1. Introduction

Leishmaniasis is a complex of vector borne diseases caused by protozoan parasites belonging to subfamily Leishmaniinae (Espinosa et al., 2016). The disease is endemic in 98 countries located in tropical and subtropical areas and recent data indicate that 1.2 million cases of visceral and cutaneous leishmaniasis occur worldwide every year (Alvar et al., 2012). Some 24-different named species of the subfamily Leishmaniinae occur worldwide every year (Espinosa et al., 2016), this includes one that does not belong to the subgenus L. (Viannia) such as L. (V.) guyanensis, L. (V.) naiffi, L. (V.) lainsoni, L. (V.) shawi and L. (V) lindenberghi also cause the disease. Additionally, L. (V) utingensis is also found in Brazil, but so far it has not been recorded in humans (Braga et al., 2003). The identification and classification of these species was originally based on multilocus enzyme electrophoresis (MLEE) (Cupolillo et al., 1994).

Several molecular assays for identifying Leishmania species have been developed based on either kinetoplast or genomic DNA. Various targets have been used such as the ribosomal DNA internal transcribed spacer (Cupolillo et al., 1995; Schonian et al., 2003), the minixxon (Marfurt et al., 2003), cytochrome B (Asato et al., 2009), and heat-shock protein 70 (hsp70) (Fraga et al., 2012) encoding genes.

What drew attention to hsp70 was that its identifications matched almost perfectly those of MLEE, which is still considered as the gold standard for Leishmania species identification (Cupolillo et al., 1994; Rioux et al., 1990). This cytoplasmic heat-shock protein, is encoded by a multicopy tandemly repeated gene in Leishmania spp., present in 5 to 10 copies in the parasite genome (Foguereira et al., 2007; Ramirez et al., 2011). Garcia et al. (2004) developed a PCR assay employing RFLP with primers designed from the conserved common hsp70 sequences of L. (V) braziliensis and L. (L.) mexicana. In subsequent studies this method differentiated 15 Leishmania species belonging to the two subgenera including the principal species found in Brazil’s
endemic regions (da Silva et al., 2010; Montalvo et al., 2012; Van der Auwera and Dujardin, 2015). However, in one analysis (da Silva et al., 2010), using the sequences of the PCR products, a strain of L. (V.) braziliensis from the Mato Grosso State group with a low bootstrap value with isolates of L. (V.) shawi.

In our laboratory we routinely use the hsp70 PCR-F-RFLP described by Montalvo et al. (2012) to identify isolates from patients. In this paper, we report polymorphisms of the hsp70 genes in isolates of L. (L.) amazonensis and in strains of the subgenus L. (V.) that should be taken into consideration when identifying or typing these parasites.

2. Material and methods

2.1. Reference strains and clinical isolates

Leishmania promastigotes were grown in medium 199 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum, 0.25% hemin, 12 mM NaHCO3, 50 U/ml penicillin and 50 μg/ml streptomycin at 25 °C. For the cultivation of Leishmania (Viannia) spp., 2% sterile human urine was added to the medium. The reference strains and isolates used are described in Table 1. Apart from isolate MHOM/BR/2002/ACVJ.

Table 1
Leishmania strains and isolates used in this study.

<table>
<thead>
<tr>
<th>International code</th>
<th>Species</th>
<th>Clinical form and origin (Brazilian State)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHOM/BR/73/M2269</td>
<td>L. (L.) amazonensis</td>
<td>CL (Pará)</td>
<td>(Miles et al., 1980)</td>
</tr>
<tr>
<td>MPRO/BR/27/M1841-LV79</td>
<td>L. (L.) amazonensis</td>
<td>LCL</td>
<td>(Chance et al., 1974)</td>
</tr>
<tr>
<td>IFLA/BR/67/PH8</td>
<td>L. (L.) amazonensis</td>
<td>- (Pará)</td>
<td>(Miles et al., 1980)</td>
</tr>
<tr>
<td>MHOM/BR/2008/2506</td>
<td>L. (L.) amazonensis</td>
<td>DCL (Paul)</td>
<td>(Coelho et al., 2014)</td>
</tr>
<tr>
<td>MHOM/BR/87/BA19</td>
<td>L. (L.) amazonensis</td>
<td>VL (Bahi)</td>
<td>(de Oliveira et al., 2007)</td>
</tr>
<tr>
<td>MHOM/BR/89/BA19</td>
<td>L. (L.) amazonensis</td>
<td>DCL (Bahi)</td>
<td>(de Oliveira et al., 2007)</td>
</tr>
<tr>
<td>MHOM/BR/75/M2903</td>
<td>L. (V.) braziliensis</td>
<td>CL (Pará)</td>
<td>(Laison et al., 1973)</td>
</tr>
<tr>
<td>MHOM/BR/75/M1417</td>
<td>L. (V.) guyanensis</td>
<td>CL (Pará)</td>
<td>(Laison et al., 1979)</td>
</tr>
<tr>
<td>MHOM/BR/2002/ACVJ</td>
<td>L. (V.) guyanensis</td>
<td>CL (Amazonas)</td>
<td>(Silveira et al., 1987)</td>
</tr>
<tr>
<td>MHOM/BR/1981/M4426</td>
<td>L. (V.) lainsoni</td>
<td>CL (Pará)</td>
<td>(Silveira et al., 2002)</td>
</tr>
<tr>
<td>MHOM/BR/96/M15732</td>
<td>L. (V.) Lindenbergeri</td>
<td>CL (Pará)</td>
<td>(Silveira et al., 2002)</td>
</tr>
<tr>
<td>IAYR/BR/6/E0337</td>
<td>L. (V.) naffi</td>
<td>(Bahi)</td>
<td>(This study)</td>
</tr>
<tr>
<td>MDAS/BR/79/M533</td>
<td>L. (V.) naffi</td>
<td>(Laison and Shaw, 1989)</td>
<td></td>
</tr>
<tr>
<td>MCEB/BR/84/M8408</td>
<td>L. (V.) shawi</td>
<td>(Pará)</td>
<td>(da Silva et al., 2010)</td>
</tr>
<tr>
<td>ITUB/BR/77/M4964</td>
<td>L. (V.) utingensis</td>
<td>*</td>
<td>(Braga et al., 2003)</td>
</tr>
</tbody>
</table>

CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; LCL, localized cutaneous leishmaniasis.

* Isolated from Lutzomyia tuberculata and have not been found in humans (Braga et al., 2003).

Table 2
In silico analysis of the 1,286 bp hsp70 amplified product in Leishmania spp. Size of products generated by digestion with selected restriction enzymes.

<table>
<thead>
<tr>
<th>Leishmania sp.</th>
<th>Restriction enzyme</th>
<th>Eco RII</th>
<th>Hae III</th>
<th>Mbo I</th>
<th>Sdu I</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. (L.) amazonensis M2269 allele 1*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (L.) amazonensis M2269 allele 2*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (V.) lainsoni C1M71*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (V.) braziliensis M2905*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (V.) peruviana LH2684*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (V.) naffi M533*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (V.) shawi M2408*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (V.) Lindenbergeri M15732*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. (V.) utingensis M4964*</td>
<td>338, 307, 246, 233, 62, 47, 40, 13</td>
<td>390, 258, 177, 141, 114, 81, 42, 38, 21, 18, 6</td>
<td>525, 321, 273, 111, 56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Partial hsp70 sequences (1,286 bp) of strains and isolates of the Leishmania spp. were determined in this study or retrieved from GenBank. The table indicates the size of fragments (in bp) upon digestion. Polymorphic fragments amongst Leishmania (Viannia) species are indicated in bold. Polymorphic fragments in L. (L.) amazonensis alleles 1 and 2 are indicated in bold and underlined.

* GenBank accession number: MG029123
* GenBank accession number: FN95047
* GenBank accession number: XM_001566275
* GenBank accession number: FN95044
* GenBank accession number: FN95056
* GenBank accession number: FR872767
* GenBank accession number: MG029126
* GenBank accession number: FN95055
* GenBank accession number: EU599093
* GenBank accession number: MG029128
* GenBank accession number: MG029127
* GenBank accession number: MG029124
all parasites used here were also typed by isoenzymes or molecular methods (references in Table 1).

2.2. PCR amplification of hsp70

Genomic DNAs of Leishmania parasites were purified using DNAzol according to the manufacturer's instructions and quantified by spectrophotometry in a microplate reader (POLARstar Omega, BMG Labtech, Ortenberg, Germany). For each PCR reaction, 100 ng of DNA was used and PCRs were performed according to Montalvo et al. (2012). The primers used were F25 (5′-GGACGCCGCGACCTTCT-3′) and R1310 (5′-CTGTTGTTTGTGACCCACTC-3′), which amplify the hsp70 PCR-F DNA fragment of 1,286 bp. The PCR amplifications were performed in a final volume of 50 μL containing 0.2 μM of each primer, 200 μM dNTPs, 1.6 mM MgCl2 and 2.5 U Taq DNA polymerase (Sinapse Biotechnologia, São Paulo, Brazil) using the following amplification cycle: 94°C for 5 min followed by 30 cycles of 94°C for 40 sec, 60°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min (Montalvo et al., 2012). The amplified products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide.

2.3. PCR-RFLPs analysis

PCR products were digested with Hae III (New England Biolabs) after DNA quantification in agarose gel electrophoresis. Digestion products were separated in 2% or 3% agarose gels stained with ethidium bromide. The GeneRuler 100 bp DNA ladder (Life Technologies) was used as molecular weight marker.

2.4. DNA sequencing

The amplified products were purified from agarose gels using the GenElute Gel Extraction kit (Sigma-Aldrich, St. Louis, USA) and cloned in the pGEM-T easy vector (Promega Corporation, Madison, USA). The nucleotide sequence of several independent positive clones containing the PCR-F fragment was determined in an automated DNA sequencer (ABI PRISM Big Dye Terminators Cycle Sequencing) using the Big Dye Terminator v3.1 Cycle Sequencing kit (Life Technologies). Primers used for sequencing were M13 primers, F25 and R1310. Consensus sequences were generated from at least three forward and three reverse sequences. Nucleotide sequence analyses were performed using Lasergene Software (DNASTAR) and Clone Manager 9.0 Software. Sequences determined in this study are listed in Table 2 and are available in the GenBank.

2.5. Data analysis and phylogenetic inferences using hsp70 sequences

The partial nucleotide sequences of 1,286 bp of the hsp70 gene were aligned using ClustalW and then edited using the program GeneDoc 2.6 (Nicholas et al., 1997). Phylogenies were inferred using the maximum likelihood (ML) and maximum parsimony (MP) analyses. The parsimony and their respective bootstrap analyses were carried out using PAUP version 4.0b10 (Swofford, 2002) with 100 replicates of random addition sequences followed by branch swapping (RAS-TBR). The ML analyses were performed using RAxML version 2.2.3 (Stamatakis, 2006), with tree searches performed with GTR model with gamma-distributed rate variation across sites and proportion of invariable sites (GTR+G model) and nodal supports were estimated with 100 bootstrap replicates in RAxML using GTR+Gamma and maximum parsimony starting trees.

3. Results

3.1. The L. (L.) amazonensis hsp70 gene is polymorphic

The PCR-F-RFLP protocol described by (Montalvo et al., 2012) has been recently adopted in our laboratory as a tool for species identification of Leishmania clinical isolates. Amongst isolates and reference strains of L. (L.) amazonensis, specific amplification of the expected hsp70 fragment of approximately 1.3 kb was observed (Fig. 1A). However, the digestion of the fragment obtained from the L. (L.) amazonensis M2269 reference strain with the restriction enzyme Hae III generated an unexpected 580 bp band (Fig. 1B) that had not been reported previously in isolates of L. (L.) amazonensis and L. (L.) mexicana (Fraga et al., 2012; Montalvo et al., 2012). Interestingly, this approximately 580 bp fragment was also observed in Hae III digested products obtained from clinical isolates 2506 and BA-199, but was absent in other clinical isolates of the same species (BA-109 and BA-276) and was not observed in the reference strains LV79 and PH8 (Fig. 1B).

To characterize this unexpected digestion pattern, the 1.3 kb M2269 amplified fragment was cloned in the pGEM-T easy vector and 8 independent positive clones were screened by digestion with Hae III. Two different patterns of amplified fragments were found: containing or not the fragment of 580 bp (3 and 5 clones respectively) (Table 2). Representative clones of the two distinct patterns had their nucleotide sequence determined revealing the absence of a synonymous polymorphism at position 894 (GCC/GCT → A/A) of the hsp70 open reading frame in one of the alleles. In addition, another SNP (a non-synonymous SNP) at position 578 (AAG/ATG → K/M) was also found in the second allele of this L. (L.) amazonensis strain. The

Fig. 1. Identification of two L. (L.) amazonensis hsp70 alleles (1 and 2) by PCR-RFLP. (A) hsp70 gene amplification (1,286 bp fragment) using the PCR-F protocol described by (Fraga et al., 2012). (B) Digestion of the amplified products shown in (A) with Hae III and size-separation in ethidium bromide stained 2% agarose gel. (−) Negative control; 1 - L. (L.) amazonensis M2269; 2 - L. (L.) amazonensis 2506 isolate; 3 - L. (L.) amazonensis LV79; 4 - L. (L.) amazonensis PH8; 5 - L. (L.) amazonensis BA109 isolate; 6 - L. (L.) amazonensis BA159 isolate; 7 - L. (L.) amazonensis BA276 isolate; 8 - L. (V.) braziliensis M2903. (C) Restriction map analysis of the two L. (L.) amazonensis (strain M2269) hsp70 alleles. The Hae III restriction sites and the 584 bp fragment from allele 2 are indicated.
Hae III restriction map for the two alleles is shown in Fig. 1C. In silico analysis of restriction patterns upon digestion with Eco RI, Mbo I and Sdu I, enzymes previously described as useful for hsp70 PCR-RFLP typing (da Silva et al., 2010; Fraga et al., 2013; Montalvo et al., 2012), confirmed that both alleles have a conserved restriction profile for these three restriction enzymes (Table 2).

3.2. Polymorphisms on L. (V.) naiffi and L. (V.) shawi hsp70 genes

A variety of species of the L. (Viannia) subgenus has been implicated in tegumentary leishmaniasis in Brazil. Aiming to implement the hsp70 PCR-F-RFLP followed by Hae III digestion as a routine to identify Leishmania species in our laboratory, we employed a panel of reference strains from the Viannia subgenus (Table 1). Apart from the most widespread species, the panel also included L. (V.) naiffi, L. (V.) shawi, L. (V.) lainsoni, L. (V.) lindenbergi, for which the hsp70 gene had not been previously studied, and L. (V.) utingensis, which has not yet been found in humans. The RFLP profile obtained after Hae III digestion for L. (V.) braziliensis M2903 was, as expected, indistinguishable from L. (V.) naiffi M5533 (Fig. 2A). As some degree of heterogeneity amongst L. (V.) naiffi isolates was detected by Montalvo et al. (2012), we added to the analysis, together with the reference strain L. (V.) naiffi M5533, the isolate L. (V.) naiffi EO337 for a more accurate comparison. A different pattern of Hae III digestion was identified in the EO337 strain, previously typed as L. (V.) naiffi. This new restriction pattern was due to the presence of an additional Hae III restriction site located at the extremity of the amplified fragment (Fig. 2B) and confirmed a degree of intra-species heterogeneity in L. (V.) naiffi. Nevertheless, in silico analyses indicated that these two strains of L. (V.) naiffi (M5533 and EO337) and also M5210 of this same species displayed the same restriction profile when digested with Eco RI, Mbo I or Sdu I (Table 2). L. (V.) lainsoni M6426 profile was unique amongst the species tested (Fig. 2A).

RFLP profiles for L. (V.) shawi, L. (V.) braziliensis M2903, L. (V.) naiffi M4964 and L. (V.) utingensis M4964 contain fragments in the region of 120-140 bp, of similar sizes, which can be distinguished only in highly stringent electrophoresis conditions (Fig. 2 and Table 2). The nucleotide sequence of L. (V.) shawi M8408 hsp70 PCR-F 1,286 bp fragment was determined. The sequence (Genbank no. MG029127) showed 99.8% identity with the L. (V.) guyanensis M4147 strain homologous sequence, while 99.4% identity was found with L. (V.) braziliensis M2903 (data not shown). Therefore and as expected, sequence data can be used to rule out typing L. (V.) shawi as L. (V.) braziliensis, in cases where the electrophoresis patterns cannot be relied upon.

In order to increase the panel of restriction enzymes that could be useful to discriminate these L. (Viannia) species and strains, we performed in silico analyses of the PCR-F 1,286 bp fragment using the following restriction enzymes: Eco RI, Mbo I, and Sdu I. Unlike Hae III, the restriction enzymes Eco RI and Mbo I were useful to discriminate L. (V.) shawi M8408 from L. (V.) braziliensis/L. (V.) peruviana, L. (V.) naiffi and L. (V.) utingensis (Table 2). On the other hand, Eco RI, Mbo I and Sdu I restriction patterns of L. (V.) guyanensis/L. (V.) panamensis and L. (V.) shawi M8408 are identical (Table 2). Besides that, the PCR-F RFLP
using Sdu I was shown to be useful to discriminate $L. (V.)$ naiffi from $L. (V.)$ guyanensis/$L. (V.)$ panamensis, $L. (V.)$ braziliensis/$L. (V.)$ peruviana or $L. (V.)$ shawi M8408 (Table 2).

3.3. Analysis of $L. (V.)$ lindenbergi and $L. (V.)$ utingensis hsp70 gene

We also characterized for the first time the partial hsp70 sequences of $L. (V.)$ lindenbergi and added a new partial sequence for $L. (V.)$ utingensis. At the time of writing, only a fragmentary annotation of this species hsp70 gene was available. $L. (V.)$ lindenbergi Hae III PCR-F-RFLP was indistinguishable from $L. (V.)$ guyanensis/$L. (V.)$ panamensis strains and different from $L. (V.)$ shawi (Table 2). The sequence analyses indicated that PCR-F RFLPs of $L. (V.)$ lindenbergi can only be discriminated from other $L. (Viannia)$ species using Eco RII, since Mbo I and Sdu I produced the same restriction profile of $L. (V.)$ naiffi (Table 2).

![Phylogenetic tree inferred by Maximum Likelihood (ML) and Maximum Parsimony (MP) of sequences of the 1,286 bp hsp70 amplified product of 13 species of Leishmania representative of the subgenera $L. (Viannia)$ and $L. (Leishmania). The numbers at the nodes correspond respectively to ML and MP bootstrap values (100 replicates). Asterisks (***) at the nodes denotes bootstrap values below 50%. Sequences obtained in this work are represented by stars (✦/✦), while triangles at the end of branches represent sequences obtained from several clones for the same isolate. The other sequences were obtained from Genbank (Fraga et al., 2010). The sequences are: $L. (L.)$ mexicana (MNYC/BZ/62/M379) GenBank number: EU599091; $L. (L.)$ mexicana (MHOM/BR/73/M269) GenBank number: EU599090; $L. (L.)$ garnhami (MHOM/VE/76/AP7) GenBank number: EU599092; $L. (L.)$ infantum (MHOM/BR/79/DD7) GenBank number: FN395026; $L. (L.)$ panamensis (MHOM/BR/07/029-ZAV) GenBank number: FN395055; $L. (L.)$ donovani (MHOM/IN/00/DEVI) GenBank number: FN395024; $L. (L.)$ tropica (MHOM/BR/75/M2903) GenBank number: FN395043; $L. (L.)$ aethiopica (MHOM/ET/89/GERE) GenBank number: FN395018.1; $L. (L.)$ major (MHOM/IL/81/Friedlin) GenBank number: XM_001684511.1; $L. (V.)$ lainsoni (MHOM/BO/95/CUM71) GenBank number: FN395047; $L. (V.)$ naiffi (MHOM/BR/06/LH2372) GenBank number: FN395048; $L. (V.)$ peruviana (MHOM/PE/02/LH2864) GenBank number: FN395049; $L. (V.)$ braziliensis (MHOM/BR/75/M2904) GenBank number: FN395044; $L. (V.)$ shawi (MHOM/BR/75/M2905) GenBank number: XM_001566275; $L. (V.)$ braziliensis (MHOM/BO/75/M2906) GenBank number: EU599088; $L. (V.)$ braziliensis (MHOM/BO/91/LH2864) GenBank number: FN395042; $L. (V.)$ braziliensis (MHOM/PE/02/LH2372) GenBank number: FN395051; $L. (V.)$ braziliensis (MHOM/BR/75/M2903) GenBank number: M87878; $L. (V.)$ braziliensis (MHOM/BR/75/M2905) GenBank number: FN395040.**
Finally for \( L. (V.) utingensis \), the PCR-F-RFLP of this species has the same restriction profile of \( L. (V.) braziliensis/L. (V.) peruviana \) for the 4 enzymes analyzed and so none of the enzymes are useful to discriminate it from other \( L. (Viannia) \) species (Fig. 2 and Table 2).

3.4. Phylogenetic analyses

The phylogenetic tree using the hsp70 gene sequences of several \( Leishmania \) species indicated the presence of the main groups of the \( Leishmania \) genus (Fig. 3). As reported previously, we observed that \( L. (V. ) guyanensis \) is placed in the same cluster of \( L. (V. ) panamensis \) and \( L. (V. ) shawi \) (Boite et al., 2012). Similarly, \( L. (V. ) braziliensis/L. (V.) peruviana \) are also located in the same cluster (Fig. 3), while \( L. (V. ) lainsoni/L. (V. ) lindenbergi \) are the most divergent species. Finally, \( L. (V. ) naffi \) strains MS210, M5533 and E0337 are in the same cluster, despite the distinct \( Hae \) III PCR-F RFLPs of the E0337 strain (Fig. 2B). Our study also indicated that \( L. (V. ) utingensis \) is closely related to the \( L. (V. ) naffi \) cluster as described (Fig. 3).

4. Discussion

The use of PCR-RFLPs for molecular diagnosis has been described in recent years as a valuable tool for typing \( Leishmania \) species. By employing the hsp70 gene PCR-F-RFLP method described by Montalvo et al. (2012), we made three observations of new restriction pattern profiles that should be considered, especially when typing Brazilian strains. First, using PCR-F followed by \( Hae \) III digestion, we found two alleles of the hsp70 gene in the \( L. (L. ) amazonensis \) M2269 reference strain, which has been widely used in several studies.

The hsp70 genes are highly conserved multicycloc sequences located in tandem in \( Leishmania \) chromosome 28. Most of the polymorphisms found in hsp70 genes are located in the intergenic regions (Ramirez et al., 2011). Interestingly, the allelic variation described here in \( L. (L. ) amazonensis \) M2269 reference strain, present inside the coding region, was also found in some other isolates from Brazilian patients. These clinical isolates originated from different States in Brazil and were obtained from patients with different clinical manifestations (Coelho et al., 2014; de Oliveira et al., 2007).

A possible explanation for the presence of two sequences would be the presence of hybrids in natural populations of \( L. (L. ) amazonensis \), as already described in parasites of the \( L. (V. ) shawi \) subgenus (Dujardin et al., 1995; Nolder et al., 2007). The possibility of a mixed population or a hybrid cannot be discarded since parasites were not cloned before DNA isolation. The presence of these alleles could also be a result of hybridization between isolates, which could also not be discarded. However, sequencing and PCR-F-RFLP results do not indicate inter-species hybridization, since 99.9% sequence identity was found between both hsp70 alleles of the M2269 reference strain (Fig. 3 and data not shown).

This is, to the best of our knowledge, the first report of the presence of two alleles detected by the PCR-F-RFLP approach for the hsp70 gene in \( L. (L. ) amazonensis \), a phenomenon that could be explained by the genetic diversity of Brazilian clinical isolates of this species (de Oliveira et al., 2007; Valdivia et al., 2017). Considering that parasites in the \( L. (V. ) \) subgenus present higher genetic diversity than \( L. (L. ) \) amazonensis (Cupolillo et al., 2003; da Silva et al., 2010; Montalvo et al., 2012), it is quite possible that different allelic patterns will be found when a greater number of isolates of each \( L. (V. ) \) species are examined.

Testing this PCR-RFLP strategy as a routine approach for \( Leishmania \) species identification in our lab, we employed a panel of \( L. (V. ) \) species. This led to the observation of a second instance, in \( L. (V. ) naffi \) strains, where PCR-F-RFLP with \( Hae \) III also revealed intra-species polymorphisms. The strain E0337 presented a different profile when compared to the two other strains of \( L. (V. ) naffi \) or the other \( L. (V. ) \) species analyzed in this study. Three other enzymes used in silico as well as the phylogenetic analysis grouped these strains of \( L. (V. ) naffi \) in the same profile, ruling out the possibility of species misidentification. The different geographic origin of these strains might explain these observations.

Despite the fact that the PCR-F-RFLP with \( Hae \) III is not straightforward for the discrimination of \( L. (V. ) braziliensis/L. (V. ) peruviana \) and \( L. (V. ) naffi \) (Montalvo et al., 2012) (Fig. 2B) and also \( L. (V. ) utingensis \) (Table 2), this restriction enzyme is a useful alternative to discriminate \( L. (V. ) guyanensis \) and \( L. (V. ) braziliensis \), which together with \( L. (L. ) amazonensis \) are the main causative species of leishmaniasis in Brazil. As an alternative, in silico analysis revealed that Sdu I and Mbo I could be used to discriminate \( L. (V. ) braziliensis/L. (V. ) peruviana \) and \( L. (V. ) naffi \) but not \( L. (V. ) utingensis \) (Table 2).

\( Leishmania \) \( L. (V. ) lindenbergi \) must also be considered when Brazilian clinical isolates are typed, especially in Amazon region, where it was already reported in patients with cutaneous leishmaniasis (Silveira et al., 2002). Similarly, \( Hae \) III was not useful to discriminate \( L. (V. ) lindenbergi \) and \( L. (V. ) guayanesis/L. (V. ) panamensis \). An alternative to this approach could be the use of \( EcoR \) RII to discriminate \( L. (V. ) lindenbergi \) from other \( L. (V. ) \) species.

The third point raised during our analyses was that it was sometimes difficult to distinguish \( L. (V. ) shawi \)’s digestion profile with \( Hae \) III (Fig. 2 and Table 2) from those generated by \( L. (V. ) braziliensis \) and \( L. (V. ) naffi \). The 120 pb fragment present in \( L. (V. ) shawi \) could be difficult to separate from the 134 pb bands observed in \( L. (V. ) braziliensis \) and \( L. (V. ) naffi \), in the absence of a good electrophoresis analysis. In this case, polyacrylamide or high-resolution agarose gels must be used to discriminate these small fragments.

In conclusion, using the PCR-F-RFLP protocol with \( Hae \) III for species identification, we detected intra-species variation in some Brazilian clinical isolates of \( L. (L. ) amazonensis \) and \( L. (V. ) \) species. These polymorphisms must be taken in consideration when identifying strains of \( L. (V. ) braziliensis \) and \( L. (V. ) naffi \) as \( L. (V. ) shawi \). Our findings indicate that restriction polymorphic sites may result in parasite mistyping and that as more strains of a species are examined more polymorphic sites will be detected. On the other hand, nucleotide sequence of the 1,286 bp hsp70 amplified product completely ruled out the possibility of misidentification of all species and strains of \( Leishmania \) endemic in Brazil.

Acknowledgments

We are grateful to Carmen S. A. Takata for performing the sequencing. The authors also thank Jenicer K. U. Yoyokama-Yasunaka for technical assistance.

Funding

This work was supported by research grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, 2011/20484-7 and 2015/09080-2) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 473343/2012-6), Brazil. SRBU is the recipient of a senior researcher scholarship from CNPq. ACC and CRE were fellows supported by FAPESP (2012/14629-5 and 2016/23405-4).

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