected in December. Figure 4 is an example of the results of the polymerase chain reaction where the diagnostic band of 506 bp was observed in six samples of *Lu. spinicrassa* infected with promastigotes. The diagnostic band was not observed in other *Lutzomyia* species nor in any of the PCR carried out with other *Leishmania (Viannia)*, *Leishmania (Leishmania)*, *T. cruzi*, *T. evansi* and the non-infected sandfly DNAs used as negative controls. Hybridization with *Le. (V.) braziliensis* species specific probe also confirmed the PCR result (result not shown). The flagellates were therefore identified as *Leishmania braziliensis braziliensis* Vianna 1911.

DISCUSSION

Lutzomyia spinicrassa is a phlebotomine sandfly belonging to the species group *Verrucarum*, series *townsendi* (Kreutzer *et al.*, 1990; Feliciangeli *et al.*, 1992; Young & Duncan, 1994). Females in this series show four teeth in the cibarium and sac-like spermathecae that make them indistinguishable. However, the

males of *Lu. spinicrassa* are easily separated from other males in the series because of the thick and S-shaped terminal spine of style. Moreover, the egg chorionic ultrastructure also allows this to be distinguished from the closely allied species *Lu. townsendi* and *Lutzomyia youngi* which are present elsewhere in Venezuela (Feliciangeli *et al.*, 1993). During the year long study carried out in Catárnica, only males of *Lu. spinicrassa* were caught, so we are confident that all the females with four teeth and sac-like spermathecae were *Lu. spinicrassa*. This species is an aggressive man-biter with a geographical distribution restricted to Colombia and Venezuela where there are abundant coffee plantations (Young & Duncan, 1994). In both countries it was observed to be highly endophilic (Alexander *et al.*, 1992; Young *et al.*, 1987; Maingon *et al.*, 1994), and it has also been found naturally infected with promastigotes. However, the rate of natural infection was only 0.04 % in Colombia *vs* 1.6 % in Venezuela (this work). In Colombia, the parasite was identified as *Le. braziliensis* by isozymes analysis (Young *et al.,* 1987) while in Venezuela a PCR product of 506 bp corresponding to the predicted diagnostic band for *Le. (V.) b. braziliensis* was observed and then confirmed after hybridization of the PCR product to LbJ38 specific probe. A high hybridization signal was observed in the PCR positive samples, while no signal was observed with *Le. (Le.) mexicana* DNA sample or any other negative controls previously mentioned. PCR and hybridization are very useful techniques for species specific identification of parasites in naturally infected sandflies as was previously demonstrated by Rodriguez *et al.* (1999) mainly when parasites in the gut of the specimen are not very abundant. Here we report the utility of primers and probe derived from the nuclear DNA (Rodriguez *et al.*, 1997) in the specific identification of *Le. (V.) b. braziliensis* in *Lu. spinicrassa*, which is a reason for incriminating this sandfly species as a vector of this parasite species.

The *Leishmania* infection in *Lu. spinicrassa* in Catárnica predominates during the last months of the year. However, because of the low endemicity (0.55 cases per 100 inhabitants per year) it is hard to correlate monthly sandfly infection rate with monthly distribution of cutaneous leishmaniasis. For instance, no cases of CL were observed during the year of study.

In Táchira State, Maingon *et al.* (1994) studied several samples from human lesions of CL in the Municipality Independencia, and found that *Le. braziliensis* was the most common parasite (70 %). Therefore, because of the strong anthropophilic habits and the repeated isolation and identification of the same species of *Leishmania* from this sandfly as well as in CL patients, according to the criteria suggested by Killick-Kendrick (1990), we conclude that *Lu. spinicrassa* is a vector species of *Le. braziliensis* in the Western Andean region of Venezuela.

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