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Differentiation of *Leishmania (Viannia) panamensis and Leishmania (V.) guyanensis* using *Bcc*I for *hsp*70 PCR-RFLP

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ABSTRACT

Leishmania panamensis and *Leishmania guyanensis* are two species of the subgenus *Viannia* that are genetically very similar. Both parasites are usually associated with cutaneous leishmaniasis, but also have the potential to cause the mucocutaneous form of the disease. In addition, the study of foci and consequently the identification of vectors and probable reservoirs involved in transmission require a correct differentiation between both species, which is important at epidemiological level. We explored the possibility of identifying these species by using restriction fragment length polymorphisms (RFLP) in the gene coding for heat-shock protein 70 (*hsp*70). Previously, an *hsp*70 PCR-RFLP assay proved to be very effective in differentiating other *Leishmania* species when *Hae*III is used as restriction enzyme. Based on *hsp*70 sequences analysis, *Bcc*I was found to generate species-specific fragments that can easily be recognized by agarose gel electrophoresis. Using the analysis of biopsies, scrapings, and parasite isolates previously grouped in a cluster comprising both *L. panamensis* and *L. guyanensis*, we showed that our approach allowed differentiation of both entities. This offers the possibility not only for identification of parasites in biological samples, but also to apply molecular epidemiology in certain countries of the New World, where several *Leishmania* species could coexist.

1. Introduction

The increase in the application of molecular tools for identification of *Leishmania* has led to the recommendation that species typing should be part of the diagnostic procedure.[1–5] This is especially relevant in Latin America where different species may be encountered, even within the same foci. For some species however, taxonomic status is questioned and without robust molecular markers, it is difficult to address this issue and explore phenotypic differences associated with the respective taxa.

This is the case of *L. (V.) panamensis* and *L. (V.) guyanensis*, two species for which multilocus enzyme electrophoresis and random amplified polymorphic DNA could not establish that they correspond to distinct monophyletic lines.[6] Clinically, both entities are usually responsible for different cutaneous presentations, ranging from mild lesions to multiple nodules following a lymphatic distribution,[7] and which are able to invade mucosal tissues by dissemination via blood, lymph or by

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extension.[8–10] However, their involvement in mucosal disease is less destructive, differing from the chronic and severe form characteristically caused by *L. braziliensis*.[11]

Early reports[12] mention differences in treatment outcome, *L. (V.) guyanensis* being eliminated with difficulty by pentavalent antimonials, with patients frequently needing many courses of treatment, in contrast to *L. (V.) panamensis* which is readily eliminated by these drugs. In addition, different proven or probable vectors are responsible for the transmission of these species,[13] which is of epidemiological importance in the countries where they are encountered, e.g. Brazil,[14] Colombia,[15] Peru,[16] Panama[17] and Ecuador.[1]

In view of the necessity to differentiate these species in a reliable manner, we explored the possibility of using an *hsp*70 PCR-RFLP approach, an assay already shown to be very effective in differentiating certain species of *Leishmania*.[18] *Leishmania (V.) panamensis* and *L. (V.) guyanensis* reference strains of different geographical origins were analyzed and a restriction enzyme allowing differentiation of both entities was identified. The assay was further validated using clinical samples from human infections.

2. Materials and methods

2.1. Strains and clinical samples

Reference strains ($n = 15$) used in the study are listed in Table 1. Parasites were maintained as promastigotes at 26 °C in Schneider's medium (SIGMA, St. Louis, MO, USA), supplemented with 10% of fetal bovine serum, 250 μg/ml streptomycin and 100 U of penicillin. Biopsies ($n = 12$) and scrapings ($n = 3$) from the indurate border of the lesion were taken using a 2-mm disposable biopsy punch. All of them were taken from Colombian subjects with cutaneous leishmaniasis and analyzed by direct examination after Giemsa-staining. In addition, 12 *Leishmania* isolates from cutaneous disease were maintained in NNN medium at the 'Programa de Estudio y Control de Enfermedades Tropicales' (PECET, Universidad de Antioquia, Medellín, Colombia). All the samples analyzed in this study were previously classified as belonging to the *L. panamensis*/*L. guyanensis* group by *hsp*70 PCR-RFLP using *Hae*III as restriction endonuclease.

2.2. DNA extraction and hsp70 PCR procedure

Genomic DNA from reference strains was obtained from parasites in culture (10^7–10^9 parasites) and dissolved in a maximum of 50 μl, according to the parasite number. Phenol-chlorophorm DNA extraction was carried out as in Sambrook et al.[19] The quality of DNA extraction was verified by electrophoresis in a 0.8% agarose gel. DNA from biopsies, scrapings and *Leishmania* isolates was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Primers used were previously reported.[18] PCR conditions were adapted as follows: the reaction mix (50 μl) contained 1 × Taq polymerase buffer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.25% DMSO, 1.0 U Taq DNA polymerase (Eurogentec, Seraing, Belgium), 0.4 μM of each primer, and DNA extracted from parasites (2 μl), biopsies (2.5 μl), scrapings (2.5 μl) and isolates (1 μl). A negative control tube containing all the components except for DNA was always included, as well as a positive one, containing DNA from a reference strain. Thermal cycling was done in a cycler from MJ Research (Baltimore, MD, USA) using the following conditions: (i) initial denaturation at 94 °C for 5 min, (ii) 33 cycles, each one consisting of 94 °C for 30 sec, 61 °C for 1 min and 72 °C for 3 min and (iii) a final extension step of 10 min at 72 °C. PCR products were analyzed on a 1.2% agarose gel.

2.3. Sequencing and analysis of polymorphisms

In order to allow sequencing of *hsp*70 PCR products for *L. panamensis* and *L. guyanensis*, four primers were used for sequencing both strands of the entire amplicon in regions found to be the most conserved in different species, according to previously reported *hsp*70 sequences: *L. braziliensis* **AF291716** and **M87878**, *L. amazonensis* **L14604** and **L14605**, *L. donovani* **X52314** and **AY913843**, *L. infantum* **X58798** and **Y08020**, *L. major* **M36675**:

Table 1
Leishmania reference strains from *L. (V.) guyanensis* and *L. (V.) panamensis* used in the study.

Leishmania species	Strain code	*hsp*70 sequence Accession Number	Country of origin
Leishmania (V.) guyanensis	MHOM/PE/91/LC1446		Peru
	MHOM/PE/03/LH2966		Peru
	MHOM/PE/03/LH2524		Peru
	MHOM/PE/02/LH2270		Peru
	MHOM/PE/02/LH2372 *	**FN395051**	Peru
	MHOM/PE/91/LC1448		Peru
	MHOM/PE/02/LH2372		Peru
	MHOM/PE/02/LH2403		Peru
	MHOM/PE/02/LH2190		Peru
	MHOM/BR/2007/025-LFA		Brazil
	MHOM/GF/85/LEM699 *	**FN395052**	French Guiana
	MHOM/BR/75/M4177 *	**EU599093**	Brazil
	MHOM/BR/2007/029-ZAV *	**FN395053**	Brazil
Leishmania (V.) panamensis	MHOM/PA/71/LS94 *	**EU599094**	Panama
	MCHO/PA/00/M4039 *	**FN395055**	Panama

The strains sequenced for the *hsp*70 coding gene in this study are indicated (*) as well as their accession numbers in the EMBL and GenBank data bases.

HSP70-F335 5′CACGCTGTCGTCCGCGACG-3′, HSP70-R429 5′–AACAGGTCGCCGCACAGCTCC-3′, HSP70-F893 5′–GTTCGACCTGTCCGGCATCC-3′, HSP70-R1005 5′–GTGATCTGGTTGCGCTTGCC-3′. Automated sequencing was done using a Big Dye Terminator cycle sequencing ready-reaction kit (Perkin Elmer, Foster City, CA, USA). Sequence accuracy was guaranteed by two-directional sequencing, except for the 40 terminal nucleotides which were sequenced on one strand only. Based on the aligned sequences, restriction sites useful for typing purposes were recognized using the NEB cutter Program (http://tools.neb.com/NEBcutter2/index.php).

2.4. hsp70 PCR-RFLP analysis with BccI

*hsp*70 PCR products were ethanol precipitated and suspended in 20 μl of water. Then, 5 μl were digested with 1 U of *Bcc*I (New England Biolabs, Ipswich, MA, USA) in a total volume of 10 μl, following manufacturer's instructions. Restricted fragments (10 μl) were analyzed by electrophoresis in a 3% small fragment agarose gel (Gentaur, Brussels, Belgium), running at 50 V. First, banding patterns corresponding to reference strains of *L. panamensis* and *L. guyanensis* were established, using as markers the Gene Ruler™ 100 bp DNA Ladder (MBI, Fermentas, St Leon-Rot, Germany). The banding observed was compared with the expected theoretical profile in each case, considering the sequence analysis. Biopsies, scrapings and isolates were analyzed using the same procedure; whereby the patterns obtained were compared with the reference strains.

3. Results

PCR amplification of the gene coding *hsp*70 was possible using the DNA extracted from reference strains of both parasite species. The band size corresponded to the expected one, according to the report of García et al.[18] Sequencing revealed an amplicon size of 1422 bp in all cases.

Two species-specific single nucleotide polymorphisms (SNPs) were identified from analysis of the *hsp*70 gene fragment from *L. guyanensis* (**FN395051**; **FN395052**; **EU599093**; **FN395053**) and *L. panamensis* (**EU599094**; **FN395055**): C/T at position 1060 and A/G at position 1280 respectively. The presence of C at position 1060 results in the *Bcc*I recognition site CCATC$(N)_4$ in *L. guyanensis*, suited to generate a species-specific banding pattern for *L. guyanensis* (104/346/428/544 bp) and *L. panamensis* (104/428/890 bp).

All reference strains were analyzed by PCR-RFLP with *Bcc*I, comparing the bands obtained with the ones expected. No intraspecific differences were encountered among *L. guyanensis* strains despite their diverse geographical origin (Supplementary Figure 1). For *L. panamensis* no differences were encountered among either of the two strains analyzed.

In a last step, we examined whether this genotyping method can be used to characterize different biological samples. From a total of 27 samples the amplified *hsp*70 fragment was studied, showing a pattern shared by *L. panamensis* and *L. guyanensis* with *Hae*III digestion (not shown). Using *hsp*70 PCR-RFLP/*Bcc*I, 24 of them were classified as *L. panamensis*, and 3 as *L. guyanensis* (Table 2). The species differentiation was possible using DNA obtained from biopsies, scrapings and isolates.

Table 2
Characterization of the infecting species in biological samples analyzed. *hsp*70 PCR-RFLP/*Hae*III pattern represents previous grouping of the samples, with respect to the enzymatic restriction of *hsp* 70PCR products using *Hae*III. The *hsp*70 PCR-RFLP/*Bcc*I pattern allowed individual differentiation.

Biological sample	Number analyzed	*hsp*70 PCR-RFLP *Hae*III pattern	*hsp*70 PCR-RFLP *Bcc*I pattern	
		L. pan/*L. guy*	*L. pan*	*L. guy*
Biopsies	12	12	12	–
Scrapings	3	3	2	1
Isolates	12	12	10	2
Total	**27**	**27**	**24**	**3**

4. Discussion

We have demonstrated that *hsp*70 PCR-RFLP with *Bcc*I is a useful tool for differentiation of *L. panamensis* and *L. guyanensis*. Whether the two taxa at hand should taxonomically be considered species or rather sub-species is a matter of interpretation and debate,[6] but this discussion lies outside the scope of the current paper.

In our study, most of the samples were identified as *L. panamensis*, which is consistent with previous reports concerning the distribution of this parasite in Colombia.[15,20] Nevertheless, a few were typed as *L. guyanensis* (3/27), usually a sylvatic species, that was recently identified as being responsible for a major epidemic of cutaneous leishmaniasis in that country.[21]

No intraspecific variations were found among the reference strains analyzed for *L. guyanensis*, despite the differences in their geographical origin (i.e., Brazil, Peru, French Guiana). However, it could be necessary to analyze additional *L. panamensis* strains to confirm our results, because the two strains studied come from Panama. In addition, typing in different samples (scraping, biopsy, isolate) point to the applicability of the method in various sampling situations.

Taking into account that it was already demonstrated that *hsp*70 PCR is successful on DNA obtained from vectors,[22] it is reasonable to expect that probable or proven reservoirs can also be investigated. Further studies addressing the applicability of this assay to a specific epidemiological or clinical situation should be done, but it is conclusive that *hsp*70 PCR-RFLP with *Bcc*I offers the possibility for specific typing of *L. panamensis* and *L. guyanensis*, which could be helpful in several Latin American areas. Our tool would indeed allow exploring on a more rational basis the epidemiological, clinical, and treatment features associated with these two taxa.

Authors' contributions: AMMA, JCD, IDVB conceived the study; AMMA, JFN, JCD, IDVB and CM designed the study; MM and CM selected the patients; AMMA, JFN, IMG, LMF, MM, GVdA and CM performed the laboratory work; AMMA, JFN, IMG, LMF, MM, JCD and CM analyzed and

interpreted the results; AMMA, IMG and LMF drafted the manuscript; JFN, MM, GVdA, JCD, IDVB and CM critically revised the manuscript. All authors read and approved the final manuscript. AMM and CM are guarantors of the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.trstmh.2009.12.002.

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Conflicts of interest: None declared.

Ethical approval: The use of human biopsies was approved by the Ethics Committee from Antioquia University, Medellín, Colombia. Informed consent was obtained from all patients. Samples were taken following the institutional indications for this purpose and in agreement with the Helsinki declaration.

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