Genetic homogeneity within *Leishmania* (*L.*) *infantum* isolated from human and dogs: the relationship with the sandfly fauna distribution in endemic areas of Nueva Esparta State, Venezuela

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SUMMARY

Leishmania infantum has been described as a highly polymorphic group of parasites, responsible for visceral leishmaniasis and cutaneous leishmaniasis. In this paper we report the life-cycle of *L.* (*L.*) *infantum* in an endemic area of visceral leishmaniasis in Venezuela, by using molecular diagnosis and characterization of parasites isolated from dogs, humans with visceral leishmaniasis and sand flies. The molecular characterization was carried out by use of kDNA restriction analysis, dot-blot hybridization with species-specific probes and RFLP of the PCR products. The results demonstrated that *L.* (*L.*) *infantum* is the parasite responsible for VL in the island. The parasites were revealed to be genetically homogeneous with no intra-specific differences between isolates from different individuals. The highest homology of the isolates was with *L.* (*L.*) *infantum* from the Old World rather than with *L.* (*L.*) *chagasi* from the New World. Additionally, we report the geographical distribution of *Lutzomyia longipalpis*, and the relationship with the transmission of *L.* (*L.*) *infantum* in the studied area.

Key words: *Leishmania infantum*, *Lutzomyia longipalpis*, homogeneity, molecular characterization.

INTRODUCTION

In the New World visceral leishmaniasis is caused by *L.* (*L.*) *infantum* (syn. *Chagasi*) with the domestic dog as the main reservoir (Mauricio, Stohard & Miles, 2000). It has been reported by Mauricio *et al.* (1999) that *L.* (*L.*) *infantum* is the species infecting dogs in Latin America but not the only one. Agents of cutaneous leishmaniasis (CL) of the *Viannia* subgenus such as *L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana* and *L.* (*V.*) *panamensis* are frequently found in dogs resulting in non-fatal cutaneous infections. An estimated number of 100 000–500 000 cases of visceral leishmaniasis occurs annually, with 90% of the cases occurring in India, Sudan and Brazil. In America, the highest incidence in humans has been reported in Brazil with an estimated number of 3000 cases each year.

According to the Ministry of Health, the high endemic area for VL in Venezuela is found in Sucre state, where 398 cases have been recorded in the last 10 years. Other states with low incidence are Yaracuy (21 cases) and Nueva Esparta with 54 cases from 1998 to 2002. However, in recent years, there has been an increase of the disease in humans throughout the country, with the re-emergence in rural communities (Delgado *et al.* 1998; Feliciangeli *et al.* 1998) and the appearance in urban and suburban communities (Aguilar *et al.* 1998). Recently, an epidemiological evaluation of the situation of VL in Eastern Venezuela, revealed an increase in the number of human cases and domestic dogs (Zulueta *et al.* 1999), the same situation has been observed in the Nueva Esparta State where the visceral leishmaniasis in humans has increased from 8 cases between 1990 and 1997 (1·25 cases/year) to an average of 10·4 cases per year in the past 5 years. From the total cases reported in Nueva Esparta, 85% occurred in children aged from 1 to 2 years. This increment of VL in humans in that part of the country, has motivated an epidemiological study to evaluate the risk to the population. The study included clinical, serological, and parasitological evaluations of humans and dogs in different localities, where human cases have been reported during the last 5 years, as well as an entomological survey. In a previous study (Zerpa *et al.* 2000) the presence of a very significant number of

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infected dogs was demonstrated by serological testing in different communities of the State. Regarding sand fly vectors, *Lutzomyia longipalpis* has long been recognized as the sole vector of the AVL. However, after Mangabeira (1969) drew attention to the morphological differences between males of *Lu. longipalpis* from Pará and Ceará, North Eastern Brazil, the study of the genetic variability led to the conclusion that this taxon is a complex of sibling species (Lanzaro *et al*. 1993). Outside Venezuela this is composed of at least 3 species (Uribe, 1999; Mutebi *et al*. 2002). In Venezuela, 2 species were identified by isoenzyme diagnostic markers (Lampo *et al*. 1999; Arrivillaga *et al*. 2000). In this study we report the natural cycle of *L*. (*L*.) *infantum* in the study area with the molecular characterization of the parasites from humans, dogs and sand flies, which resulted in a homogeneous group of parasites, not previously reported. We discuss the possibilities of an imported leishmaniasis in the island as well as the VL case distribution in relation to the sand fly fauna in different ecological situations.

MATERIALS AND METHODS

Study area

This study was carried out in 2 small villages in Nueva Esparta State, Venezuela. The island of Margarita, Venezuela (10°51′50″–11°11′06N and 63°46′06″–64°24′32″W), has an extension of 1085 km^2 and a population of 373 851 inhabitants (INE, 2001). Climate on the island is characterized by an average temperature of 26–28 °C and a bimodal pattern of annual precipitation (400–1100 mm) with 2 peaks (June–August and December–February).

So far, cases of visceral leishmaniasis have only been reported in the eastern area of the island, concentrated in dry zones at low altitude, while no cases have been recorded in the life-zone described as humid pre-mountain forest at the foothills of the massif El Copey (910 m a.s.l).

Sample collection and parasite isolation

Parasites were isolated from human cases and serologically positive dogs by inoculation of bone marrow, blood or lymph node infected samples in blood agar culture medium (DIFCO) containing 10% defibrinated rabbit blood and 1000 units of penicillin/ml. For parasitological studies biopsies were taken from dogs under anaesthetic conditions. Twenty-five cultures were isolated from infected dogs from 56 clinically suspicious or serologically positive animals, but only 2 positive cultures were isolated from patients in the studied endemic areas. Twelve isolates from dogs grew vigorously in the culture medium and were mass cultured in blood/agar plates at 26 °C for genetic studies. Sand fly sampling was carried out in localities where AVL cases have been detected. Additionally, where AVL had not been reported it was also screened for sand fly fauna. Collections were carried out using CDC traps placed between 19 p.m. and 7 a.m. in the main bedroom and outdoors in chicken houses or close to the dogs' resting houses.

Total DNA and kDNA isolation

Parasites were collected in the stationary phase by centrifuging at 5000 ***g*** for 10 min, washed 3 times with phosphate buffer pH 7·2 and lysed in buffer (0·05 M EDTA, 0·2 M NaCl, 10% SDS and 10 μg/ml of proteinase K) for 1 h at 56 °C. kDNA was obtained as previously described (Barrios *et al*. 1994). Briefly, after phenol/chloroform extraction, the aqueous phase was collected in a clean tube and centrifuged at 30 800 ***g*** for 45 min at 4 °C. Finally, the kDNA was resuspended in 100 μl of TE buffer (10 mMTris HCl, 1 mM EDTA, pH 8) and storage at −20 °C.

Total DNA was obtained from 1×10^9 promastigotes as previously described (Rodriguez *et al*. 1997).

Chromosomal DNA preparation and PFG electrophoresis

For chromosome analysis, promastigotes were washed once in phosphate-buffered saline, pH 7·0. Chromosomal DNA was prepared as previously described (Rodriguez *et al*. 2000). Agarose plugs, containing approximately 1×10^7 promastigotes were placed in the well of a 1% (w/v) agarose gel (100 ml), poured on a 14 cm (5·2″) wide × 13 cm (5″) long surface. The gel was run in a Chef-DR II Pulsed Field Electrophoresis System (Bio-Rad), using TBE buffer 0·5 × (45 mM Tris, 45 mM boric acid and 1·0 mM EDTA, pH 8) at a constant voltage of 3·5 v/cm and a ramp of 110–120 s pulse frequency for 80 h. After running, the gel was stained with ethidium bromide (0·5 μl/ml) and photographed with Polaroid film 665.

DNA digestion and hybridization

Five μg of kDNA from each isolate were digested with 10 Units of *Msp*1 endonuclease according to the manufacturer's instructions (New England Biolabs, Beverly, MA and Bethesda Research Laboratories, Gaithersburg, MD). The kDNA-digested products were separated by electrophoresis in 4·5–10% linear-gradient polyacrylamide gels, run at 7 mA overnight. The gels were stained with silver nitrate and photographed. Dot blot hybridization was carried out using a kDNA probe labelled with digoxigenin, using a non-radioactive labelling kit (Boehringer–Mannheim). Southern blot hybridization was carried

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out using Gp63 probes labelled with dCT^{32}P (Amersham) 3000 Ci/mmol in hybridization buffer (5 × SSC, 1% SDS, 0·5% Denhardt's and 50 μg/ml salmon sperm) at 42 °C, washed at 65 °C 3 times with 2 × SSC, 0·5% SDS and finally exposed to photographic film (GRI Ltd) overnight at −80 °C.

Polymerase Chain Reaction (PCR)

For PCR the DNA was extracted from bone marrow aspirates obtained from patients, dogs, and from the digestive tract of *Lu. longipalpis*. Samples were collected in TE modified buffer (10 mM Tris pH 8, 10 mM EDTA), DNA was extracted as we previously described (Rodriguez *et al*. 2002). The polymerase chain reaction was carried out with AJS-31 and DBY primers, 5′GGGGTTGGTGTAAAATAG and 5′CCAGTTTCCCGCCCCGGA derived from kDNAs of *L. infantum* which are specific for the *L. donovani* complex (Lambson, Smyth & Barker, 1999, 2000).The PCR reaction mix containing 5 μl (10 ng) of total DNA, 10 mM Tris-HCl (pH 8), 1·5 M $MgCl_2$, 0·01% gelatin, 0·2 mM dNTP mix, 100 ng of each primer, 10 μl of water, and 2·0 units of *Taq* polymerase (Promega) in a total volume of 25 μl, and overlayed with 20 μl of mineral oil. Amplification took place during 35 cycles, the annealing temperature was at 67 °C for 1 min, extension at 72 °C for 1 min and denaturation at 95 °C for 1min, except the first cycle of denaturation which was for 5 min; finally there was an extension cycle for 10 min. The programme was run on a thermocycler (MJ Research PT 100). Ten μl of the PCR product was run in a 1% agarose gel and the gel stained with ethidium bromide (10 mg/ml). DNA was purified from PCR products by ethanol precipitation, and finally digested with 10 Units of *Hae*III restriction enzyme according to the instructions of the manufactures.

RESULTS

Sandfly collection and abundance

In Table 1 we give the results of the entomological sampling in the 11 localities surveyed, located in different life-zones in the western area of the Margarita Island. Because of a variable effort of capture, we give the total numbers of males and females collected and the relative numbers per trap used. We also give the results of the natural search for *Leishmania* spp. that was more than 1% in the 2 samples of females dissected, one from Achipano, the suburb of Porlamar, the capital of the Island, and the other from the village La Vecindad.

Lutzomyia longiplapis predominated in the driest areas, tropical thorn woodland, the tropical very dry forest and the pre-mountain dry forest, while *Lu. gomezi* and, to a lesser extent *Lu. evansi*, were found in the pre-mountain moist forest, which is limited to Cerro Copey and the area at the foothills.

Diagnosis and culturing of the parasites

In Fig. 1 we show (i) endemic localities where human cases have been reported and the parasites have been isolated from humans, dogs and sand flies and (ii) non-endemic localities where entomological surveys were also carried out.

Parasites were diagnosed in 56 biopsies from different tissues by PCR, using primers DBY and AJS-1. Fig. 2 is an example of a PCR reaction. The diagnostic band of 805 bp was obtained in biopsies from bone marrow isolated from dogs and humans, as well as samples obtained from the digestive tract of *Lu. longipalpis* captured in the endemic area. The isolate from *L. evansi* used in this experiment was previously described as *L. chagasi* by Feliciangeli *et al*. (1999), this isolate gave a band of 780 bp similar to *L.* (*L.*) *chagasi* (PP75, international reference strain).

Leishmania parasites were isolated from 12/24 samples obtained from dogs (Table 2), from 2 human samples but no parasites were isolated from sand flies. Nine isolates were adapted to artificial culture medium. Massive culture of parasites was obtained in blood agar base artificial medium for molecular characterization purposes.

Molecular characterization of parasites

The isolated parasites were characterized by different molecular techniques. Fig. 3 shows the restriction pattern after digestion of the PCR product with *Hae*III restriction enzyme. The new isolates run in the gel (lanes 2–8) showed at least 4 bands under 1018 bp; corresponding to the pattern obtained for *L.* (*L.*) *infantum* (MHOM/TN/80/IPT1, lane 11), but different from the pattern obtained for *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75, lane 12) and *L.* (*L.*) *donovani* (MHOM/IN/80/DD8, lane 13).

Fig. 4 shows the results after digestion of the kDNA with the restriction enzyme *Msp*I, this enzyme cuts the kDNA and produces 4 fragments in the majority of the new isolates (lanes 1–9), the restriction fragments share homologies with the same size of 3 fragments obtained for *L.* (*L.*) *infantum* (lane 12) and with 2 fragments of *L.* (*L.*) *chagasi* (lane 13), other enzymes such as *Eco*RI and *Pst*I gave the same homologies between the dog isolates and *L.* (*L.*) *infantum* (results not shown). Dot blot hybridization of the kDNA to kDNA probe from *L.* (*L.*) *infantum*, (IPT1), gave a high hybridization signal (Fig. 5A) with the kDNA–PCR samples from dogs, humans and sand flies, which is similar to the hybridization signal obtained when the *L.* (*L.*) *donovani* (DD8) probe was used (Fig. 5B). However, a very low

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Table 1. Geographical distribution of sandfly fauna

(Phlebotomine sandflies caught indoors (*) and outdoors in localities of the Island of Margarita where VL cases have been reported (●) or not (○), situated in different life zones (Ttw = Tropical thorn woodland, Tvdf = Tropical very dry forest, P-mdf = Pre-mountain dry forest, P-mmf = Pre-mountain moist forest).)

		Males		Females	
Locality	Species	Total	No/trap/night	Total	No/trap/night
Porlamar (Ttw)	*L. longipalpis**	101	0·66	108	0·70
(Achipano) (●)	*L. longipalpis*	181	3·29	144***	2·07
La Guardia (Ttw) (●)	*L. longipalpis*	76	38·00	14	7·00
La Vecindad (Tvdf) (●)	*L. longipalpis**	118	0·72	216**	1·32
La Pista (Tvdf) (●)	*L. longipalpis**	5	0·38	11	0·85
El Espinal (Tvdf) (●)	*L. longipalpis**	11	0·13	4	0·05
El Piache (●)	*L. longipalpis**	110	4·40	152	6·08
(Tvdf)	*L. longipalpis*	2	2·00	0	0·00
	L. evansi	0	0·00	0	0·00
	L. cayennensis	0	0·00	1	1·00
Fuentidueño (○)	*L. longipalpis*	2	2·00	0	0·00
(P-mdf)	*L. evansi*	10	10·00	10	10·00
	L. gomezi	0	0·00	1	1·00
	L. atroclavata	0	0·00	1	1·00
La Fuente (○)	*L. longipalpis*	2	2·00	0	0·00
(P-mdf)	*L. evansi*	9	9·00	35	35·00
	L. atroclavata	1	1·00	1	1·00
El Valle (○)	*L. gomezi**	7	1·40	18	3·60
(P-mdf)	*L. longipalpis**	2	0·40	1	0·20
	*L. atroclavata**	1	0·20	0	0·00
	L. gomezi	12	0·35	22	0·65
	L. evansi	1	0·03	0	0·00
	L. dubitans	1	0·03	1	0·03
	L. trinidadensis	5	0·15	8	0·24
	L. cayennensis	1	0·03	10	0·29
Cerro Copey (○)	*L. gomezi*	5	5·00	17	17·00
(P-mmf)	*L. evansi*	0	0·00	2	2·00
	L. dubitans	1	1·00	1	1·00
	B. devenanzi	1	1·00	2	2·00
La Sierra (○)	*L. gomezi**	27	0·36	240	3·20
(P-mmf)	*L. gomezi*	52	0·55	290	3·09
	L. lichyi	0	0·00	3	0·03
	L. trinidadensis	3	0·03	13	0·14
	L. dubitans	1	0·01	1	0·01
	L. cayennensis	0	0·00	3	0·03
	L. atroclavata	1	0·01	2	0·02

*** 1/81 (1·2%) positive to *Leishmania infantum*.
** 1/91 (1·1%) positive to *Leishmania infantum*.

hybridization signal was obtained when the hybridization was to *L.* (*L.*) *chagasi* (PP75) kDNA probe (Fig. 5C).

Southern blotted chromosomal DNA separated by pulse field electrophoresis was hybridized to GP63 probe labelled with dCT^{32}P. GP63 probe is a 2 kb *Eco*RI/*Xba* fragment representing the coding region of the gene in *L. infantum*. The results in Fig. 6 show a high hybridization signal corresponding to a chromosomal band of 945 kb in samples of new isolates from dogs (lanes 1–5), which is the same signal obtained for *L.* (*L.*) *infantum* (lane 8) an additional hybridization band over 945 Mb was observed in *L.* (*V.*) *braziliensis* (lane 6). The signal is different for *L.* (*L.*) *chagasi* with 2 hybridization signals of 825 and 945 kbp and *L.* (*L.*) *donovani* with 2 bands of 1020 and 945 kbp.

DISCUSSION

This article describes for the first time, to our knowledge, the epidemiological cycle of *L. infantum* in an endemic area of visceral leishmaniasis in the island of Margarita, Nueva Esparta State in the North of Venezuela, where cutaneous leishmaniasis has not been reported. Human cases, infected dogs and infected sand flies were diagnosed by conventional methods (smear, culture, ELISA) and PCR using AJS and DBY primers (Lambson *et al.* 1999, 2000). The PCR product revealed the diagnostic

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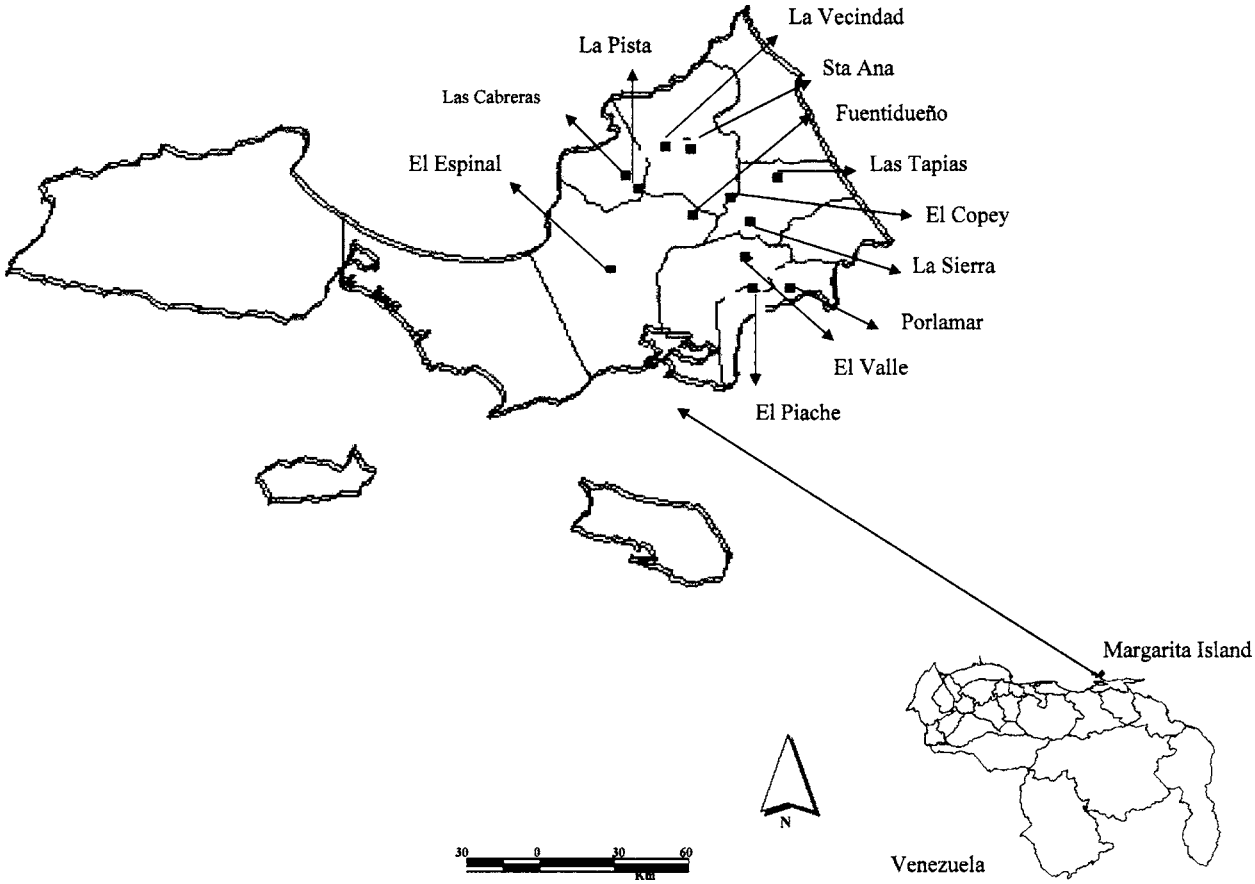

Fig. 1. Map of Nueva Esparta State, showing the localities where the entomological and clinical studies were carried out.

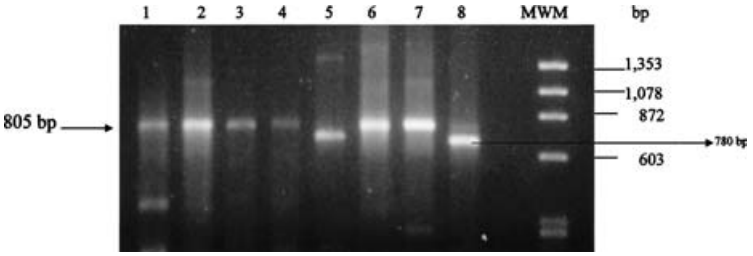

Fig. 2. Gel electrophoresis of PCR products obtained after 35 amplification cycles using DNA extracted from human, dogs and sandfly. Lane 1, human DNA from bone marrow; lanes 2 and 3, dog DNA from bone marrow; lane 4, *Lu. longipalpis* midgut; lane 5, DNA from parasites isolated from *Lu. evansi* and previously identify as *L. chagasi*; lane 6, *L.* (*L.*) *infantum* (MHOM/TN/80/IPT1; lane 7, *L.* (*L.*) *donovani* (MHOM/IN/80/DD8); lane 8, *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75).

expected band of 805 bp in human, sandflies and dog samples, and the amplified product was the same as that obtained for the Old World reference strains; *L.* (*L.*) *infantum* (MHOM/TN/80/IPT1) and *L.* (*L.*) *donovani* (MHOM/IN/80/DD8), but was different from *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75), with a size band of 780 bp. The digested PCR products, also demonstrated high homology within the field isolates and *L.* (*L.*) *infantum*, (IPT1) rather than with *L.* (*L.*) *chagasi* (PP75).

The highest homology within the studied isolates with Old World international reference strains was corroborated by dot blot hybridization of total kDNA and the Southern blot hybridization of chromosomal DNA to GP63 probe (Victoir *et al.* 1995), the results indicated that the Gp63 gene is located in a chromosome of approximately 975 kb which is in the range of that previously described for *L.* (*L.*) *infantum* from the Mediterranean Basin (Guerbouj *et al.* 2001). The results presented here also demonstrated the presence of *L.* (*L.*) *infantum* in a specimen of *Lu. longipalpis*, in 23 females captured, no parasites were observed in other species captured in AVL endemic and non-endemic areas. The presence of promastigotes in the digestive tract of *Lu. longipalpis* captured on the island was previously

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Table 2. Number of cultures isolated from different tissues

Number of samples	Tissue	Culture	
		Positive	Negative
15	Blood	10	5
4	Lymphatic node	2	2
2	Liver	1	1
13	Bone marrow	13	0
2	Spleen	1	1

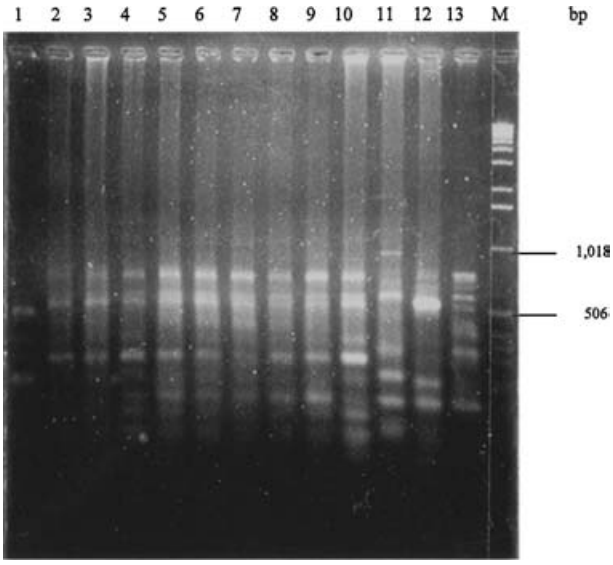

Fig. 3. Gel electrophoresis in 1% agarose of the PCR products digested with *Hae*III restriction enzyme. Lane 1, *Leishmania* (*V.*) *braziliensis* (LTB300); lane 2, human bone marrow; lane 3, *Lu. longipalpis*; lanes 4–9, dog bone marrow; lane 10, *L.* (*L.*) *infantum* (IPT1); lane 11, *L.* (*L.*) *chagasi* (PP75); lane 12, *L.* (*L.*) *donovani* (DD8); lane 13, *L.* (*L.*) *mexicana* (Bel21); lane 14, molecular weight markers (1 kb ladder, Gibco-BRL).

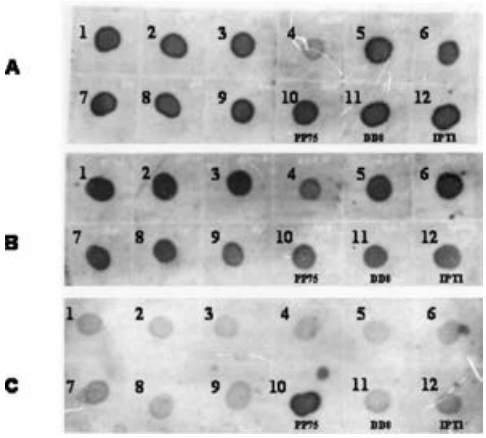

Fig. 5. Dot blot of kDNA isolated from dog isolates hybridized to total kDNA probes from international reference strains. (A) *Leishmania* (*L.*) *donovani* kDNA probe; (B) *L.* (*L.*) *infantum* kDNA probe; (C) *L.* (*L.*) *chagasi* kDNA probe.

reported by Feliciangeli *et al.* (1998), but the parasite was not identified until the present work. The above-mentioned species has been described as a vector for *L.* (*L.*) *chagasi* that is considered to be the aetiological agent of American visceral leishmaniasis (Grimaldi, Tesh & McMahon-Prat, 1989; Cupolillo, Grimaldi & Momen, 1994; Tesh, 1995). However, this species of sand fly has a wide geographical distribution in the New World, occurring in tropical areas from Mexico to Argentina (Lainson *et al.* 1985; Ward, 1985; Young & Duncan, 1994). A recent report from foci of cutaneous leishmaniasis in Colombia demonstrates the role of this species in the transmission of cutaneous leishmaniasis, but the association with visceral leishmaniasis was not proven (Alexander & Young, 1982; Lopez *et al.* 1996). Cutaneous leishmaniasis has not been reported on the island of Margarita. However, visceral leishmaniasis was reported earlier by Pifano & Romero (1964) but the parasite was not identified and *Lu. evansi* was incriminated as the vector in the absence of

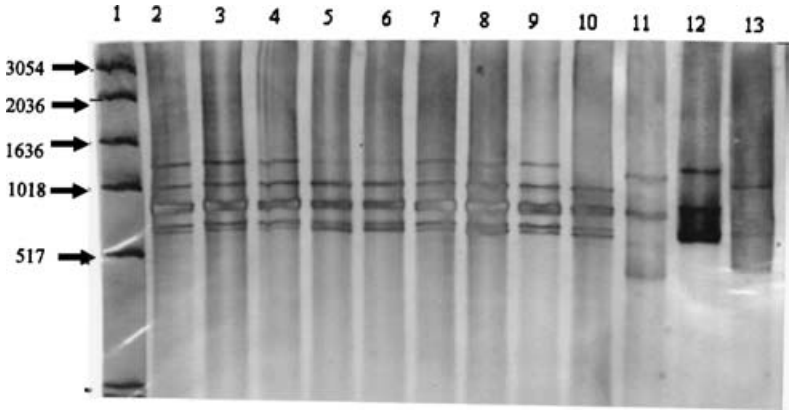

Fig. 4. Polyacrylamide gel electrophoresis of total kDNA digested with *Msp*I restriction enzyme. Lane 1, molecular weight markers (1 kb ladder, Gibco-BRL); lanes 2 and 3, kDNA from human isolate; lanes 4–10, kDNA from dogs isolates; lane 11, *L.* (*L.*) *donovani* (DD8); lane 12, *L.* (*L.*) *infantum* (IPT1); lane 13, *L.* (*L.*) *chagasi* (PP75).

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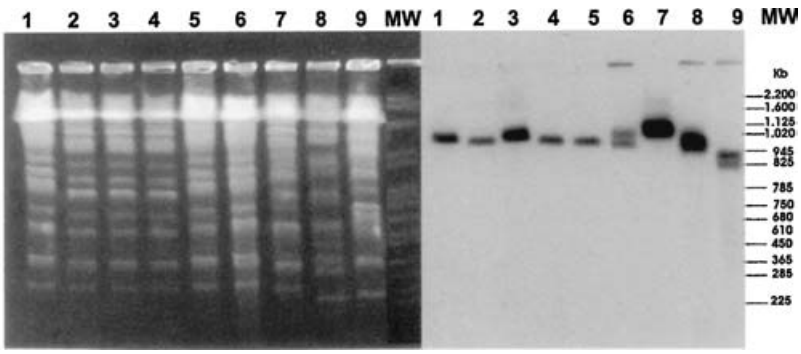

Fig. 6. Pulsed field electrophoresis hybridized to GP63 (coding region) probe. Lanes 1 and 2, human isolates; lanes 3–6, dog isolates; lane 7, *Leishmania* (*L.*) *donovani* (DD8); lane 8, *L.* (*L.*) *infantum* (IPT1); lane 9, *L.* (*L.*) *chagasi* (PP75).

Lu. longipalpis. More recently, 2 isolates from the Island were identified as *L.* (*L.*) *infantum* zymodeme MON-1 (Zerpa, 2001) which is in agreement with the early results reported by Moreno *et al.* (1990) in Trujillo isolates from humans and dogs. Our results are in concordance with the previous report about the relationship of *L.* (*L.*) *infantum* with the outcome of visceral leishmaniasis in Venezuela; however, *Leishmania infantum*, zymodeme MON-1 is highly polymorphic, (Hide, Bañuls & Tibayrenc, 2001). The results shown in this paper suggest that the group of studied isolates is genetically homogeneous, and even there was no difference in isolates from different foci in the same or different municipalities. As far as we know, this is the first report where the molecular characterization of the parasites revealed the natural epidemiological cycle of *L.* (*L.*) *infantum* in an area of visceral leishmaniasis in the country, and the genetic homogeneity of isolates from human, with no HIV history, dogs and sand fly vector. This result is not in agreement with a previous report about the heterogeneity of *L. infantum* described by Guerbouj *et al.* (2001). The genetic characterization of the isolates compare with the international reference strains (IPT1), provide evidence for the existence of *L.* (*L.*) *infantum* on the island of Margarita, genetically homologous to *L.* (*L.*) *infantum* from the Old World but with minor homology to *L.* (*L.*) *chagasi* (PP75, international reference strain) from the New World. This finding, together with the clinical manifestations of the disease in infants aged from 1 to 2 years (Zerpa *et al.* 2002), suggests that this disease will be imported in dogs coming with tourists, visiting the island from the Mediterranean Basin and have been adapted to the ecological conditions of the island.

On the island of Margarita the parasite found a good vector, *Lu. longipalpis s.l.* In fact, the entomological study showed that, in all the localities where VL cases have been recorded, *Lu. longipalpis s.l.* was present. Additionally, this species was found naturally infected and the parasites were indistinguishable from those of dogs and humans, which proves its role as possible vector of *L. infantum* in the area. As in other foci in Colombia, (Travi *et al.* 1990) and Venezuela (Feliciangeli *et al.* 1998), *Lu. evansi* seems to predominate in most humid areas that do not seem to be endemic for VL on the island of Margarita. However, the potential to be incriminated as a possible vector has not to be discarded. On the other hand, it is also interesting to notice that in the eastern area of the island, which is almost completely covered by tropical thorn woodland, no VL cases have been recorded, but anti-*Leishmania* antibodies have been detected in the population (Zerpa *et al.* 2002).

A vector control trial targeted to *L. longipalpis s.l.*, has recently been carried out on the island, using indoor spraying of λ-cyhalotrin. The population density of *Lu. longipalpis* was significantly reduced in Santa Ana in comparison to the population density in a control village, Las Cabreras (Feliciangeli *et al.* 2003). However, it is necessary to evaluate the impact of this measure on the incidence of the disease, and further studies are needed to fully understand the transmission dynamics of the disease in order to design opportune strategies of prevention and control on the island of Margarita, which is the most important tourist area in Venezuela. The high incidence of serologically positive dogs (43% in 35 households) and co-habitants, together with the low incidence of sand fly vectors, have motivated an epidemiological surveillance and other studies concerning the transmission of visceral leishmaniasis in that area.

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