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REFERÊNCIA

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Development and validation of PCR-based assays for diagnosis of American cutaneous leishmaniasis and identification of the parasite species

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In this study, PCR assays targeting different Leishmania *heat-shock protein 70 gene (*hsp70*) regions, producing fragments ranging in size from 230-390 bp were developed and evaluated to determine their potential as a tool for the specific molecular diagnosis of cutaneous leishmaniasis (CL). A total of 70* Leishmania *strains were analysed, including seven reference strains (RS) and 63 previously typed strains. Analysis of the RS indicated a specific region of 234 bp in the* hsp70 *gene as a valid target that was highly sensitive for detection of* Leishmania *species DNA with capacity of distinguishing all analyzed species, after polymerase chain reaction-restriction fragment length polymorfism (PCR-RFLP). This PCR assay was compared with other PCR targets used for the molecular diagnosis of leishmaniasis:* hsp70 *(1400-bp region), internal transcribed spacer (ITS)1 and glucose-6-phosphate dehydrogenase (*G6pd*). A good agreement among the methods was observed concerning the* Leishmania *species identification. Moreover, to evaluate the potential for molecular diagnosis, we compared the PCR targets* hsp70*-234 bp, ITS1,* G6pd *and mkDNA using a panel of 99 DNA samples from tissue fragments collected from patients with confirmed CL. Both PCR-*hsp70*-234 bp and PCR-ITS1 detected* Leishmania *DNA in more than 70% of the samples. However, using* hsp70*-234 bp PCR-RFLP, identification of all of the* Leishmania *species associated with CL in Brazil can be achieved employing a simpler and cheaper electrophoresis protocol.*

Key words: leishmaniasis - molecular diagnosis - species identification - polymerase chain reaction - RFLPs - *hsp70*

Leishmaniasis are parasitic diseases caused by a heteroxenous protozoan of the genus *Leishmania* (Ross 1903). Leishmaniasis are complex diseases showing significant clinical pleomorphism and epidemiological diversity. The genus *Leishmania* is comprised of approximately 30 species, approximately 20 of which are pathogenic for humans (Desjeux 2004).

Definitive diagnosis of cutaneous leishmaniasis (CL) can be challenging, especially in endemic areas where other diseases with similar clinical symptoms occur. The currently accepted gold standard for diagnosis requires isolation of the parasites involved and microscopic visualisation of amastigotes in tissue fragments from a lesion, though identification of the etiological agent at the species level is only possible if the parasite is successfully cultured (Herwaldt 1999, Desjeux et al. 2004, Reithinger et al. 2007).

The identification of *Leishmania* species in infected patients is crucial for achieving a correct prognosis and diagnosis (Arévalo et al. 2007). However, because of morphological similarities among the species of this genus, common parasitological assessments cannot discriminate between them (Weigle et al. 1986, Wilson et al. 1995, Aviles et al. 1999, Romero et al. 2001). Similarly, immunological tests, though useful for diagnosis, do not allow discrimination between *Leishmania* species.

Multilocus enzyme electrophoresis (MLEE) (Rioux et al. 1990), which is the gold standard for the identification of *Leishmania* species, requires prior isolation and mass culturing of the parasites. However, isolation can be complicated by the occurrence of secondary infections and the protocol is costly and time consuming. Therefore, a method that ensures the direct diagnosis and identification of pathogenic *Leishmania* species is still required for an appropriate therapy to be developed, as well as to contribute to epidemiological studies.

The diversity of the species associated with human CL in Brazil further necessitates the development of a method for the diagnosis of CL and determination of pathogenic *Leishmania* species. In Brazil, there are two attributes of *Leishmania braziliensis* that warrant particular attention: (i) the species is widely distributed and

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is often the sole or primary etiological agent of CL in several endemic areas and (ii) it is associated with the mucocutaneous form of the disease. However, this situation regarding *Leishmania* species is not static, as a few studies have revealed the presence of some species in areas where they had not previously been recorded (Azeredo-Coutinho et al. 2007, Azpurua et al. 2010, van Thiel et al. 2010), and other pathogenic species, such as *Leishmania guyanensis*, have been demonstrated to play an important role in the epidemiology of mucocutaneous leishmaniasis (Guerra et al. 2011).

Developing suitable detection methods for *Leishmania* infections that also distinguish between the pathogenic species involved has been a major challenge. Polymerase chain reaction (PCR)-based methods involving different targets have been proposed to enable characterisation at the species level; these protocols have been tested in *Leishmania* promastigotes and for direct detection of the parasites in infected human or other animal tissues, including phlebotomine sandflies [see Reithinger and Dujardin (2007), Goto and Lindoso (2010), Schönian et al. (2011) for more details].

Whereas PCR assays targeted at amplification of internal transcribed spacer (ITS)1-rDNA are among the most commonly used methods for the diagnosis and identification of *Leishmania* species in the Old World (Schönian et al. 2010a), PCR targeted at amplification of the conserved *locus* of kDNA minicircles (mkDNA) has been widely used in Brazil (Ampuero et al. 2009). Digestion of the ITS1 amplicon using the restriction enzyme *Hae*III has been demonstrated to be able to distinguish between nearly all *Leishmania* species, although distinction of some species, such as *L. braziliensis* and *L. guyanensis*, is only possible via sequence analysis of this region (Schönian et al. 2003). Few methods have been developed for the identification of specific species; one such protocol employs a PCR protocol that targets amplification of the glucose-6-phosphate dehydrogenase (*G6pd*) gene of *L. braziliensis*, which is the main etiological agent of mucocutaneous leishmaniasis in the Americas (Castilho et al. 2003).

A PCR-restriction fragment length polymorfism (RFLP) assay for the heat-shock protein 70 gene (*hsp70*) has recently been presented as a simple, universal tool for discrimination of *Leishmania* species in both the New and Old Worlds (Montalvo et al. 2010). This tool has been successfully used to distinguish human pathogenic *Leishmania* species circulating in Brazil (da Silva et al. 2010). Although this technique has already been used for direct diagnosis of CL (Garcia et al. 2004), the sensitivity of this assay is compromised by the large size (1400-bp) of the target region. Therefore, in this study, a region of the *hsp70* gene of *Leishmania* spp was analysed and primers flanking regions ranging from 234-384 bp in length were designed for amplification of smaller fragments of this gene. The ability of these regions to distinguish between *Leishmania* species was assessed by digesting each PCR product with restriction enzymes. A panel of 70 strains (including reference strains) of the *Leishmania* species circulating in Brazil was used for this purpose. In addition, the feasibility of this method was evaluated, including comparison with other PCR-based assays for the molecular diagnosis of CL using a panel of clinical samples from several endemic areas in Brazil.

SUBJECTS, MATERIALS AND METHODS

Leishmania strains - References strains of *L. braziliensis*, *L. guyanensis*, *Leishmania naiffi*, *Leishmania shawi*, *Leishmania lainsoni*, *Leishmania amazonensis* and *Leishmania infantum,* in addition to 63 other strains representative of these species, were used in this study (n = 70) (Table I). The stocks were frozen in liquid nitrogen and are part of the archive of the *Leishmania* Collection at the Oswaldo Cruz Institute (CLIOC); all of the strains were previously identified using MLEE. After thawing, the parasites were cultured in a two-phase culture medium (NNN-Schneider) (Cupolillo et al. 1994). DNA from these strains was extracted using the Wizard™ Genomic DNA Purification kit (Promega, WI, USA) according to the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop® spectrophotometer.

Clinical samples - A total of 99 DNA samples from tissue fragments collected from patients with a confirmed diagnosis of CL were used in this study. Biopsies were performed by physicians as part of the routine procedure for the diagnosis of leishmaniasis, according to ethical protocols, including obtaining patient consent and approval from the respective ethics committees. A total of 50, 29 and 20 samples were used from patients who resided in the states of Acre (AC), Rio de Janeiro (RJ) and Mato Grosso, respectively (Table II).

The biopsies were frozen and stored at -20ºC prior to DNA extraction using the Wizard™ Genomic DNA Purification kit according to the manufacturer's instructions, which included a prior phase of digestion with proteinase K.

PCR and RFLP assays - New primers were designed for amplification of smaller fragments corresponding to regions of the *hsp70* gene (Fig. 1, Table III). Primer design was accomplished via analysis of sequences from the *Leishmania hsp70* gene [which were deposited in GenBank (GU071172.1 - GU071188.1)] using the Primer-Blast tool available from the National Center for Biotechnology Information. Seven primers were designed (forward: *hsp70*F1, *hsp70*F2, *hsp70*F3 and *hsp70*F4; reverse: *hsp70*R1, *hsp70*R2 and *hsp70*R3) and combined to amplify four different fragments: *hsp70*A (230 bp), *hsp70*B (384 bp), *hsp70*C (234 bp) and *hsp70*D (379 bp) (Fig. 1, Table III). These regions were selected based on the inter-species polymorphisms. The PCR amplifications were performed in a final volume of 25 µL containing 1 µL of DNA, 20 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 1 U GoTaq® DNA polymerase (M3005, Promega). The PCR assays used the following amplification cycle: 94ºC for 5 min followed by 30 cycles of 94ºC for 30 sec, 61ºC for 1 min and 72ºC for 3 min and a final extension at 72ºC for 8 min.

PCR assays targeting different genomic DNA regions were performed using DNA extracted from promastigotes of the *Leishmania* strains (Table I) employing the following, previously described, methods: (i) PCR amplification of the *G6pd* gene *locus* (PCR-*G6pd*) us-

TABLE I

Leishmania strains (n = 70) previously characterized by multilocus enzyme electrophoresis (MLEE) and used to validate the ability of polymerase chain reaction (PCR) assays to differentiate *Leishmania* species

Species identification by MLEE	Reference strains	IOC/L	Country (state)	Zymodeme (IOC/Z)	*G6pd* PCR	ITS1 PCR-RFLP	1400 bp *hsp70* PCR-RFLP	234 bp *hsp70* PCR-RFLP
Leishmania amazonensis	**IFLA/BR/1967/PH8**	**575**	**BR (PA)**	**7**	**L(L)**	**La**	**La**	**La**
(n = 8)		114, 185, 569, 615, 930, 982, 2571	VE, BR (AM, BA, MS, PA)	7	L(L)	La	La	La
Leishmania infantum	**MHOM/BR/1974/PP75**	**579**	**BR (PA)**	**1**	**L(L)**	**Li**	**Li**	**Li**
(n = 6)		2565, 2566, 2568, 2569, 2570	(MS)	1	L(L)	Li	Li	Li
Leishmania braziliensis	**MHOM/BR/1975/M2903**	**566**	**BR (PA)**	**27**	**Lb**	**Lb**	**Lb**	**Lb**
(n = 32)		2152, 2419, 2420, 2427, 2492, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2202, 2203, 2237, 2251, 2254, 2501, 2538, 2823, 2824, 2825, 2918, 3072, 2502	BR (AC, PE, RJ)	27, 73, 74, 78, 79, 82	Lb	Lb	Lb	Lb
		1731[a]	BR (RO)	53	NLb	Lg	Lb	Lb
		2492, 2498[a]	BR (AC)	83, 84	NLb	Lb	Ln	Lb
		2495, 2499[a], 2501[a]	BR (AC)	78, 79, 81	Lb	Lb	Ln	Lb
Leishmania guyanensis	**MHOM/BR/1975/M4147**	**565**	**BR (PA)**	**23**	**NLb**	**Lg**	**Lg**	**Lg**
(n = 7)		2366, 2369, 2380, 2383, 2393	BR (AM)	23	NLb	Lg	Lg	Lg
		2493[a]	BR (AC)	110	[b]	Lg	Lg	Lg
Leishmania lainsoni	**MHOM/BR/1981/M6426**	**1023**	**BR (PA)**	**15**	**NLb**	**Ll**	**Ll**	**Ll**
(n = 6)		1058, 2490, 2496, 2500, 2503	BR (AC, RO)	15, 86, 87				
Leishmania naiffi	**MDAS/BR/1979/M5533**	**1365**	**BR (PA)**	**36**	**NLb**	**Ln**	**Ln**	**Ln**
(n = 8)		992, 993, 995, 1123, 1871, 1939, 1953	BR (AM, PA)	36, 42, 49	NLb	Ln	Ln	Ln
Leishmania shawi	**MCEB/BR/1984/M8408**	**1545**	**BR (PA)**	**26**	**NLb**	**Ls**	**Ls**	**Ls**
(n = 3)		1067, 1069	BR (PA)	26	NLb	Ls	Ls	Ls

a: strain (s) whose identification results were discordant between markers; *b*: sample positive for PCR-glucose-6-phosphate dehydrogenase *(G6pd)* with the specific primer combination for *L.* (*Viannia*), the specific primer combination for *L. braziliensis* and the specific primer combination to amplify non-*L. braziliensis* species; AC: Acre; AM: Amazonas; BA: Bahia; BR: Brazil; hsp: heat-shock protein; IOC: Oswaldo Cruz Institute; ITS: internal transcribed spacer; La: *L. amazonensis*; Lb: *L. braziliensis*; Lg: *L. guyanensis*; Li: *L. infantum*; Ll: *L. lainsoni*; L(L): samples positive for PCR-g6p with the specific primer combination for *L.* (*Leishmania*); Ln: *L. naiffi*; Ls: *L. shawi*; MS: Mato Grosso do Sul; NLb: samples positive for PCR-g6p with the specific primer combination for *L.* (*Viannia*) and the specific primer combination to amplify non-*L. braziliensis* species; PA: Pará; PE: Pernambuco; RFLP: restriction fragment length polymorphism; RJ: Rio de Janeiro; RO: Rondônia; VE: Venezuela. The strains were grouped accordingly to the profiles observed for all markers employed. Reference strains for each species are highlighted in bold.

ing a combination of primers (Castilho et al. 2003, Tojal da Silva et al. 2006); (ii) PCR targeting the ITS1 rDNA gene (PCR-ITS1) (Schönian et al. 2003, Tojal da Silva et al. 2006), (iii) PCR targeting a 1400-bp fragment of the *hsp70* gene from *Leishmania* (PCR-*hsp70*) (Garcia et al. 2004, da Silva et al. 2010) and (iv) PCR targeting a 234-bp region of the *hsp70* gene (see above for details).

Four PCR protocols were applied to DNA extracted from tissue fragments collected from lesions of patients who were diagnosed with CL. PCR targeting the conserved region of the mkDNA (120-bp PCR-mkDNA) (Volpini et al. 2004) was used only for molecular diagnostics and not for species identification. PCR-*G6pd*, PCR-ITS1 and 234-bp PCR-*hsp70* were used as described above.

The PCR products obtained from the different assays were visualised after electrophoresis on a 6% polyacrylamide gel, either by silver staining using a specific kit (DNA Silver Staining, GE Healthcare, 17-6000-30) or with ultraviolet light after being stained with ethidium bromide.

TABLE II

Detection and identification of *Leishmania* spp by different polymerase chain reaction (PCR)-based methods using DNA extracted from 99 tissue samples collected from lesions of patients with clinical diagnosis of leishmaniasis

Profile	Kdna	ITS1	ITS1 PCR-RFLP	234 bp *hsp70*	234 bp hsp70 PCR-RFLP	*G6pd*[a] [L(V)]	*G6pd*[a] (Lb)	*G6pd*[a] (non-Lb)	n[b]	Geographic origin
1	+	+	Lb	+	Lb	+	+	-	10	AC, RJ
2	+	+	Lb	+	Lb	-	+	-	2	AC
3	**+**	**+**	**Lb**	**+**	**Ln**	**+**	**+**	**-**	**2**	**AC**
4	**+**	**+**	**Lg**	**+**	**Lg**	**+**	**+**	**+**	**1**	**AC**
5	**+**	**+**	**Lg**	**+**	**Lg**	**+**	**+**	**-**	**1**	**AC**
6	+	+	Ls	+	Ls	NP	NP	NP	1	MT
7	+	+	Lb	-	_	NP	NP	NP	3	MT
8	+	+	Lb	+	Lb	-	-	-	15	AC, RJ
9	+	+	Lb	+	Lb	+	-	-	1	AC
10	+	+	Lb	+	Lb	NP	NP	NP	11	MT
11	+	+	La	+	La	NP	NP	NP	1	MT
12	**+**	**+**	**Ln/Ll**	**+**	**Ll**	**-**	**-**	**+**	**1**	**AC**
13	+	+	Ll	+	Ll	+	-	-	1	AC
14	+	+	Ll	+	Ll	-	-	-	5	AC
15	+	+	Ll	+	Ll	-	-	+	4	AC
16	+	+	Ll	+	Ll	NP	NP	NP	1	MT
17	+	+	Ll	-	-	-		-	2	AC
18	+	+	Ln	+	Ln	-	-	-	2	AC
19	+	+	Ln	+	Ln	+	-	+	1	RJ
20	**+**	**+**	**Ln**	**+**	**Lb**	**+**	**-**	**-**	**1**	**AC**
21	+	+	Lb	-	-	+	+	-	5	AC, RJ
22	+	+	Lb	-	-	-	-	-	5	AC, RJ
23	+	+	Lb	-	-	NP	NP	NP	3	MT
24	+	-	-	+	Ll	+	-	-	1	AC
25	+	-	-	-	-	-	-	-	1	AC
26	+	-	-	+	Lb	+	+	-	1	RJ
27	+	-	-	+	Lb	-	-	-	8	AC, RJ
28	+	-	-	-	-	+	+	-	1	RJ
29	+	-	-	-	-	-	-	-	6	AC, RJ
30	-	+	Lb	-	-	-	-	-	1	RJ
31	-	+	Lb	+	Lb	-	-	-	1	RJ
Total	97	81	-	72	-	26	24	7	99	-

a: see Subjects, Materials and Methods for details; *b*: total of samples presenting this result; AC: Acre; hsp: heat-shock protein; ITS: internal transcribed spacer; La: *Leishmania amazonensis*; Lb: *Leishmania braziliensis*; Lg: *Leishmania guyanensis*; Ll: *Leishmania lainsoni*; Ln: *Leishmania naiffi*; Ls: *Leishmania shawi*; L(V): *L.* (*Viannia*); MT: Mato Grosso; NP: not performed; RFLP: restriction fragment length polymorphism; RJ: Rio de Janeiro; +: positive PCR; -: negative PCR. Samples presenting discordant species identification results were highlighted in bold.

The PCR products from the amplification of ITS1 and *hsp70* (1400 bp or smaller) were subjected to digestion with restriction enzymes. For *G6pd*-PCR, a combination of subgenus or species-specific primers was used for identification of strains according to previously described protocols (Castilho et al. 2003, Tojal da Silva et al. 2006). The objective was to identify the subgenus and if the subgenus was *L.* (*Viannia*), to identify *L. braziliensis.*

The restriction enzyme *Sau*3AI was used for digestion of the ITS1 product based on previous results from our group (unpublished observations). *Hae*III, *Mbo*I *and Bst*UI were used to digest the products of amplifications that targeted *hsp70* (da Silva et al. 2010). The amplification products for *hsp70*A, *hsp70*B, *hsp70*C and *hsp70*D from the reference strains were subjected to digestion with *Hae*III, *Mbo*I, *Bcc*I, *Rsa*I, *Ava*I, *Bstu*I, *Kpn*I and *Sph*I. Based on the results obtained, only *Hae*III and *Bstu*I were used to digest the products amplified (with hsp70C as the target) from DNA extracted from other samples. The digestion reactions with different restriction enzymes were performed according to the manufacturer's instructions. All of the enzymes used in this study were from New England BioLabs.

The obtained restriction products were electrophoresed on a 6% polyacrylamide gel, followed by silver staining using a specific kit, with the exception of ITS1, which was electrophoresed in a specific machine (GenePhor electrophoresis unit, GE Healthcare) using a high-resolution gel (GeneGel Excel 12.5/24 kit, GE Healthcare) and the same silver-staining kit mentioned above.

Molecular identification of the *Leishmania* strains using DNA from promastigotes was performed as a blind assay, without prior knowledge of the identity determined via MLEE.

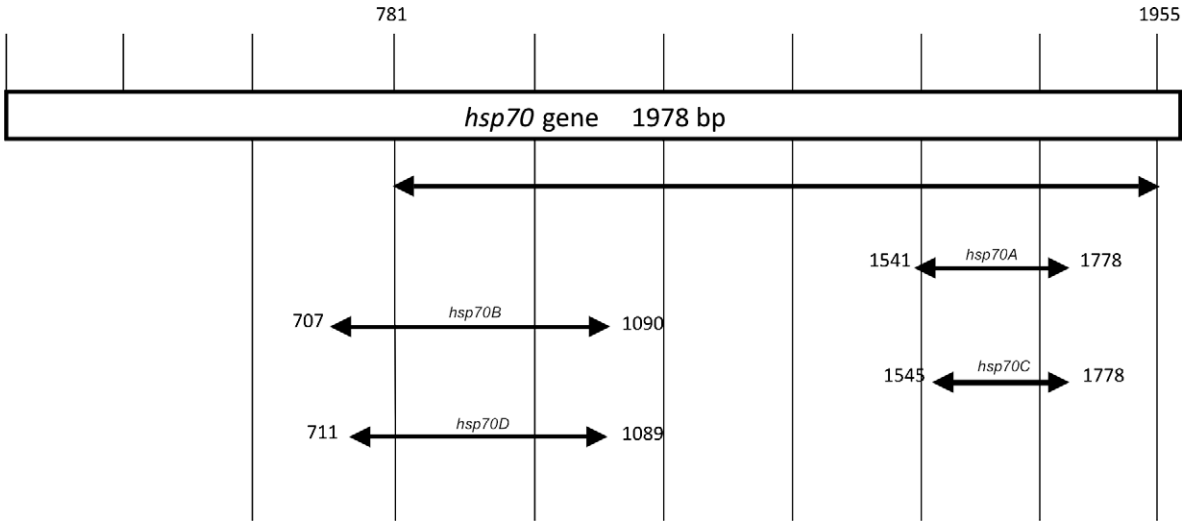

Fig. 1: position of polymerase chain reaction products from different primer combinations in relation to the heat-shock protein (*hsp*)*70* gene on chromosome 28 of *Leishmania major* [strain Friedlin (ncbi.nlm.nih.gov) accession FR796424 CT005266]. *hsp70*A: 230 bp; *hsp70*B: 384 bp; *hsp70*C: 234 bp; *hsp70*D: 379 bp.

TABLE III

Primers used for amplification of different regions of the *hsp70* gene

PCR assay	Amplicon size	Primer sequence (5'>3')	Tm	GC (%)
*hsp70*A (230 bp *hsp70*)	230 bp	GCAAGGACGAGATCGAGCGCA	59.6	61.9
		TCCTTCGACGCCTCCTGGTTG	58.2	61.9
*hsp70*B (384 bp *hsp70*)	384 bp	ACAACCGCCTCGTCACGTTCT	58.7	57.1
		TGTTCAGCTCCTTGCCGCCG	59.9	65
*hsp70*C (234 bp *hsp70*)	234 bp	GGA CGAGATCGAGCGCATGGT	59.7	61.9
		TCCTTCGACGCCTCCTGGTTG	58.5	61.9
*hsp70*D (379 bp *hsp70*)	379 bp	CCGCCTCGTCACGTTCTTCAGC	60.3	63.6
		GTTCAGCTCCTTGCCGCCGA	59.7	65

GC: guanine-cytosine; hsp: heat-shock protein; PCR: polymerase chain reaction; Tm: melting temperature.

A fragment of hamster tissue that was not infected with *Leishmania* was included as a negative control during the extraction of each clinical sample. A no DNA negative control was included in each PCR run.

As a positive control, a reaction containing DNA extracted from tissue fragments of a hamster that had been experimentally infected with *L. amazonensis* was included in each PCR run.

To avoid sample contamination, separate workstations were used to extract DNA from the promastigotes and clinical samples, to prepare the master mix solution for the addition of DNA to the master mix and to verify the amplicons via electrophoresis.

Sequencing - The products obtained for the *hsp70*C (234 bp) target from reference strains were visualised on a 2% agarose gel and purified using the Wizard SV Gel kit and PCR Clean-up System kit (Promega). The products were then sequenced with the same primers used for the PCR assay. Sequencing was performed on an automated sequencer (ABI PRISM® BigDye™ Terminator Cycle Sequencing) at the facilities of Oswaldo Cruz Foundation (IOC) (Genomic Platform - DNA sequencing, PDTIS-FIOCRUZ).

Statistical analysis - The number of positive clinical samples was calculated as a percentage using the results obtained for the different molecular markers evaluated in this study for detection of *Leishmania* DNA.

The Kappa index was used to determine the agreement between the results obtained for each molecular marker evaluated in the study (ITS1, *G6pd* and 234-bp *hsp70*). This calculation was performed using the software available at lee.dante.br/pesquisa/kappa/index.html.

The sensitivity of each method was determined based on comparison with the results of the PCR-mkDNA assay, which was considered as the gold standard because of its high sensitivity for CL diagnosis.

RESULTS

Identification of Leishmania spp reference strains - PCR-RFLP of the ITS-1 product using *Sau*3AI differentiated between the analysed *Leishmania* species, although it was possible to distinguish between *L. guyanensis* and *L. braziliensis* only when electrophoresis of the digested product was performed on a high-resolution gel (Fig. 2).

PCR-RFLP of the 1400-bp *hsp70* region differentiated between all of the species when *Bst*UI, *Mbo*I and *Hae*III were used, which supported the findings of previous studies (da Silva et al. 2010). The results could be visualised by electrophoresis using either a high-resolution gel or a 6% polyacrylamide gel (results not shown).

Neither PCR-*hsp70*B nor PCR-*hsp70*D were useful for identification of the *Leishmania* species. The same profiles were observed for all of the species analysed, whether PCR-RFLP of the *hsp70*B or the *hsp70*D targets was employed, with any of the restriction enzymes tested.

The assessment of the different *hsp70* fragments revealed that PCR-*hsp70*C and PCR-*hsp70*A yielded identical results (i.e., the same RFLP profile), reflecting the fact that the primers for *hsp70*A and *hsp70*C are targeted at flanking or neighbouring regions (Fig. 1). PCR-RFLP of either *hsp70*A or *hsp70*C discriminated between all of the analysed *Leishmania* species when a maximum of three restriction enzymes (*Hae*III, *Mbo*I and *Bst*UI) were used. *Hae*III generated identical profiles for *L. lainsoni* and *L. shawi* as well as for *L. braziliensis* and *L. naiffi*, whereas *L. guyanensis*, *L. amazonensis and L. infantum* exhibited unique profiles. *Bst*UI differentiated *L. naiffi* from *L. braziliensis* and *L. shawi* from *L. lainsoni;* these species could also be differentiated using *Mbo*I (Fig. 3).

Because the same result was achieved via PCR-RFLP of either *hsp70* A or C, the PCR *hsp70*C product (234 bp) for all of the reference strains was sequenced to determine whether it matched the expected region of the *hsp70* gene (Fig. 1). Sequence analysis revealed that this fragment corresponded to the region of the gene between base pairs 1545-1778, which is a portion of the *hsp70* gene on chromosome 28 of *Leishmania major* [strain Friedlin (ncbi.nlm.nih.gov) accession FR796424 CT005266]. The sequences obtained were identical to a portion of the *hsp70* gene (region between sites 1346-1580) that was already deposited in GenBank for the same strains sequenced in the present study (*L. naiffi*: GU071183.1; *L. shawi*: GU071177.1; *L. lainsoni*: GU071174.1; *L. braziliensis*: GU071173.1; *L. guyanensis*: GU071172.1; *L. amazonensis*: EU599090.1), with polymorphisms observed between species.

Validation of species identification assays - *A*ll of the strains (Table I) were included in a blind assay to assess the reliability of the various targets for discriminating between *Leishmania* species.

The results of ITS1 PCR-RFLP validated the MLEE identification, with one exception. The PCR-RFLP profiles for the 1400-bp *hsp70* and 234-bp *hsp70* fragments from the different *Leishmania* species were repeated for the panel assembled with 63 strains and the identification obtained at the species level supported the MLEE results, although six exceptions were observed for the 1400-bp *hsp70* PCR-RFLP (Table I).

Strain identification was also confirmed by the PCR-*G6pd* results, although this technique only discriminated between the subgenera *L.* (*Leishmania*) and *L.* (*Viannia*) and, within the latter, between *L. braziliensis* and non-*L. braziliensis*.

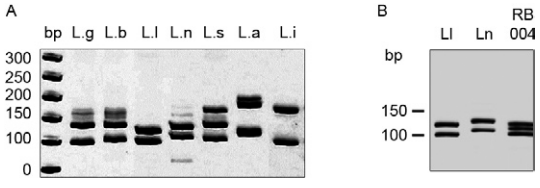

Fig. 2: internal transcribed spacer 1 polymerase chain reaction-restriction fragment length polymorphism profiles of different *Leishmania* species after digestion of products with *Sau*3AI. A: panel of profiles for reference strains; B: pattern for the sample RB004 from a patient diagnosed with cutaneous leishmaniasis, exhibiting a mixed profile between the species *Leishmania lainsoni* (Ll) and *Leismania naiffi* (Ln). Silver-stained 12.5% polyacrylamide gel (Genephor®). bp: 50 bp molecular weight marker; La: *Leishmania amazonensis*; Lb: *Leishmania braziliensis*; Lg: *Leishmania guyanensis*; Li: *Leishmania infantum*; Ls: *Leishmania shawi*.

Discrepancies were only observed for seven strains (Table I). Strain IOC/L1731, which was characterised by MLEE as *L. braziliensis*, exhibited an *L. guyanensis* profile when ITS1 PCR-RFLP was used and a non-*L. braziliensis* profile when PCR-*G6pd* was used, although the 1400-bp *hsp70* and 234-bp *hsp70*C PCR-RFLP results validated the MLEE identification. Strains L2492 and L2498, which were characterised as *L. braziliensis* by MLEE and ITS1 PCR-RFLP, showed a non-*L. braziliensis* profile when PCR-*G6pd* was used and a *L. naiffi* profile when 1400-bp *hsp70* PCR-RFLP was used. Strains L2495, L2499 and L2501, which were characterised as *L. braziliensis* by MLEE, ITS1 PCR-RFLP, PCR-*G6pd* and 234-bp *hsp70*C PCR-RFLP, each presented an *L. naiffi* profile when 1400-bp *hsp70* PCR-RFLP was used. Finally, strain L2493, which was characterised as *L. guyanensis* by MLEE and most of the molecular methods employed in this study, was positive in all of the *G6pd* PCR assays tested, meaning that it could not be identified as *L. braziliensis* or non-*L. braziliensis*.

Sensitivity of PCR methods for the diagnosis of leishmaniasis and the identification of Leishmania species in clinical samples - The DNA used in these assays was isolated from tissue fragments collected from cutaneous lesions of patients who had been diagnosed with CL based on clinical examination, parasitological analysis and/or their therapeutic response (Table II). High DNA concentrations can inhibit PCR and the DNA extracted from clinical samples can contain an amount of patient DNA that is much higher than the quantity of DNA from *Leishmania;* therefore, it is critical to determine the maximum DNA concentration to be used in each PCR assay. Although the effect of the concentration of human DNA on the PCR results was not evaluated in this study, quantities equal to or greater than 20 ng hindered the amplification of the target DNA (unpublished observations). Consequently, 200 pg of total DNA (from human tissue) per reaction was used.

Only two of the 99 samples analysed were negative for PCR-mkDNA (98% positivity), so this assay was considered to be the gold standard for determination of the sensitivity of the other PCR assays. The positivity was 81.8% (81/99) for PCR-ITS1, 41.8% (33/79) for PCR-*g6p* and 72.7% (72/99) for PCR-*hsp70*-234 bp. PCR-*G6pd* was not performed for samples from MT because sufficient DNA was not available. Both samples negative for kDNA were positive for ITS1; only one was positive for *hsp70*-234 bp and both were negative for g6p (Table II).

Of the 99 samples analysed, 62 were positive for ITS1 and *hsp70*-234 bp and only eight were negative for both targets. A total 19 of samples were positive for ITS1 alone and 10 were positive for *hsp70*-234 bp alone. The agreement between the PCR-ITS1 and PCR-*hsp70*-234 bp results was low according to the Kappa index (Kappa = 0.176, p = 0.071). Combining the two assays (PCR-ITS1 and PCR-*hsp70*-234 bp), 91.9% (91/99) positivity was achieved.

The sensitivities of PCR-ITS1, PCR-*hsp70*-234 bp and PCR-*G6pd* were 81.4% (79/97), 73.2% (71/97) and 41.6% (32/77), respectively, compared with the mkDNA PCR assay.

Whether clinical samples for which amplification products were obtained in every PCR assay could be differentiated at the species level depended on the target, as observed with the cultured strains (Table I). ITS1 PCR-RFLP allowed identification of the various *Leishmania* species in the clinical samples, as follows (number of samples in parentheses): *L. braziliensis* (58), *L. lainsoni* (13), *L. guyanensis* (2), *L. naiffi* (2), *L. shawi* (1), *L. amazonensis* (1) and one sample with a mixed profile between *L. lainsoni* and *L. naiffi* (Fig. 2). PCR-*G6pd* identified samples infected with *L. braziliensis* (22) vs. non-*L. braziliensis* (6). Finally, *hsp70*-234 bp PCR-RFLP identified the following parasite species in the samples: *L. braziliensis* (50), *L. lainsoni* (13), *L. naiffi* (5), *L. guyanensis* (2), *L. shawi* (1) and *L. amazonensis* (1).

Regarding species identification in the 62 samples that were positive for both PCR-ITS1 and PCR-*hsp70*-234 bp,

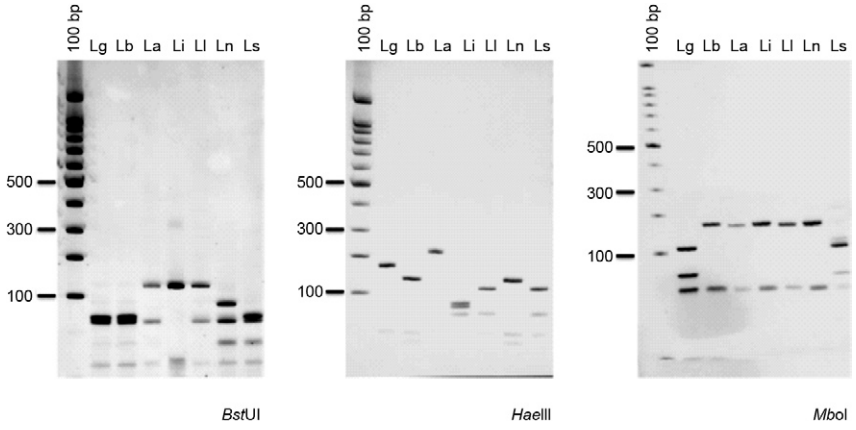

Fig. 3: 234 bp heat-shock protein *70* (*hsp70*C) polymerase chain reaction-restriction fragment length polymorphism profiles for reference strains of different *Leishmania* species after digestion of products with *Bst*UI, *Hae*III and *Mbo*I. Silver-stained 12.5% polyacrylamide gel (Genephor®). La: *Leishmania amazonensis*; Lb: *Leishmania braziliensis*; Lg: *Leishmania guyanensis*; Li: *Leishmania infantum*; *Ll*: *Leishmania lainsoni*; Ln: *Leishmania naiffi*; Ls: *Leishmania shawi*; 100 bp: molecular weight marker.

only four samples yielded conflicting results, which are presented below. Twenty-three samples were identified by ITS1 PCR-RFLP, PCR-*hsp70*-234 bp and PCR-*G6pd* (Table II). Eighteen samples yielded consistent results. Two samples (RB005 and RB011) yielded conflicting results and were characterised as *L. braziliensis* by ITS1 PCR-RFLP and PCR-*G6pd*, but as *L. naiffi* by *hsp70*-234 bp PCR-RFLP. One sample (RB053) was characterised as *L. naiffi* by ITS1 PCR-RFLP, but as *L. braziliensis* by *hsp70*-234 bp PCR-RFLP; this sample was positive for PCR-*G6pd*, which distinguished the subgenus *L. (Viannia)*. Sample RB004 was characterised as *L. lainsoni* by *hsp70*-234 bp PCR-RFLP, but as non-*L. braziliensis* by PCR-*G6pd*, whereas the ITS1 PCR-RFLP profile suggested a combination between *L. lainsoni* and *L. naiffi* (Fig. 2). These results could indicate either combined infection by *L. naiffi* and *L. lainsoni* or a hybrid between these two species, as a combined infection would not explain the hybrid profile observed with MLEE.

Sample RB013 demonstrated a *L. guyanensis* profile based on ITS1 PCR-RFLP and *hsp70*C PCR-RFLP, but was positive in all PCR-*G6pd* assays, thereby precluding identification of this sample as *L. braziliensis* or non-*L. braziliensis* using this methodology. Strain IOC/L2493 was isolated from sample RB013 and was characterised as *L. guyanensis* based on all of the markers (Table I). However, after analysis of the *L.* (*Viannia*) strains with a battery of microsatellites, strain IOC/L2493 was included in the same cluster as *Leishmania panamensis* and *L. shawi* (Oddone et al. 2009).

DISCUSSION

The main objective of this study was to develop a PCR-based method that can be used for diagnosis of CL, to both the infection and allowing identification of the species involved. Previous research indicates that PCR-RFLP of a 1400-bp fragment of the *Leishmania hsp70* gene can differentiate all of the species circulating in Brazil. Through analysis of 70 strains, the present study validated the previously demonstrated ability of this methodology to discriminate between the various *Leishmania* species associated with human leishmaniasis in Brazil (da Silva et al. 2010). This portion of the *hsp70* gene has also been used in other studies and its potential to distinguish *Leishmania* species has been discussed. Furthermore, the effectiveness of this target for molecular diagnosis of leishmaniasis has previously been suggested (Garcia et al. 2004, 2007, Fraga et al. 2010).

However, given the large size of the 1400-bp *hsp70* fragment, a lower level of success is expected for this target when amplifying small amounts of DNA, such as those obtained from clinical samples. Therefore, new primers were designed to amplify smaller fragments of the *Leishmania hsp70* gene. A 234-bp region of the *Leishmania hsp70* gene (designated 234-bp PCR-*hsp70* in this study) was deemed promising, given its utility for detecting and identifying *Leishmania* species in clinical samples. The ability of several PCR assays, either following an RFLP approach or not, to discriminate between species was evaluated using a panel of reference strains representing the *Leishmania* species circulating in Brazil. Although 120-bp mkDNA PCR-RFLP has been demonstrated to be appropriate for identifying some *Leishmania* species (Volpini et al. 2004), this approach was not used in the present study because most of the species analysed here belong to the *L. (Viannia)* subgenus and a conserved region would be not appropriate for distinguishing between closely related species. Furthermore, profile reproducibility and identification of *Leishmania* species using mkDNA PCR-RFLP could be hindered by variability in the minicircle classes (Brewster & Barker 2002). ITS1 PCR-RFLP using *Sau*3AI generated the same profiles as are obtained with *Hae*III (E Cupolillo, unpublished observations), which has been used in several studies employing ITS1 PCR-RFLP to diagnose leishmaniasis (Schönian et al. 2003, Nasereddin et al. 2008, Gelanew et al. 2010, Hajjaran et al. 2011). As reported elsewhere, the differences between *L. braziliensis* and *L. guyanensis* are not easily detected, although there are polymorphisms in the DNA sequences of both species. Therefore, high-resolution gel electrophoresis, which can resolve fragment size differences as small as 4 bp, was successfully employed to distinguish between these two species.

A total of 63 strains were used to validate the identification of *Leishmania* species from different PCR-RFLP and PCR-*G6pd* assays. Notably, the panel of isolates, which represented the diversity of the genus *Leishmania*, demonstrated the risk of using only reference strains for evaluating the ability of a method to discriminate between species, considering the observed disagreement in the identification of some strains based on the different methods.

Profile reproducibility was satisfactory for ITS1 PCR-RFLP, 234-bp *hsp70* PCR-RFLP and PCR-*G6pd* among the assayed strains, though there were a few exceptions. The target associated with the most conflicting results compared with MLEE was 1400-bp *hsp70* PCR-RFLP, while 234-bp *hsp70* PCR-RFLP corroborated the MLEE identification for all of the evaluated strains. Some strains characterised as *L. braziliensis* by MLEE presented a non-*L. braziliensis* profile for PCR-*g6p*. These strains exhibited an atypical electromorphic profile for the g6PDH enzyme, which was different from the profile observed for the reference strains and the vast majority of strains that were characterised as *L. braziliensis* (Cupolillo et al. 1994, 1995). This result corroborates recent data obtained by our group, which indicate the presence of different alleles for the *G6pd* gene among *L. braziliensis* strains (unpublished observations). These findings should be considered prior to employing this type of PCR analysis in samples collected from regions where *L. braziliensis* variants have already been described.

Discrepancies among the results related to the identification of *Leishmania* species were also observed when using clinical samples and the possibility that there were mixed infections among the specimens cannot be ruled out (Camara-Coelho et al. 2011). One explanation for this finding is the differential amplification of each marker, whereby different primers may preferentially amplify DNA from different species.

It has previously been reported that *Leishmania* strains from various species might exhibit similar pro-

files, depending on the marker used (Almeida et al. 2011). For the subgenus *L. (Viannia)*, this is expected because the species from this group show high genetic similarity (Cupolillo et al. 1995, 1997, Cupolillo & Momen 2000), share epitopes (Grimaldi & MacMahon-Pratt 1996) and exhibit high similarity among partial sequences of certain genes (Croan et al. 1997, Asato et al. 2009, Fraga et al. 2010). Moreover, the mechanisms of genetic recombination have been described for natural populations of *Leishmania* (Rougeron et al. 2010).

To assess the potential of different targets for molecular diagnosis of leishmaniasis, including species discrimination, the ITS1, *G6pd* and 234-bp *hsp70* regions were employed. The capacity to detect *Leishmania* spp was compared with the PCR-mkDNA results. Although employing PCR-RFLP mkDNA for species identification appears to be inappropriate (see above), the high sensitivity of this method justifies its use for the molecular diagnosis of leishmaniasis (Pereira et al. 2008, Ampuero et al. 2009, Romero et al. 2009, Kobets et al. 2010, Azmi et al. 2011).

The targets with the highest positive rates were 120-bp mkDNA-PCR, followed by ITS1-PCR, 234-bp *hsp70*-PCR and *G6pd*-PCR. A better performance for mkDNA-PCR compared with ITS1-PCR has also been observed in previous studies (Bensoussan et al. 2006, Azmi et al. 2011). This result was expected because primer sets that target sequences with higher copy numbers in the genome will be more sensitive. mkDNA PCR has been considered to represent an excellent alternative for the molecular diagnosis of leishmaniasis because of its high sensitivity, given the approximately 10,000-20,000 minicircles per *Leishmania* cell (Simpson 1973). Many copies of the ribosomal gene have been observed per *Leishmania* diploid genome. For example, the presence of 20-40 copies per cell has been reported for *L. (Viannia)* species (Inga et al. 1998). In at least some *Leishmania* species, the *hsp7*0 genes are arranged in a single genomic cluster that contains five or six HSP70-I copies followed by one HSP70-II copy (Ramirez et al. 2011), whereas *g6p* is a single-copy gene.

Amplification of the ITS1-PCR and 234-bp *hsp70*-PCR DNA fragments yielded approximately 17% agreement between the genes. Several studies comparing the use of PCR-based assays for molecular diagnosis of leishmaniasis have demonstrated poor agreement between different methods applied to the same samples (Marfurt et al. 2003, Azmi et al. 2011), but the cause of this disparity has not been established. Discrepant results obtained from different PCR assays can be explained by various factors, such as an unequal distribution of DNA in solution (Chandler 1998). Furthermore, although the primers used in this study were designed to amplify conserved regions, there could be failures in the annealing of the primer sequence with the target DNA, thereby hampering amplification by PCR. Moreover, the presence of a secondary infection with other microorganisms (such as fungi and bacteria) in leishmaniasis lesions is common (Formiga et al. 1986, Edrissian et al. 1990, Vera et al. 2001, Markle & Makhoul 2004, Bhutto et al. 2009). Some primers may share similarities with DNA sequences of these microorganisms, thus affecting PCR performance.

ITS1 PCR-RFLP and 234-bp *hsp70* PCR-RFLP yielded the best results because these methods differentiate between etiological agents at the species level. These findings confirmed the circulation of *L. braziliensis*, *L. lainsoni and L. guyanensis* in AC (Tojal da Silva et al. 2006) and suggested the presence of *L. naiffi* in this region. Although a strain showing a hybrid profile between *L. lainsoni* and *L. naiffi* had previously been isolated, the latter species had not yet been identified in this region (Tojal da Silva et al. 2006). As expected, only *L. braziliensis* was observed in patients from RJ (de Oliveira-Neto et al. 2000) and the single sample characterised as *L. naiffi* was isolated from a patient who had been infected in the Amazon. *L. braziliensis*, *L. amazonensis*, *L. shawi and L. lainsoni* were found in samples from MT and the first two species have previously been reported in this region (Carvalho et al. 2006). To our knowledge, there are no existing reports of *L. shawi* and *L. lainsoni* in MT. Analysing the records of the patient infected with *L. lainsoni*, it was noted that he was most likely infected in an area where this *Leishmania* species is circulating (AC) (Tojal da Silva et al. 2006). The patient infected with *L. shawi* acquired the infection in MT, in a region that is north of the state, an area of occurrence of the Amazon Rain Forest. Although the aim of this analysis was not to determine which species are circulating the studied regions (AC, RJ and MT), the results indicate the importance of combining diagnosis with the identification of species for the surveillance of leishmaniasis.

The discrimination of *Leishmania* species should be addressed to understand the discrepancies among the various markers. The biological species concept cannot be applied to *Leishmania* and there is currently no agreement regarding the definition of the species boundaries within this genus (Banuls et al. 1999, Schönian et al. 2010b). Therefore, none of the available genotyping systems can currently serve as the gold standard for the identification of a particular species and a combination of different methods is required. However, the use of different protocols is impractical and may be unnecessary when the objective is to combine molecular diagnosis with the identification of an etiological agent or to assist in epidemiological surveillance, prognosis and definition of therapeutic regimens. ITS1 PCR-RFLP delivered promising results and other studies have demonstrated that this assay performs well for the identification of *Leishmania* species circulating in the Old World (Schönian et al. 2003, Bensoussan et al. 2006, Shahbazi et al. 2008). However, the finding that it was only possible to distinguish between *L. braziliensis* and *L. guyanensis* via sequencing or high-resolution gel electrophoresis limits the utility of ITS1 PCR-RFLP in the areas where these species occur. The 234-bp *hsp70* PCR-RFLP assay, which targeted a region of the *Leishmania hsp70* gene, combined the ability to detect *Leishmania* in clinical samples with the ability to discriminate between all of the species circulating in Brazil, which could make this technique particularly useful in areas of sympatry.

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