



ARTÍCULO ORIGINAL

Molecular typing of *Leishmania (Leishmania) amazonensis* and species of the subgenus *Viannia* associated with cutaneous and mucosal leishmaniasis in Colombia: A concordance study

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Introduction: Multilocus enzyme electrophoresis (MLEE) is the reference standard for the characterization of *Leishmania* species. The test is restricted to specialized laboratories due to its technical complexity, cost, and time required to obtain results. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is used to identify *Leishmania* species.

Objective: To establish the concordance between the two tests as identifying methods for circulating species in Colombia.

Materials and methods: A total of 96 isolates from patients with cutaneous or mucosal leishmaniasis were selected and identified by MLEE and PCR-RFLP with miniexon and hsp70 as the molecular targets, which were used sequentially. Restriction enzymes HaeIII and BclI were similarly applied. Cohen's kappa coefficient and the 95% confidence interval (CI) were calculated.

Results: The kappa coefficient and the 95% CI between MLEE and PCR-RFLP displayed "very good" concordance with a coefficient of 0.98 (CI95%: 0.98 to 1.00). The identified species were *Leishmania Viannia braziliensis*, *Leishmania Viannia panamensis*, *Leishmania Viannia guyanensis* and *Leishmania Leishmania amazonensis*. A total of 80 of the 96 isolates were sequenced and the results obtained by PCR-RFLP were confirmed.

Conclusion: Due to the concordance obtained between tests results with the amplification of the genes miniexon and hsp70, PCR-RFLP is proposed as an alternative for identifying circulating *Leishmania* species in Colombia.

Key words: *Leishmania*; leishmaniasis; molecular typing; isoenzymes; polymerase chain reaction; polymorphism, restriction fragment length.

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Tipificación molecular de *Leishmania (Leishmania) amazonensis* y especies del subgénero *Viannia* asociadas a la leishmaniasis cutánea y la mucosa en Colombia: un estudio de concordancia

Introducción. La electroforesis de enzimas multilocus (*Multilocus Enzyme Electrophoresis*, MLEE) es el estándar de referencia para la tipificación de las especies de *Leishmania*. La prueba está restringida a laboratorios especializados por su complejidad técnica, sus costos y el tiempo necesario para obtener resultados. La PCR-RFLP (*Polymerase Chain Reaction-Restriction Fragment Length Polymorphism*) se utiliza para tipificar especies de *Leishmania*.

Objetivo. Establecer la concordancia entre las dos pruebas como métodos de tipificación de las especies circulantes en Colombia.

Materiales y métodos. Se seleccionaron 96 aislamientos de pacientes con leishmaniasis cutánea o mucocutánea y se tipificaron mediante MLEE y PCR-RFLP con los blancos moleculares miniexon y hsp70 usados en serie. Las enzimas de restricción aplicadas fueron la HaeIII y la BclI, respectivamente. Se calculó el coeficiente kappa y un intervalo de confianza (IC) de 95 %.

Resultados. Se determinó que la concordancia fue "muy buena" al obtener un coeficiente de 0,98 (IC_{95%}: 0,98-1,00). Las especies identificadas fueron: *Leishmania Viannia braziliensis*, *L. (V.) panamensis*, *L. (V.) guyanensis* y *L. (L.) amazonensis*. De los 96 aislamientos, 80 se enviaron a secuenciación y se confirmaron los resultados obtenidos mediante PCR-RFLP.

Conclusión. Dada la concordancia obtenida con la PCR-RFLP amplificando los genes miniexon y hsp70, se propone esta prueba como alternativa para la tipificación de especies de *Leishmania* circulantes en Colombia.

Palabras clave: *Leishmania*; leishmaniasis; tipificación molecular; isoenzimas; reacción en cadena de la polimerasa; polimorfismo de longitud del fragmento de restricción.

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Author's contributions:

Clemencia Ovalle-Bracho: conception and design of the study and acquisition of the data

Carolina Camargo and Yira Díaz-Toro: development of the experimental aspects

All authors participated in the analysis and interpretation of the data and in the writing of the manuscript.

Leishmaniasis is a group of endemic diseases in 98 countries, affecting approximately 12 million people, with approximately 350 million more at risk. These diseases are produced by a protozoan of the *Leishmania* genus with clinical manifestations in the skin, mucous membranes, and organs (1). The presentation of the disease, its evolution, and its response to treatment in the host vary according to the infective species. Therefore, identification of the diseases is relevant when determining the prognosis of the disease and when making an effective treatment choice. Species identification also allows for an understanding of the epidemiological behavior of the disease and contributes to the formulation of the control and prevention strategies appropriate to each region (2-5).

Currently, multilocus enzyme electrophoresis (MLEE) is considered the reference standard for the identification of *Leishmania* species due to its widespread use and ability to discriminate species in both the New and Old World (6-8). This methodology allows the characterization of the microorganisms by the electrophoretic mobility of a wide range of intracellular enzymes (9,10). Worldwide, nine reference centers provide species identification by MLEE, and only three of them are in the Americas (1). This, added to the complexity of the technique, limits the frequent application of this methodology to characterize *Leishmania* species in Colombia and the world (10-12).

Polymerase chain reaction (PCR) followed by an analysis of restriction fragment length polymorphism (RFLP) offers an alternative for identifying *Leishmania* species by analyzing the characteristic patterns of the different species obtained using restriction enzymes on amplified products of the genetic material of interest. This methodology has been expanded in the last two decades due to its speed in obtaining results, its ability to discriminate between species, and to adapt it to somewhat complex conditions regarding available technical resources, as well as the possibility of identifying *Leishmania* species directly from clinical samples (4,11,13,14).

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For identifying *Leishmania* species via PCR-RFLP, different molecular targets have been used. Some of the most frequently used have been kinetoplast DNA (kDNA) (15-18), the GP63 gene (4), the β -tubulin gene (19), ITS rDNA (18,20), the miniexon gene (21-24), and the gene that encodes heat shock protein 70 (hsp70) (2,25).

Previous studies from our group have focused on identifying circulating species of *Leishmania* in Colombia by using MLEE, monoclonal antibodies and PCR with kDNA, ITS rDNA, miniexon and hsp70 as targets, with the last two being those that allow differentiation of all subgenus *Leishmania* and *Viannia* species circulating in Colombia (18,26-29).

Amplification of the miniexon gene permits to identify the species *L. (L.) amazonensis*, *L. (L.) mexicana*, and *L. (L.) infantum* by differences in amplified product size (22,23). When amplification is followed by restriction using the HaeIII enzyme, it is possible to identify *L. (V.) braziliensis* but not the complex *L. (V.) panamensis/L. (V.) guyanensis* (22-24,30). The hsp70 gene amplification followed by RFLP with the BclI enzyme allows to differentiate between *L. (V.) panamensis* and *L. (V.) guyanensis* (25,31,32).

The aim of this study was to establish the concordance between PCR-RFLP using miniexon and hsp70 as molecular targets and the reference standard MLEE for the identification of circulating *Leishmania* species in Colombia.

Materials and methods

Type of design

We conducted a concordance study that used MLEE and PCR-RFLP for the identification of the *Leishmania* species circulating in Colombia.

Study population

Isolates came from patients diagnosed with cutaneous, diffuse and mucosal leishmaniasis who visited the *Centro Dermatológico Federico Lleras Acosta E.S.E.* between 1999 and 2011.

Sample

We included 96 samples out of the 800 available at the biological bank. The inclusion criteria was strains previously identified under the indirect fluorescent antibody test and classified like null phenotype.

Reference strains

The reference strains included *L. (V.) braziliensis* (MHOM/BR/75/M2903), *L. (V.) panamensis* (MHOM/PA/71/LS94), *L. (V.) guyanensis* (MHOM/BR/75/

M4147), *L. (L.) amazonensis* (IFLA/BR/67/PH8), and *L. (L.) mexicana* (MNCY/BZ/62/M379). The electrophoretic and restricted patterns of the reference strains were used to determine the species of the isolates.

Parasite culture

Parasites were cultured in Senekjie's medium and incubated at 26°C until we obtained a primary culture. Subsequently, a mass culture was performed using Schneider's medium supplemented with 10% fetal bovine serum (33).

Species identification

Identification by MLEE and PCR-RFLP were performed from the same isolated sample. Samples were processed by different professionals for each of the tests. Isoenzyme identification was performed in a reference center of the Pan American Health Organization (PAHO).

Identification by MLEE

The preparation of the extract for analysis and the isoenzyme was performed according to the methods described by Saravia, *et al.* (34,35). Isoenzyme electrophoresis of the clinical isolates and reference strains was performed on a cellulose support according to the method described by Godfrey, *et al.* (36).

DNA extraction

The cetyltrimethylammonium bromide method was performed as described by Wagner, *et al.*, in 1987 and used for both the reference strains and the clinical isolates (37).

Minixon gene PCR

For gene amplification we used the primers reported by Marfurt, *et al.* (22,23). Each PCR reaction contained 20 mM Tris-HCl pH 8.4; 50 mM KCl; 1 mM MgCl₂; 200 μM dATP, dCTP, dTTP and dGTP; 0.5 μM of each primer (Fme 5'-TAT TGG TATGCG AAA CTT CCG-3' and Rme 5'-ACA GAA ACT GAT ACT TAT ATA GCG-3') and DMSO 6%, 2 μl of DNA at 10 ng/μl and 0.05 units of Taq Polymerase Platinum (Invitrogen, São Paulo, Brasil) at a final volume of 50 μl.

Amplification was performed with the following conditions: An initial DNA denaturation at 95°C for 8 minutes; 40 amplification cycles of 95°C for 20 s, 55°C for 30 s and 72°C for 30 s; and a final extension cycle at 72°C for 5 minutes.

hsp70 gene PCR

Amplification was performed following the protocol proposed by García, *et al.* (25). Each PCR reaction contained 20 mM Tris-HCl pH 8.4; 50 mM KCl; 2 mM MgCl₂; 200 μM dATP, dCTP, dTTP and dGTP; 0.4 μM of each primer (hsp70 sen 5'-GAC GGT GCC TGC CTA CTT CAA-3' and hsp70 ant 5'-CCG CCC ATG CTC TGG TAC ATC-3'); 10% DMSO; 2 μl of DNA at 10 ng/μl and 0.05 units of Taq Polymerase Platinum (Invitrogen, São Paulo, Brazil) at a final volume of 50 μl.

Amplification was performed using the following protocol: An initial DNA denaturation at 94°C for 5 minutes; 33 amplification cycles at 94°C for 1 minute, 64°C for 1 minute and 72°C for 3 minutes; and a final extension cycle at 72°C for 10 minutes.

Visualization of the minixon and hsp70 PCR products

Amplicons of the minixon and hsp70 genes were analyzed by electrophoresis in 1% and 2% agarose gels, respectively. For the identification of species through PCR-RFLP, the molecular weights of the amplicons from the minixon gene must be considered: 308 bp for *L. (L.) mexicana*, 283 bp for *L. (L.) amazonensis*, and 418 bp for the *L. (L.) chagasi* control, and between 255 and 227 bp for the *Viannia* subgenus (22, 23). The hsp70 gene presents a 1422-bp band for the *Viannia* subgenus (38).

Restriction fragment length polymorphism (RFLP)

Samples showing an amplification profile of the minixon gene compatible with the *Viannia* subgenus were subjected to restriction with the HaeIII enzyme (Invitrogen, São Paulo, Brazil).

The hsp70 gene of the samples that were identified as *L. (V.) panamensis*/*L. (V.) guyanensis* through the minixon gene PCR-RFLP was also amplified and digested with the BclI enzyme (New England Biolabs, Ipswich, England).

Amplicons of the minixon and hsp70 genes were purified using QIA quick PCR Purification kits (Qiagen, Düsseldorf, Germany). A total of 12.5 μl of purified PCR product was used for the digestion, with the addition of 1.4 μl of reaction buffer and 0.5 μl of restriction enzymes HaeIII (10 U/μl) and BclI (10 U/μl); the mix was incubated at 37°C for 120 minutes and at 45 volts.

Restriction products were analyzed by electrophoresis using high-resolution 2.5% agarose gels (Sigma-Aldrich, St. Louis, Missouri, USA) at 2.5 V/cm for 1.5 hours; a 100-bp molecular-weight marker was used (Biolone, London, England).

The samples that presented a restriction pattern of two bands of 108 and 118 bp with the HaeIII enzyme were identified as *L. (V.) braziliensis*. Amplicons that did not show a restriction pattern were identified as *L. (V.) panamensis*/*L. (V.) guyanensis*.

The restriction products obtained through the BcclI enzyme in the reference strains *L. (V.) panamensis* (MHOM/PA/71/LS94) and *L. (V.) guyanensis* (MHOM/BR/75/M4147) showed the following patterns: Two bands of 428 and 890 bp and three bands of approximately 346, 428 and 544 bp. Species of clinical isolates were determined based on the restriction patterns obtained with reference strains.

Sequencing

We selected 80 isolates by simple random sampling and sequenced them to confirm the species identified by PCR and PCR-RFLP, including the samples identified as *L. (V.) panamensis*, *L. (V.) braziliensis*, *L. (V.) guyanensis*, and *L. (V.) amazonensis*. For sequencing we used a BigDye Terminator, v3.1, Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730xl DNA Analyzer system (Applied Biosystems, Foster City, CA, USA). For the analysis we used the software Geneious Pro 5.5.6 (Biomatters Ltd, Auckland, New Zealand) to align the sequences obtained with the sequences available at NCBI of the particular genes of the species of interest.

Statistical analysis

The results of the MLEE and PCR-RFLP of the species identified were recorded in an Excel database (Microsoft Office XP). Cohen's kappa coefficient and the 95% CI were calculated using the software Stata®, version 13 (Stata Corp LP, College Station, Texas, USA) to determine the concordance between the MLEE and PCR-RFLP results.

Ethical considerations

This study was classified as safe and approved by Centro Dermatológico Federico Lleras Acosta E.S.E. Ethical Committee for the use of cryopreserved samples according to the ethical guidelines established by the Ethics Committee in Research, which are based on the Declaration of Helsinki and current Colombian regulations.

Results

The identification of the species for the isolates was performed according to the results obtained by PCR, PCR-RFLP and MLEE: *L. (V.) braziliensis*: 63.5%, *L. (V.) panamensis*: 21.9%, *L. (V.) guyanensis*: 5.2%

and *L. (L.) amazonensis*: 9.4%. Sequencing confirmed the results obtained by PCR and PCR-RFLP. Figures 1 and 2A show PCR–miniexon gene and PCR-hsp70 gen amplification products from different *Leishmania* reference strains and clinical samples, respectively. The figure 2B shows restriction endonuclease DNA fragment patterns from amplicons of the PCR-hsp70 gene using *BcclI* enzyme. Table 1 shows the identification results obtained by MLEE, miniexon gene PCR-RFLP, and hsp70 gene PCR-RFLP, and table 2 shows the results obtained by sequencing.

The concordance between the results of *Leishmania* species identification by PCR-RFLP and MLEE was “very good” according to the scale of Landis, *et al.* (39), for obtaining a kappa of 0.98 (CI95%: 0.98-1).

Among the 96 samples analyzed by both molecular tests (miniexon gene PCR-RFLP and hsp70 gene PCR-RFLP) and by MLEE, only one discordant result was observed. One of the isolates was identified through molecular tests as *L. (V.) braziliensis* but as *L. (V.) panamensis* by MLEE.

We obtained 96 isolates from patients whose ages ranged between 1.4 and 73 years and who had been diagnosed with cutaneous, diffuse cutaneous or mucosal leishmaniasis.

The patients came from 51 municipalities endemic for leishmaniasis in 15 different departments: Antioquia (n=1), Boyacá (n=4), Caldas (n=4), Caquetá (n=15), Casanare (n=2), Cundinamarca (n=24), Guainía (n=1), Guaviare (n=6), Meta (n=21), Nariño (n=1), Risaralda (n=1), Santander (n=4), Tolima (n=6), Valle del Cauca (n=2), and Vichada (n=4) (figure 3).

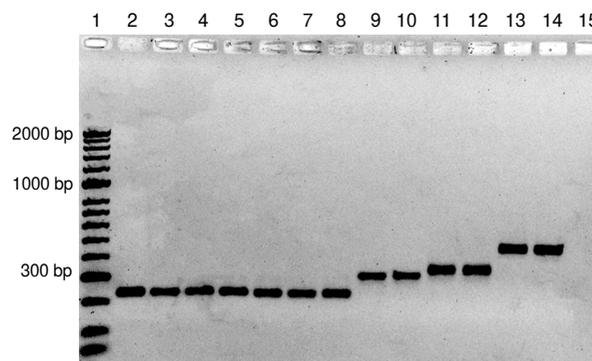


Figure 1. A. Line 1, molecular weight marker; Line 2, *L. (V.) braziliensis* reference strain DNA; Line 3, *L. (V.) panamensis* reference strain; Line 4, *L. (V.) guyanensis* reference strain; Lines 5-8, DNA from clinical samples of leishmaniasis cases typed as *Leishmania Viannia* subgenus; Lines 9-10 *L. (L.) amazonensis* reference strain; Lines 11-12, *L. (L.) mexicana* reference strain; Lines 13-14, *L. (L.) infantum*; Line 15, negative control

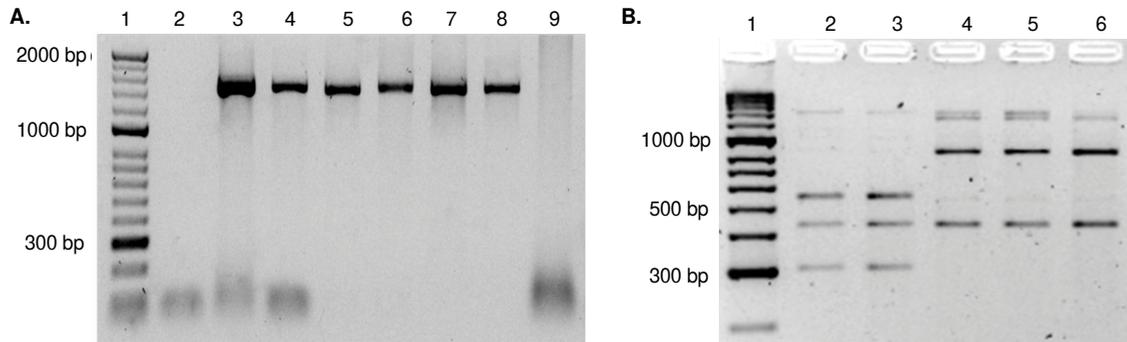


Figure 2. **A.** Line 1, molecular weight marker; Line 2, negative control; Line 3 *L. (V.) panamensis* reference strain DNA; Line 4, *L. (V.) guyanensis* reference strain DNA; Lines 5-8, DNA from clinical samples of leishmaniasis cases; Line 9, clinical sample from patient with suggested diagnosis of mucosal leishmaniasis and rhinoscleroma confirmed diagnosis. **B.** Line 1, molecular weight marker; Line 2, *L. (V.) guyanensis* reference strain; Line 3, clinical sample typed as *L. (V.) guyanensis*; Line 4, *L. (V.) panamensis* reference strain; Lines 5-6, clinical samples typed as *L. (V.) panamensis*

Table 1. Typing results of 96 isolates of *Leishmania* spp. by MLEE and PCR-RFLP methods

	MLEE	PCR-RFLP miniexon gene	PCR-RFLP hsp70 gene [‡]
<i>L. (V.) braziliensis</i>	60	61	-
<i>L. (V.) panamensis</i>	22	-	21
<i>L. (V.) guyanensis</i>	5	-	5
<i>L. (V.) panamensis/L. (V.) guyanensis</i>	-	26	-
<i>L. (L.) amazonensis</i>	9	9	-
Total	96	96	26

[‡]: Method applied only to 26 samples classified as *L. (V.) panamensis/L. (V.) guyanensis* by PCR-RFLP with the miniexon gene

Table 2. Typing results of 80 isolates of *Leishmania* spp. by MLEE and PCR-RFLP miniexon/hsp70 methods evaluated by sequencing

	Sequencing	MLEE	PCR-RFLP miniexon/hsp70
<i>L. (V.) braziliensis</i>	47	46	47
<i>L. (V.) panamensis</i>	20	21	20
<i>L. (V.) guyanensis</i>	4	4	4
<i>L. (L.) amazonensis</i>	9	9	9
Total	80	80	80

Discussion

According to the criteria of Landis, *et al.* (39), this study achieved a “very good” concordance between MLEE and PCR-RFLP of the miniexon and hsp70 genes for characterizing circulating *Leishmania* species in Colombia from clinical isolates. These results indicate that those obtained using PCR-RFLP for the species under study are equivalent to the results obtained using MLEE, which is considered the reference standard (1,6). The tests were

applied on samples from the five geographical regions of the country, suggesting that it is possible to identify species despite the intraspecies variability associated with geographical distribution.

MLEE is a useful technique for characterizing species, as it permits the analysis of genetic variability between and among species, as well as their taxonomies and phylogenies (40,41). However, this methodology does not permit the direct identification of clinical specimens, the culture is required and it has low sensibility for samples from patients with mucosal and chronic cutaneous leishmaniasis, which prevents obtaining large amounts of parasites. Besides, MLEE is technically demanding and requires prolonged times for obtaining results (10-12,41). An alternative to discriminate between *Leishmania* species is PCR-RFLP, a widely used test that is performed with different molecular targets to determine the ability to differentiate species (42).

The miniexon and hsp70 genes were selected to identify circulating species in Colombia as they have several advantages: The number of copies of the genes allows for good test sensitivity, and these genes have been validated on a global scale with Old and New World species.

Recently, van der Auwera, *et al.*, concluded after the analysis of several publications that the miniexon and hsp70 genes, compared to other molecular targets, exhibit the best resolution to identify medically relevant *Leishmania* species (43). This claim is supported by experimental studies of the miniexon gene performed by Marfur, *et al.*, as well as studies performed by Serin, *et al.*, who evaluated the functionality of the miniexon gene as a phylogenetic marker of 17 species of the New

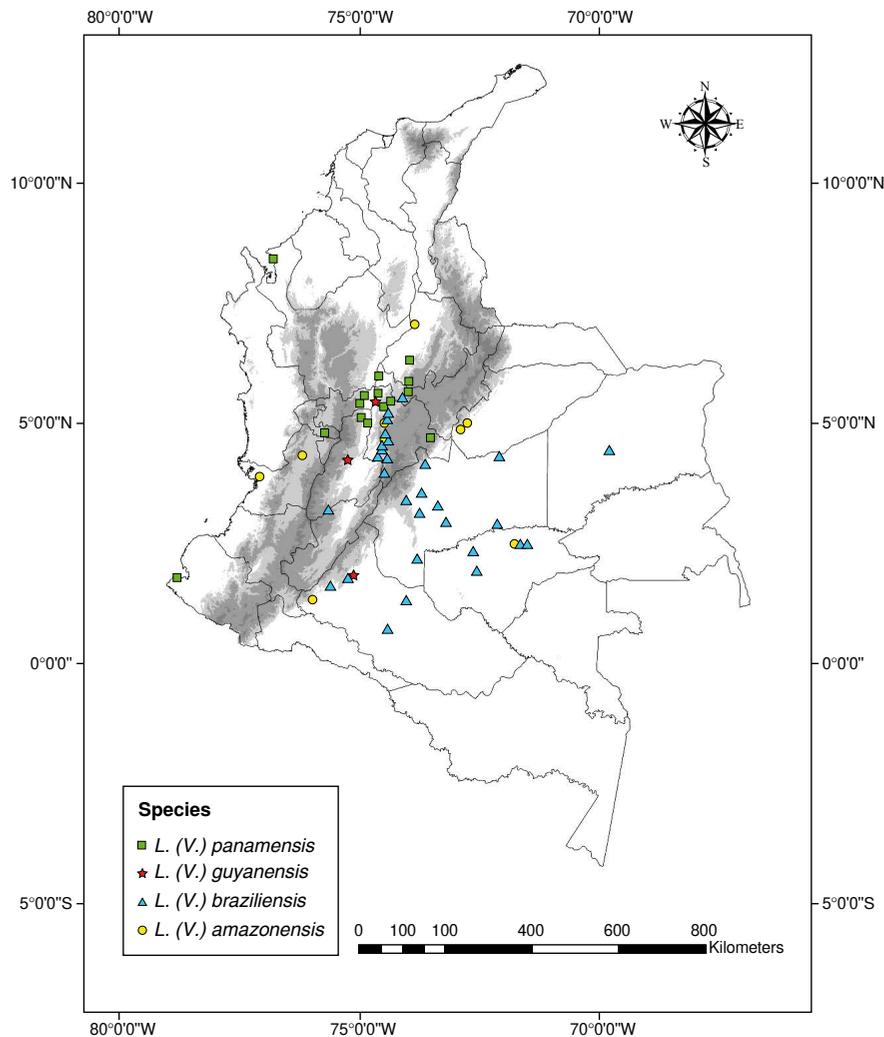


Figure 3. Distribution of *Leishmania* species identified by PCR-RFLP with miniexon and hsp70as molecular targets. Map done using ArcGis vr. 10.2 program; the altitude was obtained from the WorldClim database with 1 km resolution (<http://www.worldclim.org/>).

and the Old World (22-24). The hsp70 gene has been evaluated for almost all circulating species in the world, with results that demonstrate its discriminatory capacity (28,32,44).

In our study, we identified *L. (V.) braziliensis* using PCR-RFLP, and *L. (L.) amazonensis* using only PCR based on amplicon size, results that were confirmed by sequencing. In Colombia, even though the prevalent species are associated with the *Viannia* subgenus, *L. (L.) amazonensis* recently was associated with cutaneous, diffuse cutaneous, and mucosal leishmaniasis. PCR of the miniexon gene allows the identification of *L. (L.) amazonensis*, *L. (L.) mexicana*, and *L. (L.) chagasi* only by amplicon size. This approach is very useful and less expensive than other methods that require additional tests such as RFLP or sequencing.

The hsp70 gene has also been extensively evaluated in Old and New World species. García, *et al.*, were the first to identify its functionality to diagnose and differentiate species from the subgenera *Leishmania* and *Viannia*, complemented with techniques such as RFLP and sequencing (29,32,38,45-48).

This technique has also proven to be useful in differentiating species from the *L. guyanensis* complex, which includes *L. (V.) panamensis* and *L. (V.) guyanensis*. These species are of great clinical and epidemiological interest in Colombia because *L. (V.) guyanensis* was associated with the largest epidemic of leishmaniasis in the country. This species also presents differences in the *in vitro* susceptibility response to drugs compared to other species of the *Viannia* subgenus (49,50).

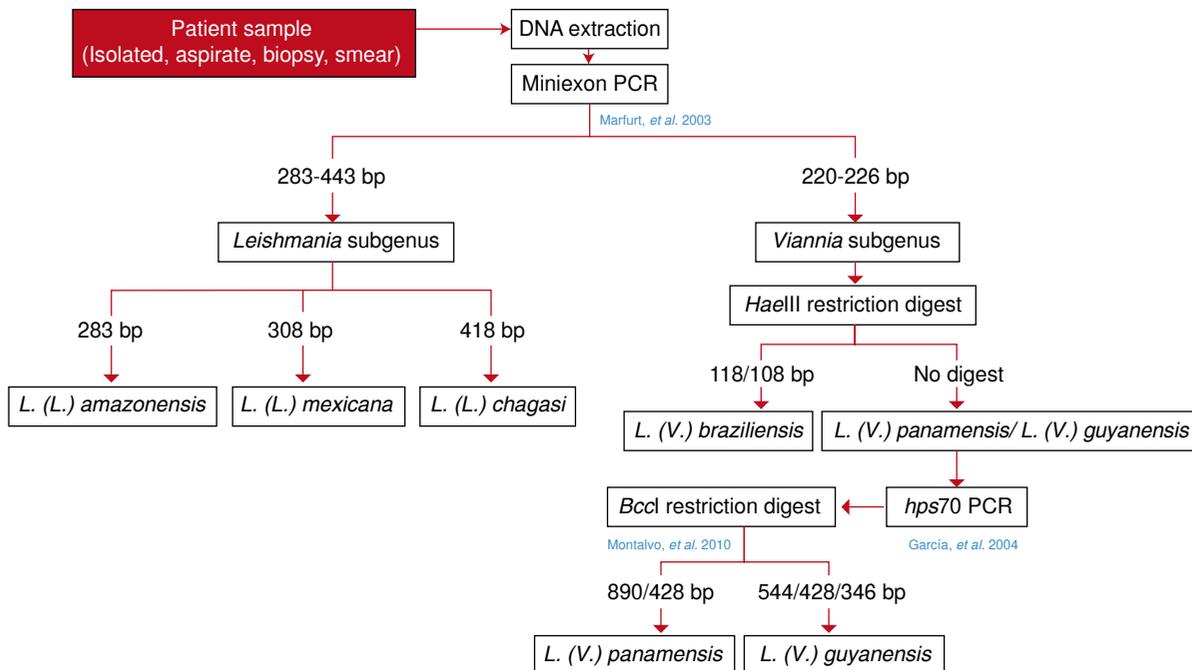


Figure 4. Identification algorithm of *Leishmania* species with miniexon and hsp70 as molecular targets

In our study, hsp70 gene amplification and RFLP using the enzyme *BclI* were very useful, as they allowed discrimination between *L. (V.) panamensis* and *L. (V.) guyanensis*. The results obtained in the concordance analysis between the two tests suggest that they are equivalent for identifying the species circulating in Colombia. Our results are consistent with those obtained by da Silva, *et al.*, who found an overall concordance between MLEE and PCR-RFLP of the hsp70 gene of the circulating species in Brazil, based on which the authors concluded that the latter method can replace MLEE in the routine identification of *Leishmania* species (51).

Another method recently used to identify species is the high-resolution analysis of dissociation using the hsp70 gene, which has demonstrated good agreement with PCR-RFLP and MLEE (52). Cardoso da Graça, *et al.*, compared the molecular targets ITS, G6PD and hsp70 with MLEE, and found very good concordance between the methodologies for the identification of *L. (L.) amazonensis*, *L. (L.) infantum*, *L. (V.) guyanensis*, *L. (L.) lanzonei*, *L. (V.) naiffi*, and *L. (L.) shawi*, but discordance for the identification by MLEE of *L. (V.) braziliensis* (53), which was the only dissenting finding of the current study.

Several hypotheses have been proposed to explain the difference between the identification results of the various methodologies, including the possibility

of mixed infection, variation in the patterns according to the markers used, and the similarity between species of the *Viannia* subgenus (53,54).

The results indicate that the PCR-RFLP of the miniexon and hsp70 genes permit the identification of circulating species in the country. This test could be used in more laboratories compared to MLEE because it is technically simpler and the cost is 50% less than for MLEE. This work includes the largest number of samples reported in the country, identified by molecular tests and with a wide geographical distribution.

The test is restricted to native species, but it would be possible to expand its spectrum using other restriction enzymes to identify new circulating or imported species. Here we propose an identification algorithm for the *Leishmania* species that are circulating in Colombia (figure 4).

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Conflicts of interest

The authors report no conflicts of interest.

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