THE RE-EMERGENCE OF AMERICAN VISCERAL LEISHMANIASIS IN AN OLD FOCUS IN VENEZUELA. II. VECTORS AND PARASITES

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Summary :

As part of an epidemiological study in an old focus of American Visceral Leishmaniasis (AVL) in Venezuela (Guayabita, Aragua State), a longitudinal entomological survey (January 1993-June 1994) was carried out. A total of 3,239 males and 6,043 females belonging to 11 phlebotomine sandfly species were collected. The two recognised vectors of AVL in the New World, *Lutzomyia evansi* and *Lu. longipalpis* were found to be sympatric. *Lutzomyia evansi* was the dominant species (86.4 %), almost ten fold times more abundant than *Lu. longipalpis* (10.6 %). The two species alternated seasonally: *Lu evansi* peaked at the end of the rainy season while *Lu. longipalpis*, almost virtually absent during such period, increased in the dry season. This species seems more greatly influenced by the temperature. Seven of 4,559 *Lutzomyia evansi* (0.15 %) and one of 353 *Lu. longipalpis* (0.28 %) were found positive for suprapyloric promastigotes. Using the polymerase chain reaction (PCR) with universal primers, all isolates were identified as *Leishmania* spp. Two cultures from *Lu. evansi*, IEVA/VE/93/UCNA-2 and IEVA/VE/93/UCNA-3, were established. k-DNA restriction analysis showed high homologies between these isolates and *Leishmania chagasi*. High hybridization signal with *L. chagasi* specific kDNA confirmed these results. These findings suggest that *Lu. evansi* may play a role as vector of visceral leishmaniasis in this area. The identity of the parasite carried by *Lu. longipalpis* needs to be confirmed.

KEY WORDS : American visceral leishmaniasis, AVL, vectors, *Lutzomyia evansi*, *Lutzomyia longipalpis*, *Leishmania chagasi*, Venezuela.

Résumé : RÉÉMERGENCE DE LA LEISHMANIOSE VISCÉRALE AMÉRICAINE DANS UN FOYER ANCIEN DU CENTRE-NORD DU VÉNÉZUELA. II. VECTEURS ET PARASITES

Une étude entomologique longitudinale (janvier 1993-juin 1994) a été réalisée dans le village de Guayabita, État Aragua, un foyer ancien de leishmaniose viscérale du centre-nord du Vénézuela (10°16'N, 67°28'W; 500 m). Au total, 3239 mâles et 6043 femelles appartenant à 11 espèces de phlébotomes ont été capturés en utilisant des pièges CDC et Shannon. Lutzomyia evansi *et* Lu. longipalpis, *les deux espèces reconnues comme vecteurs de leishmaniose viscérale dans le Nouveau Monde ont été rencontrées en sympatrie dans ce foyer.* Lutzomyia evansi *prédomine nettement (86,4 %) sur* Lu. longipalpis *(10,6 %). Les deux espèces se succèdent selon la saison :* Lu. evansi *domine à la fin de la saison des pluies alors que* Lu. longipalpis, *virtuellement absent à cette époque de l'année, augmente à la saison sèche; cette espèce serait fortement influencée par la température. Sept exemplaires sur 4559* Lutzomyia evansi *(0,15 %) et un spécimen sur 353* Lu. longipalpis *(0,28 %) étaient naturellement infectés par des promastigotes suprapyloriques. En utilisant la PCR avec des amorces universelles, toutes les souches isolées ont été identifiées comme Leishmania spp. La multiplication des parasites à partir du tube digestif de deux* Lu. evansi, *IEVA/VE/93/UCNA-2 et IEVA/VE/93/UCNA-3 a été obtenue. L'analyse de restriction du k-DNA a montré une grande homologie entre les souches isolées et* Leishmania chagasi. *Un haut signal d'hybridation avec kDNA spécifique de* Le. chagasi *a confirmé ces résultats qui ont permis de conclure au rôle vecteur de Lu. evansi dans ce foyer. L'identité du parasite transporté par Lu. longipalpis doit être confirmée.*

MOTS CLÉS : *leishmaniose viscérale, vecteurs,* Lutzomyia evansi, Lutzomyia longipalpis, Leishmania chagasi, *Vénézuela.*

INTRODUCTION

American visceral leishmaniasis (AVL) is a parasitic disease considered to occur sporadically and with very low endemicity in Venezuela (Desjeux, 1991). Since the report of the first case (Martinez-Niochet & Pons, 1941), 818 cases have so far been recorded in the files of the Department of Dermatology (1995). However, these figures are considered to be underestimated.

Lutzomyia longipalpis, the more widely distributed proved vector of AVL in the New World (see Young & Duncan, 1994) was first incriminated as the putative vector of AVL in Agua Hedionda, Guárico State, Venezuela, by Amaral *et al.* (1961*a, b, c*). Its epidemiological importance in different foci was also established by Pifano (1969) who, in the temporal absence of *Lu. longipalpis*, first suggested that *Lu. evansi* might be responsible for the transmission on AVL in Isla de Margarita (Pifano & Romero, 1964). *Lutzomyia evansi* which is distributed from Central America to Colombia, Peru and Venezuela, has been so far demonstrated as

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AVL vector only in San Andrés de Sotavento, northwestern Colombia, where *Lu. longipalpis* is not present (Travi *et al.*, 1990, 1996).

This paper deals with the results of a longitudinal entomological study carried out in Guayabita village, an old endemic AVL focus in Venezuela where *Lu. longipalpis* and *Lu. evansi* coexist. In 1992 a new visceral leishmaniasis case was registered there. Consequently, an epidemiological study on humans and dogs was carried out (Delgado *et al.*, 1998). The objectives of this work were: 1. to study the species composition and the adult sandfly population dynamics; 2. to search for natural infection to *Leishmania* spp.; 3. to identify *Leishmania* spp. isolated from naturally infected sandfly species.

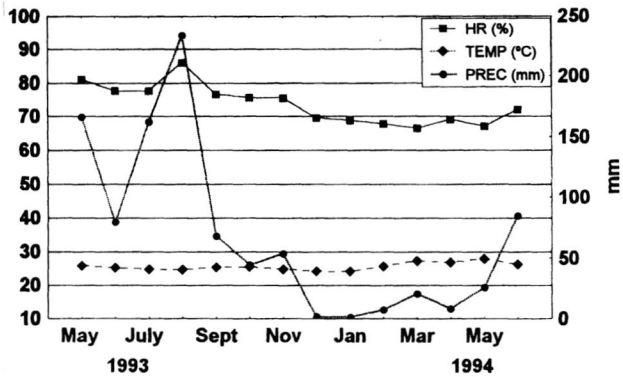

Fig. 1. – Monthly climate data recorded in the meteorological station of the Venezuelan Air Force in Maracay, 20 km away from the study village Guayabita.

MATERIALS AND METHODS

Study area

The Guayabita village, Aragua State, Venezuela is located in north-central Venezuela (10°16'N; 67°28'W; 500 m above sea level), at the foothill of the Cordillera de la Costa (Costal Mountain Range) and about 20 km from Maracay. This life zone is classified as premontane dry forest (annual mean temperature = 25°C, annual average rainfall = 700-1000 mm). Climate is markedly seasonal with six dry months (November-April) (Ewel & Madriz, 1968). The population of Guayabita is constituted by 493 inhabitants living in 65 households. The epidemiological features of this community have been described elsewhere (Delgado *et al.*, 1998).

Sandfly collections

During 2-3 consecutive nights, between January 1993 and June 1994, sandfly fortnight collections were carried out within the patient's family farm. Sandfly captures began in January 1993 with a Shannon trap between 18:30 and 21:30 and one CDC trap placed indoors overnight. In May 1993 two additional CDC traps were placed in the chicken coop and in the hog-pen from 19:00 to 07:00 h. After collection, males were kept in alcohol, females were washed in a weak detergent solution (2 %), rinsed in distilled water, and then kept in Nunc vials (1.5 ml; 20-25 females/each) in sterile 10 % DMSO in PBS pH = 7.4 for cryopreservation in liquid nitrogen. All samples were transferred to the Centro Nacional de Referencias de Flebótomos, University of Carabobo, Maracay for counting, species identification and dissection.

To assess the temporal association between sandfly numbers and weather variables, weather data [mean temperature (Tx), minimum temperature (Tmin), maximum temperature (Tmax), mean relative humidity (RHx), minimum relative humidity (RHmin), maximum relative humidity (RHmax) and rainfall] recorded during the same period of collections at the meteorological station in Maracay, were obtained from the Department of Meteorology of the Venezuelan Air Force (Fig. 1).

Sandfly data analysis

Sandfly collections were determined as number of sandflies per night per trap. As no normal distribution was observed for sandfly numbers (Kolmogorov-Smirnov one sample test: Dmax = 0.37103; $P < 0.01$), Mann-Witney U test was used to compare this variable between species and sexes and the Kruskall-Wallis test was used to compare numbers obtained with Shannon trap, CDC in chicken coop and CDC in hog-pen (May 1993-June 1994) and among months (May 1993-February 1994). These analysis were only applied to *Lu. evansi* and *Lu. longipalpis*, using the STATISTICA package (Statsoft 1993). A Canonical Correspondence Analysis (Ter Braak, 1986) was also performed using the CANOCO package (Ter Braak, 1987-1992), to detect relationship between species abundance and environmental factors.

Sandfly identification, search for natural flagellate infection and *Leishmania* spp identification

In the laboratory, male sandflies were clarified and mounted for specific identification. The technique for searching natural infection was described previously (Feliciangeli *et al.*, 1994). When flagellates were seen, culture was performed on blood agar base medium and, after multiplication, total DNA was extracted and analyzed as previously reported (Barrios *et al.*, 1994). Comparison of the restriction patterns was obtained using international reference strains from both subgenus *Viannia* and *Leishmania*, listed in Table I.

Strain designation	Classification	Abbreviation
MHOM/BZ/82/BEL21	*L. (L.) mexicana*	BEL21
MHOM/BZ/62/M379	*L. (L.) mexicana*	M379
MHOM/VE/83/H-78	*L. (L.) venezuelensis*	H78
IFLA/BR/67/PH8	*L. (L.) amazonensis*	PH8
MHOM/VE/76/LL1	*L. (L.) pifanoi*	LL1
MHOM/BR/75/M2903	*L. (V.) braziliensis*	M2903
MHOM/BR/75/M4147	*L. (V.) guyanensis*	M4147
MHOM/PE/84/LC26	*L. (V.) peruviana*	LC26
MHOM/BR/81/M6426	*L. (V.) lainsoni*	M6426
IHR/CO/85/LC500	*L. (V.) colombiensis*	LC500
MHOM/PA/71/LS94	*L. (V.) panamensis*	LS94
MHOM/VE/76/JAP78	*L .(L.) garnhami*	JAP78
MHOM/TN/80/IPT1	*L. (L.) infantum*	IPT1
MHOM/BR/69/PP75	*L. (L.) chagasi*	PP75
MHOM/IN/80/DD8	*L. (L.) donovani*	DD8
MCAV/BR/45/L88	*L. (L.) enriettii*	L88

Table I. – *Leishmania* international reference strains used for characterization of isolates from *Lu. evansi* collected in Guayabita, Venezuela.

The polymerase chain reaction (PCR), was performed using universal primers (Rogers *et al.*, 1990), and *L. donovani* complex specific primers. kDNA hybridization was performed as previously described (Rodriguez *et al.*, 1994).

RESULTS

SANDFLY DATA

A total of 9,282 sandflies (3,239 males and 6,043 females) of 11 phlebotomine sandfly species, six of them anthropophilic, were collected (Table II). *Lutzomyia evansi* numbers were significantly higher than *Lu. longipalpis* (U = 40086.50; $P < 0.001$). A significant difference was found between sexes of *Lu. evansi* (U = 13732.50; P = 0.0268) but not between sexes of *Lu. longipalpis* (U = 14787.00; P = 0.2772). The Shannon trap was highly efficient for collecting *Lu. evansi*, while the CDC trap placed in the chicken coop collected the highest number of *Lu. longipalpis*. Monthly sandfly abundance varied significantly (χ^2 = 67.465; df = 11; $P < 0.0001$). Figure 2 shows the adult population dynamics of females and males of *Lu. evansi* and *Lu. longipalpis* collected with all methods.

The Canonical Correspondence Analysis results are given in Table III. *Lutzomyia longipalpis* showed the highest score, indicating that this is the species which is more greatly influenced by the environmental variables, whereas *Lu. evansi* seems not to be affected by any specific abiotic factor. For the former species, the three temperature measurements exert a positive influence (the mean temperature exhibiting the greatest effect), whereas the relative humidity appears to be negatively correlated, the effect of mean values being the highest. Rainfall does not show a high score, however, it happened to be negatively correlated as well.

LEISHMANIA SEARCH AND IDENTIFICATION

Results of female dissections are given in Table IV. The highest infection rate of *Lu. evansi* was found in October, when this species presented the highest density. The infected specimen of *Lu. longipalpis* was caught in June 1993. In all flies parasites were located in the midgut. Two cultures from *Lu. evansi* (IEVA/VE/93/UCNA-2 and IEVA/VE/93/UCNA3) were established in NNN medium with gentamycine. Using the

	Shannon trap		House		Pigpen		Chicken coop			
	January 1993-June 1994				May 1993-June 1994					
Species	Males	Females	Males	Females	Males	Females	Males	Females	Total	%
**L. evansi*	2,075	4,894	4	9	188	375	219	252	8,016	86.36
**L. longipalpis*	189	97	5	0	27	32	399	235	984	10.60
**L. gomezi*	35	10			4	6	5	4	64	0.69
L. dubitans	36	37	1	0	12	8	38	38	170	1.83
L. trinidadensis		1				14		15	30	0.32
L. cayennensis		2				3	1	3	9	0.10
L. rangeliana	1	1							2	0.02
L. atroclavata						2			2	0.02
**L. lichyi*						2			2	0.02
**L. migonei*								1	1	0.01
**L. panamensis*		2							2	0.02
Total	**2,336**	**5,044**	**10**	**9**	**231**	**442**	**662**	**548**	**9,282**	**100,00**

* antropophilic species.

Table II. – Total sandfly species collected in a restricted focus of visceral leishmaniasis. Guayabita, Aragua State, Venezuela.

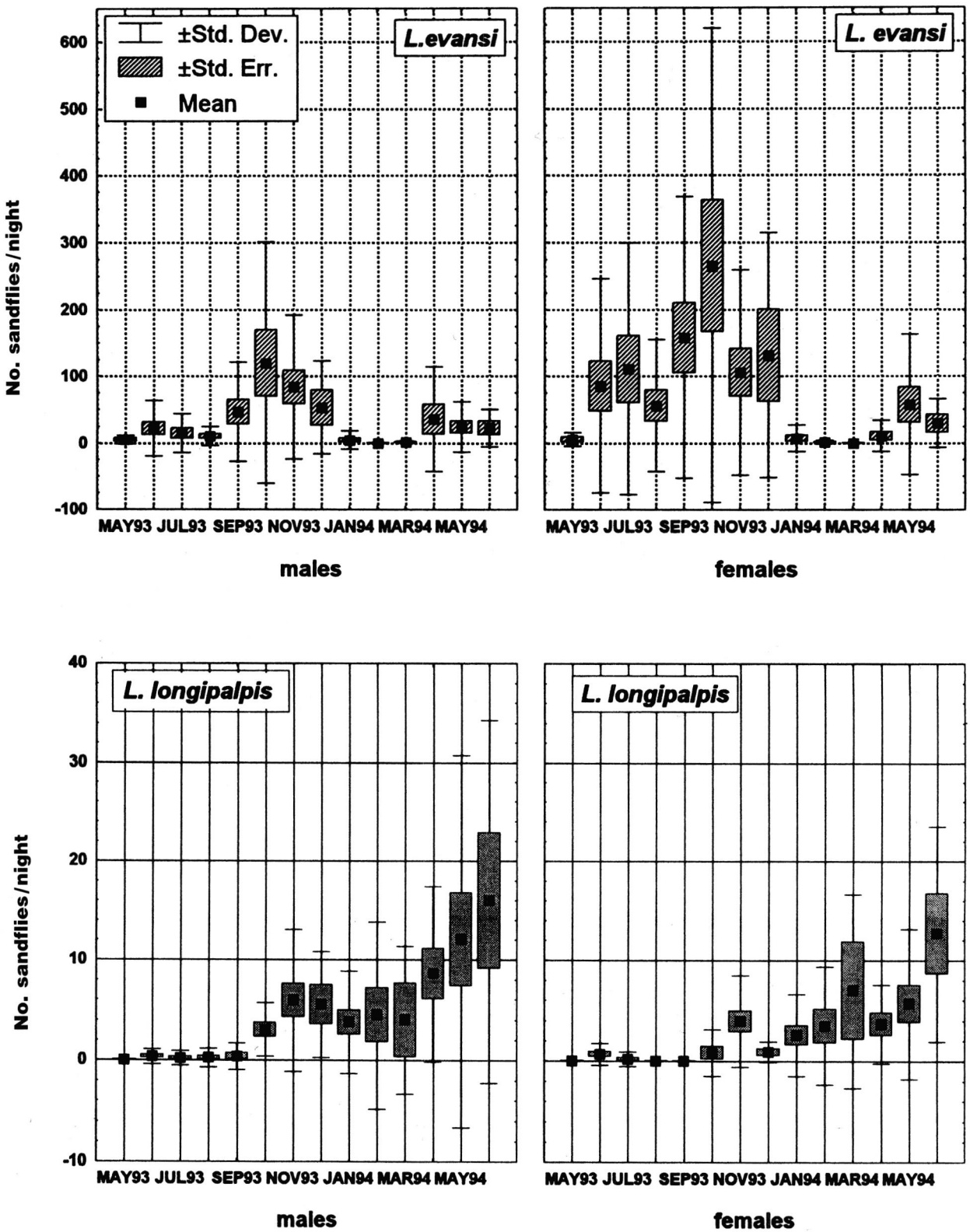

Fig. 2. – Adult population dynamics of *Lu. evansi* and *Lu. longipalpis* in an endemic focus of AVL, Guayabita, Venezuela.

	Axe 1
Eigenvalue	0.133
Cumulative percentage Variance:	
Of species data	68,6 %
Of species-environment relation	100 %
Species scores:	
L. evansi	– 0.0978
L. longipalpis	1.3622
Environmental variables scores:	
Mean Temperature	0.6663
Maximum Temperature	0.6164
Minimum Temperature	0.4028
Mean Relative Humidity	– 0.6592
Maximum Relative Humidity	– 0.5957
Minimum Relative Humidity	– 0.5218
Precipitation	– 0.3259

Table III. – Results of the Canonical Correspondence Analysis performed on two sandfly species, vectors of visceral leishmaniasis, and seven environmental variables, in Guayabita village, Venezuela.

	Total dissected	*L. evansi*	% (n)	*L. longipalpis*	% (n)
Shannon trap	4,807	3,980	0.15 (6)*	86	0
Pigpen	385	318	0	32	0
Chicken coop	548	252	0.40 (1)**	235	0.42 (1)#
House	9	9	0		
Total	**5,749**	**4,559**	**0.15 (7)**	**353**	**0.28 (1)**

* 01/06/1993 (two infected females;
05, 06, 18 and 19/10/1993 (four infected females).
** 02/02/1994 (one infected female).
# 21/06/1993 (one infected female).

Table IV. – Total sandfly females dissected for search of *Leishmania* spp. and infected with promastigotes.

PCR technique with universal primers, all isolates from sandfly females (seven *Lu. evansi* and one *Lu. longipalpis*) showed a 120 bp product characteristic of *Leishmania* spp (results not shown).

k-DNA restriction analysis (Fig. 3) showed high homologies between the isolates IEVA/VE/93/UCNA-2 and IEVA/VE/93/UCNA-3 and *L. chagasi*, minor homologies were observed with *L. donovani* and *L. infantum*. However no homologies were observed with *L. (Viannia)* and others members of the *L. (Leishmania)* subgenus. These results were confirmed after kDNA hybridization. Fig 4C shows the high hybridization signal between IEVA/VE/93/UCNA-2, IEVA/VE/93/UCNA-3 and *L. chagasi* kDNA; an hybridization signal was not observed with *L. braziliensis* and *L. mexicana* kDNA. The polymerase chain reaction (PCR) using specific *Le. donovani* primers showed at least three bands, one of those corresponding to a 805 bp, characteristic of *L. donovani* complex (Fig. 5).

Restriction fragment length polymorphism did not reveal differences between the IEVA/VE/93/UCNA-2 and IEVA/VE/93/UCNA-3 isolates and indicated no similarities between this group of isolates with other trypanosomatids tested (*Crithidia sp, Endotrypanum,*

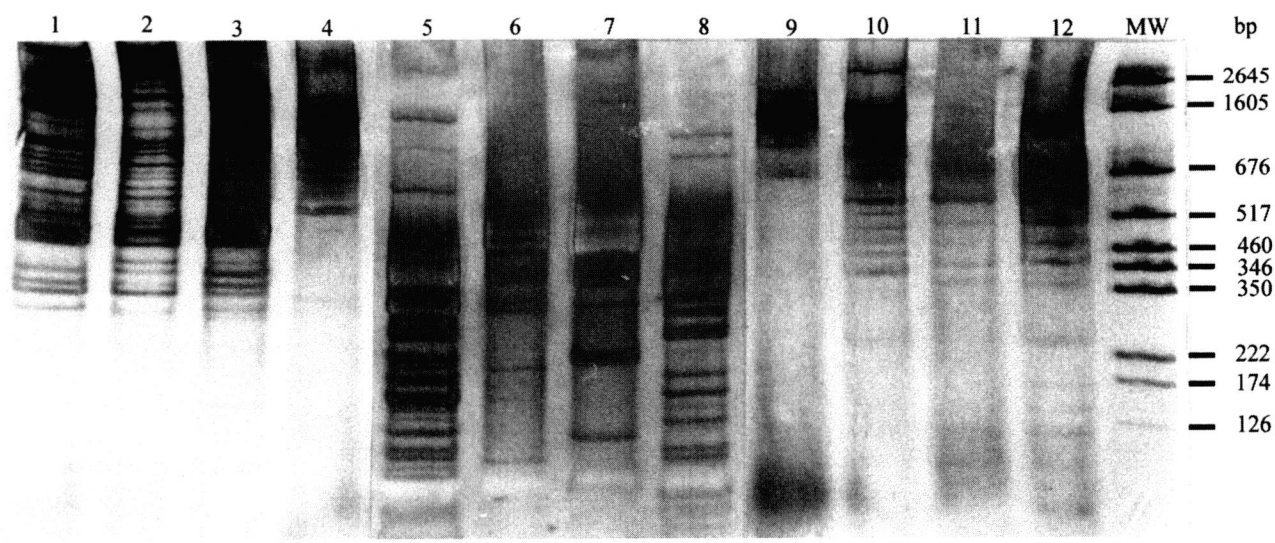

Fig. 3. – Kinetoplast DNA (kDNA) fragments patterns generated with the restriction enzyme Msp I. lane 1 - *L(L) chagasi* (PP75); lane 2 - Eva2; Lane 3 - Eva3; lane 4 - *L(V) braziliensis* (M2903); lane 5 - *L(L) amazonensis* (PH8); lane 6 - *L(V) colombiensis* (LC500); lane 7 - *L(L) pifanoi* (LL1); lane 8 - *L(L) venezuelensis*, lane 9 - *L(V) peruviana* (LC26); lane 10 - *L(L) infantum* (IPT1); lane 11 - *L(L) donovani* (DD8); lane 12 *L(L) enrrietti* (L88); lane 13 - Molecular weight markers pGEM (Promega).

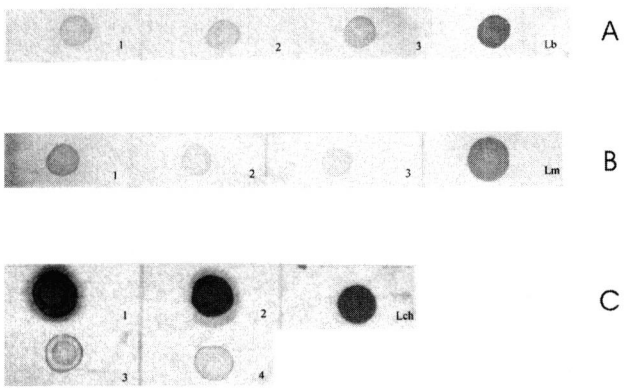

Fig. 4. – Dot-blot hybridization of kinetoplast DNA (kDNA) from the parasite culture. kDNA obtained from the isolates was hybridized to kDNA isolated from the International reference strains labelled to dATP-digoxigenin. A - L(V) braziliensis (M2903)-kDNA probe; number 1 : Eva2; 2 : Eva3; 3 : L(L) chagasi; 4 : M2903-kDNA. B - L(L) mexicana(PH8)-kDNA probe. 1 : Eva2; 2 : Eva3; 3 : L(L) chagasi; 4 : L(L) amazonensis (PH8). C - L(L) chagasi-kDNA probe. 1 : Eva2; 2 : Eva3; L(L) chagasi (PP75); 3 : L(L) amazonensis (PH8); 4.- L(V) braziliensis (M2903).

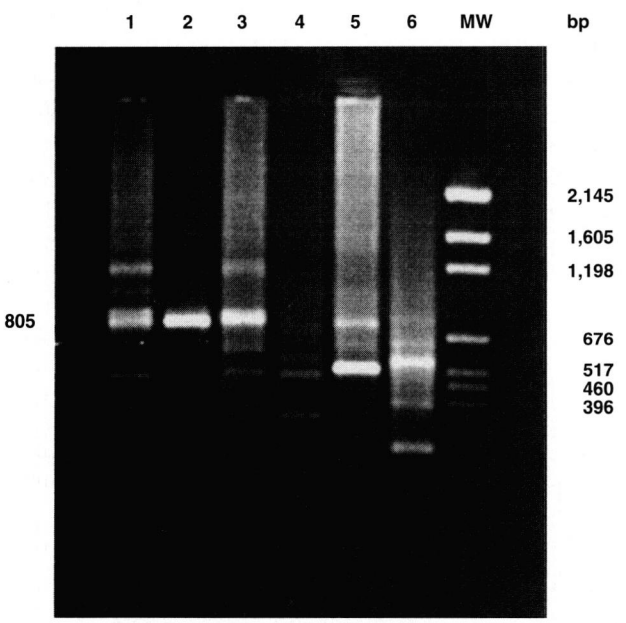

Fig. 5 – PCR products obtained after 35 cycles of amplification with AJS3/DB8 *L. donovani* species-specific primers. Lane 1 : Eva2; lane 2 : Eva3; lane 3 : *L. chagasi* (PP75); lane 4 : *L. donovani* (DD8); lane 5 : *L. infantum*; lane 6 : Talisman 2 (Venezuela strain); MW : Molecular weight markers.

Sauroleishmania, L. herreri, L. hertigi, L. aristedesi). However, this type of analysis did not distinguish among the reference strains from each of the three complexes (*L. (V.) braziliensis, L. (L.) mexicana* and *L. (L.) donovani*) (data not shown).

DISCUSSION

Information on the abundance and temporal distribution of phlebotomine sandfly vectors contributes to the understanding of the epidemiology of the leishmaniases, to the prediction of transmission seasonality, and to assist in planning control strategies. When more than one putative vector coexist in the same area, these data are specially important. This work is the first one that deals with the concomitant population dynamics of *Lu. longipalpis* and *Lu. evansi*, the two recognised vectors of American Visceral Leishmaniasis, a disease which has shown to be emerging in different foci in urban areas (Arias *et al.*, 1996; Aguilar *et al.*, 1998) and re-emerging in rural areas (Delgado *et al.*, 1998) in the New World.

Both species were present throughout the year in Guayabita, a village with flourishing agriculture and animal breeding. The *Lu. evansi* population overwhelmed the *Lu. longipalpis* population and alternated with it, peaking at the end of the rainy season (October 1993), while the low *Lu. longipalpis* population hardly increased at the end of the dry season (April-May 1994). This unimodal pattern of *Lu. evansi* was similar to that reported in San Andrés de Sotavento, Cordoba Department, Colombia (Travi *et al.*, 1996) where the amount of rainfall was also similar, with a maximum precipitation of about 200 mm. However, indoor collections of *Lu. evansi* in Guayabita were extremely scarce when compared with those obtained with the same trapping method (CDC trap) in Colombia, even though differences were observed there between two villages in the same area (Vidales and El Contento). Unfortunately Travi *et al.* (1996) did not give data on the domestic fauna in each locality; we believe that the abundance of domestic animals around the house in Guayabita may be a key factor in determining the exophilic behaviour of *Lu. evansi*. The population of *Lu. longipalpis* in Guayabita was clearly different in size and fluctuation to the population in El Callejón, Cundinamarca Department, Colombia. In Guayabita the *Lu. longipalpis* density was very low and the results of the canonical analysis allowed us to conclude that rainfall and relative humidity are determinant and negative factors in *Lu. longipalpis* density. On the contrary *Lu. longipalpis* was abundant and displayed a bimodal annual cycle positively related to humidity and rainfall in El Callejón (Morrison *et al.*, 1995). However, rainfall was erratic and low in El Callejón, while in Guayabita it was continuous during June to September 1993, reaching up to 230 mm. Comparison of population dynamics in different ecological situations is therefore of value to determine, for each climatic variable, the range in between the species is favoured and outside which it will be affected. Such information may not

only help in predicting local vector occurrence and abundance; in addition, it may be useful to attempt an ecological approach to the stratification of the visceral leishmaniasis in the neotropics.

In this study both *Lu evansi* and *Lu. longipalpis* were found naturally infected with suprapyloric promastigotes in the same focus. The nature of two isolates from *Lu. evansi* previously reported (Feliciangeli *et al.*, 1993), was investigated using molecular techniques. Traditionally, parasites isolated from different sources have been identified using biochemical techniques such as zymodemes (Momen *et al.*, 1993) and monoclonal antibodies (McMahon Pratt *et al.*, 1985). However, during the last ten years the molecular techniques are in greater use due to their sensitivity and specificity for species and subspecies identification. These technique have allowed the identification of parasites from humans (Barker, 1987; Barker & Butcher, 1993; Rodriguez *et al.*, 1994) and vectors (Barrios *et al.*, 1994) as well as taxonomic studies (Dujardin *et al.*, 1993). The sensitivity of kDNA probes has been widely demonstrated (Ready *et al.*, 1988; Barker & Butcher, 1993). kDNA contains regions which vary in homology between the different species; some regions are very conserved whereas other areas are variable, and they are different between the different complexes. IEVA/VE/93/UCNA-2, IEVA/VE/93/UCNA-3 isolates and *L. chagasi* share large areas of homology while there are very short regions of homology with *Le. braziliensis* and *Le. mexicana* complexes and others trypanosomatidae such as *T. cruzi* and *T. evansi.* Concordance within different molecular techniques which are complementary to each other, allows us to characterise our isolates from *Lu. evansi* as belonging to the *L. donovani* complex; moreover, the high homology with the *L. chagasi* kDNA suggests that these isolates are more similar to this group of parasites.

Though the suprapyloric *Leishmania* found in *Lu. longipalpis* might have been *L. chagasi*, the rate of natural infection was higher in *Lu. longipalpis* (0.28 %) than in *Lu. evansi.* (0.15 %). However, the identity of the parasite carried by *Lu. longipalpis*, which in Venezuela often shares the same ecological habitats as *Lu. evansi* (Pifano & Romero, 1964; Bendezu *et al.*, 1995; Aguilar *et al.*, 1998) needs to be confirmed.

The epidemiological situation in Venezuela seems more complicated than in Colombia where *Lu. evansi* and *Lu. longipalpis* are responsible for AVL transmission in separate foci. Natural infection rates of 0.03 % and 0.10 % *Lu. evansi* to *Le. chagasi* were reported in two hamlets in San Andrés de Sotavento, where, despite intensive collections, *Lu. longipalpis* was never caught. (Travi *et al.*, 1996). In El Callejón, where *Lu. evansi* has not been found, *Lu. longipalpis* is considered the unique vector of AVL. The natural infection rates reported there in two different periods were 8.8 % (Corredor *et al.*, 1989) and 0.29 % (Ferro *et al.*, 1995). In both foci the parasites were identified using zymodemes. Strains from naturally infected opossums (*Didelphis marsupialis*) and sentinel dogs were also identified as *L. chagasi* in El Callejón, where the prevalence of infection among the inhabitants was 51.2 % (Corredor *et al.*, 1988). The low endemicity of the disease in Guayabita with 11.4 % of leishmanin positive humans and 15.5 % of seropositive dogs (Delgado *et al.*, 1998) has not allowed, so far, the isolation and comparison with parasites from sand flies, humans and reservoir hosts. Further laboratory and field studies here and in other different defined ecotopes, are necessary in order to fully understand the vector-parasite relationship and the transmission dynamics of the AVL in Venezuela, looking for appropriate control strategies.

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