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ORIGINAL PAPER

Molecular identification of the parasites causing cutaneous leishmaniasis on the Caribbean coast of Colombia

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Abstract All clinical manifestations of leishmaniasis exist in Colombia, the cutaneous form being the most frequent in the department of Sucre, where the *Leishmania* species associated with cutaneous leishmaniasis (CL) is unknown. This study was carried out to determine which *Leishmania* species was responsible for CL in Sucre, based on amplification and sequencing of the *Cyt b* gene. Isolates of *Leishmania* were obtained after CL diagnosis of eight patients who received attention in several health care centers of the study area. The nucleotide sequences obtained from patients were compared to *Leishmania* reference strains and six of the isolates identified as *Leishmania* (*Viannia*) *braziliensis*, the remaining two being identified as *Leishmania* (*Viannia*) *panamensis* and *Leishmania* (*Viannia*) *guyanensis*. This represents the first report of the presence of *L.* (*V.*) *guyanensis* on the Caribbean coast of Colombia.

Introduction

Leishmaniasis is a disease caused by a broad species of the genus *Leishmania*. To date, 21 species have been reported to cause human infection (Herwaldt 1999). All clinical manifestations of leishmaniasis are present in Colombia, distributed throughout the country, with the cutaneous form (cutaneous leishmaniasis, CL) responsible for approximately 99% of cases (Zambrano 2006).

The geographical distribution and frequency of the etiological agents of CL in Colombia have been reviewed previously. Thus, *Leishmania* (*Viannia*) *panamensis* is known to cause 54–80% of CL cases, predominantly in Northern and Southwestern Colombia, while *Leishmania* (*Viannia*) *braziliensis* is responsible for 10–30% of cases and is distributed throughout the country. A third species, *Leishmania* (*Viannia*) *guyanensis* is involved in 1–2% of cases and is mainly present in the Amazon basin, although it was also recently detected in the department of Tolima. Finally, 1–5% of cases of *Leishmania* (*Leishmania*) *mexicana* infects have only been reported from the departments of Antioquia, Caldas, Nariño, Putumayo, and Santander (Sierra et al. 2006; Saravia et al. 2002; Ovalle et al. 2006; Rodríguez-Barraquer et al. 2008).

The department of Sucre on the Caribbean coast of Colombia presents a remarkable incidence of CL, with 206 cases of the disease diagnosed in 2008 (DASSSALUD, unpublished observations). Although CL is considered to have been endemic to Sucre since at least the 1980s, the identity of the parasite responsible remains unknown. Identification of the *Leishmania* species involved has both clinical and epidemiological relevance since it would allow improvement of both control and treatment of the disease, the latter through the provision of an appropriate therapeutic schedule. Studies have shown that the response of patients to treatment varies according to the parasite species responsible for infection (Rodríguez et al. 1994).

Traditionally, both *Leishmania* taxonomy and species typing have been based on the use of multilocus enzyme electrophoresis (MLEE) (Rioux et al. 1990) and monoclonal antibodies, respectively. However, accurate discrimination of each species is sometimes limited by interspecific phenotypic

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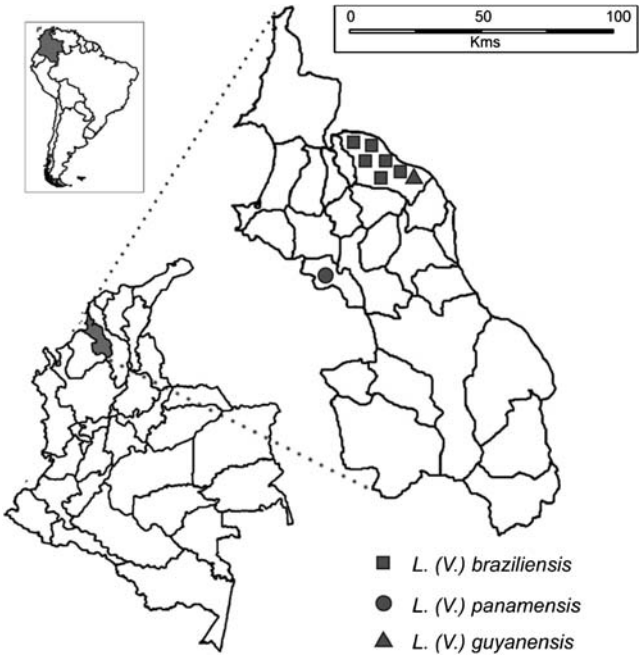

Fig. 1 Distribution of *Leishmania* clinical isolates identified in the department of Sucre

variation (Bañuls et al. 2007). In recent years, sequencing of the Cytochrome *b* (*Cyt b*) gene has begun to be used in identification and phylogenetic analyses of wild-type *Leishmania* isolates (Luyo-Acero et al. 2004; Kato et al. 2005; Marco et al. 2006; Foulet et al. 2007). In this paper, we report the identification of *Leishmania* species causing CL in Sucre using *Cyt b* nucleotide sequences and the presence of *L.* (*V.*) *guyanensis* on the Colombian Caribbean coast.

Materials and methods

Collection of samples Eight clinical isolates from patients attended in health care centers of the department of Sucre were included in this study (Fig. 1). Dermal scraping of the lesion edge with a sterile scalpel was performed on six patients from the municipality of Ovejas. Each isolate was placed in 1.5 mL vials containing 500 µL TES buffer (Tris-HCl 10 mM, EDTA 10 mM, SDS 0.1%) for DNA extraction. In addition, thin-needle aspirative biopsies were carried out after application of a local anesthetic (2% lidocaine) on two patients, one each from the municipalities of Ovejas and Sampues, was carried out. These latter two samples were placed in tubes containing NNN culture medium for parasite isolation. Four WHO *Leishmania* reference strains, *Leishmania* (*Leishmania*) *infantum* (MHOM/FR/91/LEM2259), *L.* (*L.*) *mexicana* (MHOM/MX/95/NAN1), *L.* (*V.*) *braziliensis* (MHOM/CO/88/UA301), and *L.* (*V.*) *panamensis* (MHOM/CO/87/UA140) were included in this study.

Before taking clinical samples, written informed consent was obtained from each patient or (in the case of minors) their parents or guardians. The experimental protocols satisfied the national and international policies on ethical clearances established by the declaration of Helsinki of 1983.

DNA extraction Whole DNA extraction of clinical samples and reference strains was carried out according to the protocol previously described (Watts 2001) with the following modifications. Each sample was incubated in a water bath at 65°C for 1 h, after which proteinase K (500 µg/mL) was added, and the mixture incubated at 55°C for a further 2 h. Subsequently, 150 µL of NaCl 6 M were added, followed by centrifugation at 12,000 rpm for 10 min, transfer of the supernatant to a sterile vial and precipitation of the DNA with an equal volume of absolute ethanol at −20°C overnight. Each sample was then centrifuged at 12,000 rpm for 10 min at 4°C, after which the supernatant was discarded and the precipitated material washed with 300 µL of absolute ethanol and 200 µL of

Table 1 *Leishmania* isolates and reference strains used in this study, showing species, origin, and GenBank accession numbers of the sequences of *Cyt b* gene

Species	Strain/isolate	Origin	GenBank accession number
L. (*L.*) *infantum*	MHOM/FR/91/LEM2259	France	EU499921
L. (*L.*) *mexicana*	MHOM/MX/95/NAN1	Mexico	EU499922
L. (*V.*) *braziliensis*	MHOM/CO/88/UA301	Colombia	EU499923
L. (*V.*) *panamensis*	MHOM/CO/87/UA140	Colombia	EU499924
L. (*V.*) *braziliensis*	Lbrove1	Ovejas/Sucre	EU499925
L. (*V.*) *braziliensis*	Lbrove2	Ovejas/Sucre	EU499926
L. (*V.*) *braziliensis*	Lbrove3	Ovejas/Sucre	EU499927
L. (*V.*) *braziliensis*	Lbrove4	Ovejas/Sucre	EU499928
L. (*V.*) *braziliensis*	Lbrove5	Ovejas/Sucre	EU499929
L. (*V.*) *braziliensis*	Lbrove6	Ovejas/Sucre	EU499930
L. (*V.*) *panamensis*	Lpasam1	Sampués/Sucre	EU499931
L. (*V.*) *guyanensis*	Lguove1	Ovejas/Sucre	EU499932

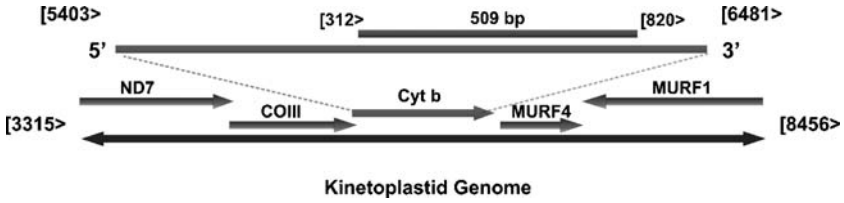

Fig. 2 Relative position of *Cyt b* gene and the 509 bp partial sequence in the kinetoplastid genome of *Leishmania tarentolae*

70% ethanol. Finally, the DNA was resuspended in 100 µL of high-purity water and stored at 4°C until used.

Amplification of the Cyt b gene of Leishmania Polymerase chain reaction (PCR) was carried out using GoTaq Flexi DNA polymerase (Promega) as follows: initial denaturation at 94°C for 1 min, followed by 35 amplification cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension cycle at 75°C for 5 min. Three microliters of whole DNA solution was used as a template. In order to amplify an 860 bp fragment of the *Leishmania Cyt b* gene both LCBF1 (5′-GGT GTA GGT TTT AGT YTA GG-3′) and LCBR2M (5′-ACA ATA AAC AAA TCA TAA TAT RCA ATT-3′) primers were used. The LCBR2M primer is a modification of the original LCBR2 one (Luyo-Acero et al. 2004). PCR yields were visualized in 1.25% agarose gels.

Nucleotide sequencing and analyses Amplified products were purified by the ethanol-precipitation method. Nucleotide sequences from purified fragments were achieved by direct sequencing using the BigDye™ Terminator Cycling Kit in an ABI 3730xl DNA Analyzer. Sequences from both ends of double-stranded DNA were edited and assembled with molecular evolutionary genetic analysis (MEGA) version 4 software (Tamura et al. 2007). Nucleotide alignment was carried out with CLUSTAL W software (Higgins et al. 1994); nucleotide and aminoacidic composition and paired distances were calculated with MEGA 4. Location of the nucleotide sequences for the *Cyt b* gene of *Leishmania tarentolae* was determined using basic local alignment search tool.

Sequences of isolates were compared to homologous sequences of *Leishmania* available in GenBank (access numbers AB095958, AB095959, AB095963, AB095967, AB095968, AB095969, EF579895, EF579900, EF579901, EF579905, and EF579915). Finally, evolutionary relationships between the obtained isolates and reference strains were inferred by means of a neighbor-joining algorithm (Saitou and Nei 1987).

Results

Eight clinical isolates were identified, obtained from patients from the region of Los Montes de María, Sucre. An 860 bp DNA fragment of the *Cyt b* gene was amplified in eight clinical isolates and four reference strains. These sequences were edited and 12 509 bp sequences were obtained and submitted to GenBank with the accession numbers EU499921 to EU499932 (Table 1). The first and the last nucleotide positions of the 509 bp DNA fragment of *Cyt b* gene of each tested isolate/strain corresponded to the 312 and 820 nucleotide of the *Cyt b* gene sequence of *L. tarentolae* (Fig. 2).

Six of these isolates were identified as *L.* (*V.*) *braziliensis*, one as *L.* (*V.*) *panamensis*, and the other as *L.* (*V.*) *guyanensis*. All *L.* (*V.*) *braziliensis* isolates and the one identified as *L.* (*V.*) *guyanensis* came from the rural area of

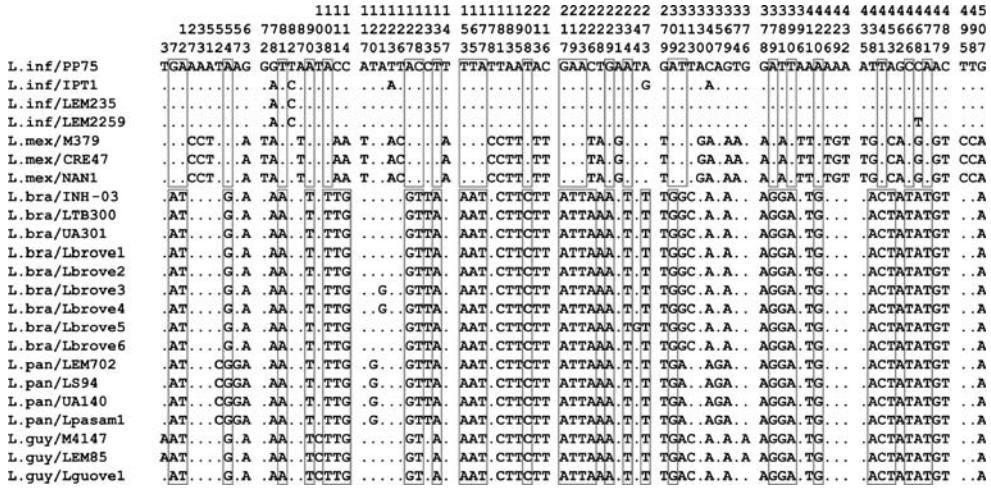

Fig. 3 Comparison of the nucleotide sequences of the *Cyt b* gene of *Leishmania* species included in this study. Nucleotide positions specific to the subgenera *Viannia* and *Leishmania* are marked with *gray squares*

the municipality of Ovejas. The isolate identified as *L.* (*V.*) *panamensis* was from the municipality of Sampués. Alignment of the partial sequences of the *Cyt b* gene (Fig. 3) revealed that 83 nucleotide positions were polymorphic of which two were singletons and 81 informative under parsimony criteria. The interspecies pairwise distances (Table 2) reached a minimum value of 0.00589 among *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis* and a maximum value of 0.11591 among *L.* (*L.*) *mexicana* and *L.* (*V.*) *panamensis*.

The interspecies variability of the clinical isolates identified as *L.* (*V.*) *braziliensis* was 0.39% due to non-synonymous substitutions: 1 A–G transition in the position 121 and 1 T–G transversion in the position 244 over the 509 bp. Comparison of the partial sequence of the *Cyt b* gene of both clinical isolates and the reference strain showed that Lbrove1, Lbrove2, Lbrove3, Lbrove4, Lbrove5, and Lbrove6 exhibited a high degree of similarity (99.8–100%) to the MHOM/CO/88/UA301 (GeneBank accession number EU499923), MHOM/EC/88/INH-03 (AB095967), and MHOM/BR/84/LTB300 (EF579900) *L.* (*V.*) *braziliensis* strain sequences. Lpasam1 showed a 100% similarity to the MHOM/CO/87/UA140 (EU499924), MHOM/PA/71/LS94 (AB095968), and MHOM/PA/71/LEM702 (EF579901) *L.* (*V.*) *panamensis* strain sequences.

Analysis of the clinical sequence Lguove1 and the MHOM/BR/75/M4147 (AB095969) and MHOM/GF/79/LEM85 (EF579905) *L.* (*V.*) *guyanensis* strain sequences showed 99.6% similarity due to synonymous and non-synonymous substitutions: 1 A/T transversion on the third position and 1 A/G transition on the position 376. All the substitutions in the amino acid sequences of the *Cyt b* gene products were to amino acids of the same electric nature, which does not involve important alterations in the polypeptide structure. The neighbor-joining tree (Fig. 4) shows the clinical sequences clustered in defined groups of species, providing evidence for evolutionary associations among the clinical isolates and the reference strains.

Discussion

The similarity percentages and the phylogenetic tree obtained from the partial sequences of the *Cyt b* gene of *Leishmania* allowed us to distinguish clearly between *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis*. Our results depart from those reported by Bañuls et al. (1999) who, using MLEE and random amplified polymorphic DNA (RAPD) data, questioned the distinctness between the two species *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis*. MLEE and RAPD data did not indicated that *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis* corresponded to distinct monophy-

Table 2 Pairwise distances based on sequences of the *Cyt b* gene among clinical isolates and reference strains of *Leishmania*

Subgenus species	*Leishmania*					*Viannia*					
	L. inf/LEM235	L. inf/IPT1	L. inf/PP75	L. inf/LEM2259	L. mex/NAN1	L. bra/UA301	L. bra/Lbrove1	L. bra/Lbrove3	L. bra/Lbrove5	L. pan/UA140	L. pan/Lpasam1	L. guy/M4147
L. inf/LEM235												
L. inf/IPT1	0.00589											
L. inf/PP75	0.00393	0.00982										
L. inf/LEM2259	0.00196	0.00786	0.00589									
L. mex/NAN1	0.08644	0.08448	0.8644	0.08644								
L. bra/UA301	0.10413	0.10413	0.10413	0.10413	0.11002							
L. bra/Lbrove1	0.10413	0.10413	0.10413	0.10413	0.11002	0.00000						
L. bra/Lbrove3	0.10609	0.10609	0.10609	0.10609	0.11198	0.00196	0.00196					
L. bra/Lbrove5	0.10609	0.10609	0.10609	0.10609	0.11198	0.00196	0.00196	0.00393				
L. pan/UA140	0.11002	0.11002	0.11002	0.11002	0.11591	0.01179	0.01179	0.01375	0.01375			
L. pan/Lpasam1	0.11002	0.11002	0.11002	0.11002	0.11591	0.01179	0.01179	0.01375	0.01375	0.00000		
L. guy/M4147	0.10806	0.10806	0.10806	0.10806	0.11395	0.00982	0.00982	0.01179	0.01179	0.01768	0.01768	
L. guy/Lguove1	0.10413	0.10413	0.10413	0.10413	0.11002	0.00589	0.00589	0.00786	0.00786	0.01375	0.01375	0.00393

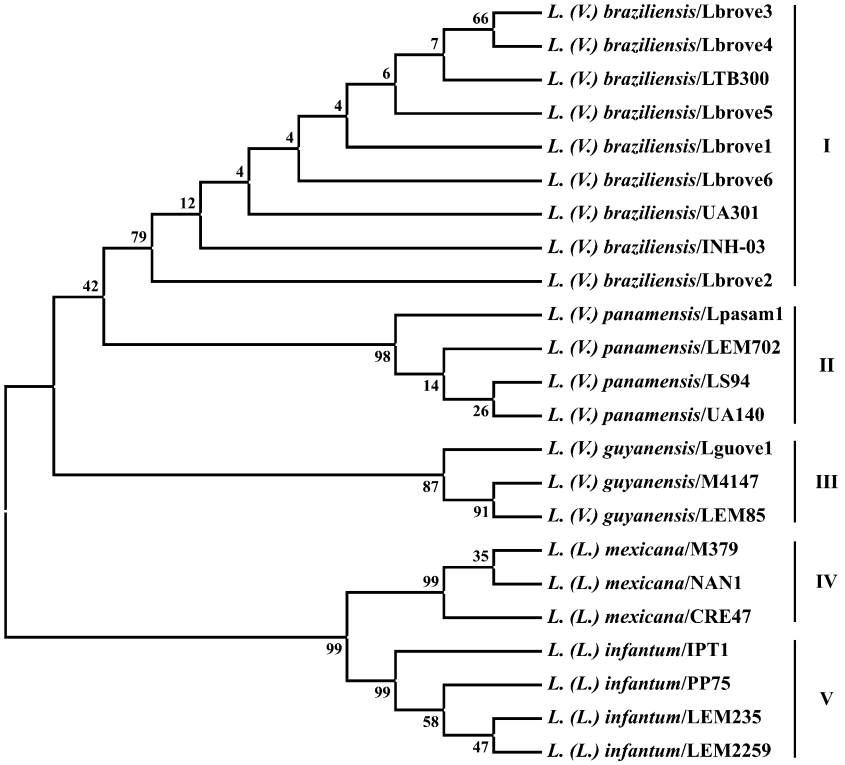

Fig. 4 Phylogenetic relationships of *Leishmania* species based on sequences of the *Cyt b* gene. *I L.* (*V.*) *braziliensis*, *II L.* (*V.*) *panamensis*, *III L.* (*V.*) *guyanensis*, *IV L.* (*L.*) *mexicana*, *V L.* (*L.*) *infantum*

letic lines. However, the limited number of isolates evaluated in the present study makes it difficult to reach a final conclusion about the taxonomic status of these species. It would be necessary to increase the sample size in order to compare both studies.

The nucleotide sequence variation of the *Cyt b* gene, 509 bp DNA fragment, allowed us to determine that *L.* (*V.*) *braziliensis*, *L.* (*V.*) *panamensis*, and *L.* (*V.*) *guyanensis* are the probable etiological agents of CL in Sucre. This finding confirms previous reports of members of the *L.* (*V.*) *braziliensis* species complex being the causative agents of human leishmaniasis in this region (López 2000; Martínez et al. 2005). Analysis of the sequences allowed us to identify 33 and 26 nucleotide positions specific to the subgenera *Viannia* and *Leishmania*, respectively (Fig. 3).

Even more important was the detection of polymorphic sites that facilitated the discrimination of different *Leishmania* species within each subgenus. In the *Viannia* subgenus, combinations of bases were identified in positions 312 and 313, which allowed us to distinguish the more epidemiologically relevant species: GC for *L.* (*V.*) *braziliensis*, AT for *L.* (*V.*) *panamensis*, and AC for *L.* (*V.*) *guyanensis*. Furthermore, our *Cyt b* gene sequences analyses showed 40 nucleotide positions key to the separation of *L.* (*L.*) *infantum* and *L.* (*L.*) *mexicana* species.

It is also important to highlight the higher numbers of nucleotide positions seen in members of the subgenus *Leishmania* (*Leishmania*) compared to those of *Leishmania* (*Viannia*), which is important in the identification of New World *Leishmania* species. This can be explained by the relatively recent evolutionary separation among *Viannia* species, which has hindered the discrimination of these taxa by some laboratory techniques. Despite the low interspecies polymorphism of the *Cyt b* gene, the key nucleotide positions described previously corroborate the potential of this gene as a molecular marker for *Leishmania* species characterization, not only in geographically related isolates, but also in widely separated regions.

From an epidemiological viewpoint, we surmise that the presence of *L.* (*V.*) *braziliensis* in Sucre could be associated with the continuous reports of CL treatment failures with Glucantime® and Miltefosine® in this area of the country. Limited effectiveness of the latter drug in the treatment of CL due to *L.* (*V.*) *braziliensis* has already been reported elsewhere (Soto et al. 2004). The presence of *L.* (*V.*) *panamensis* in the municipality of Sampués can be linked to previous findings of this species in the neighboring departments of Córdoba (Saravia et al. 2002) and Antioquia (Ovalle et al. 2006), there being no strong geographical barriers that would impede the dispersion of its potential mammal reservoirs throughout the Caribbean coastal plain of Colombia.

The clinical isolate identified as *L.* (*V.*) *guyanensis* present in the locality of Canutalito (Ovejas municipality) is the first diagnosed case of CL due to *L.* (*V.*) *guyanensis* not only in Sucre but anywhere on the Caribbean coast of Colombia. This is the second finding to suggest a possible expansion in the distribution of *L.* (*V.*) *guyanensis* beyond

its natural focus around the Orinoco and Amazon rivers (Rodríguez-Barraquer et al. 2008). This observation suggests a possible change in the pattern of transmission of this species in Colombia, since *Lutzomyia umbratilis*, the proven sand fly vector of *L.* (*V.*) *guyanensis*, has not been reported from this region.

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