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Standardization of a molecular method for epidemiologic identification of *Leishmania* strains

R.F. Rocha[1]*, E.V. Menezes[1]*, A.R.E.O. Xavier[1]*, V.A. Royo[1], D.A. Oliveira[1], A.F.M. Júnior[1], E.S. Dias[2], A.C.V.M.R. Lima[2] and E.M. Michalsky[2]

[1]Laboratório de Bioprospecção e Recursos Genéticos,
Departamento de Biologia, Centro de Ciências Biológicas e da Saúde,
Universidade Estadual de Montes Claros,
Campus Universitário Professor Darcy Ribeiro, Montes Claros, MG, Brasil
[2]Centro de Pesquisas René Rachou, Unidade da FIOCRUZ, Belo Horizonte, MG, Brasil

*These authors contributed equally to this study.
Corresponding author: E.V. Menezes
E-mail: menezes.elytania@gmail.com

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ABSTRACT. Molecular studies of the evolutionary relationships among *Leishmania* species suggest the presence of high genetic variation within this genus, which has a direct effect on public health in many countries. The coexistence of species in a particular region can result in different leishmaniasis clinical forms and treatment responses. We aimed to standardize the kinetoplast DNA (kDNA) enterobacterial repetitive intergenic consensus (ERIC) sequence polymerase chain reaction (PCR) method for molecular epidemiological identification

of *Leishmania* strains, and estimate existing inter-strain genomic differences and kDNA signatures using this technique. ERIC-PCR of genomic DNA revealed genetic polymorphisms between species, although some strains shared many DNA fragments. *Leishmania guyanensis*, *L. amazonensis*, and *L. braziliensis* clustered together in a dendrogram with similarities ranging from 42.0 to 61.0%, whereas *L. chagasi* grouped with these three species with a similarity of 28.0%. After amplification of kDNA, 780-bp bands were extracted from an agarose gel and purified for analysis of its genetic signature. kDNA ERIC-PCR electrophoretic patterns consisted of 100- to 600-bp fragments. Using these profiles, *L. braziliensis* and *L. guyanensis* grouped with a similarity of 26.0%, and *L. amazonensis* and *L. chagasi* clustered based on a similarity of 100%. The electrophoretic profiles and dendrograms showed that, for epidemiological identification by ERIC-PCR, genomic DNA had greater discriminatory power than kDNA did. More strains need to be analyzed to validate the kDNA ERIC-PCR method. The genomes of these strains should be sequenced for better epidemiological identification of *Leishmania* species.

Key words: Genetic variability; Kinetoplast; Polymorphism; Genetic similarity

INTRODUCTION

Leishmaniasis is a group of infectious and parasitic diseases caused by various species of *Leishmania* protozoa, with a wide spectrum of clinical manifestations involving the skin, mucosa, and internal organs (Alvar et al., 2012). *Leishmania* is one of several genera within the Trypanosomatidae family, and is characterized in part by the kinetoplast, an organelle composed of DNA (kDNA). This structure accounts for 15-35% of the total cellular DNA and multiplies independently of the genomic DNA (Simpson et al., 1987). Leishmaniasis is transmitted by the bite of female insects belonging to the Psychodidae family, Phlebotominae subfamily, known as sand flies (Desjeux and Alvar, 2003). *Lutzomyia longipalpis* is considered the main vector of this disease (Lainson and Rangel, 2005).

Leishmania species are divided into two subgroups, *Viannia* and *Leishmania*, and the disease into two types, cutaneous and visceral. Cutaneous leishmaniasis is principally caused by *Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*Viannia*) *guyanensis*, and *Leishmania* (*Leishmania*) *amazonensis*, and more rarely by *Leishmania* (*Viannia*) *lainsoni*, *Leishmania* (*Viannia*) *naiffi*, *Leishmania* (*Viannia*) *shawi*, and *Leishmania* (*Viannia*) *lindenbergi*, whereas *Leishmania* (*Leishmania*) *chagasi* is the causative agent of visceral leishmaniasis (Lessa et al., 2007; Ministério da Saúde, 2007; Lainson, 2010).

L. guyanensis, *L. amazonensis*, and *L. braziliensis* have been found simultaneously in the Americas, Mediterranean Basin, Middle East, and Central Asia, mainly in Afghanistan, Algeria, Colombia, the Islamic Republic of Iran, and the Syrian Arab Republic. *L. chagasi* is found in the Indian subcontinent and East Africa, particularly Bangladesh, Brazil, Ethiopia, India, and Sudan (WHO, 2015).

Leishmaniasis is endemic in 85 countries on five continents, with a total of 310 million

people at risk. An estimated 1.3 million new cases of this disease occur annually worldwide (WHO, 2015). The *Leishmania* genus includes a large number of described species. Studies having used molecular methods to analyze the evolutionary relationships between these organisms suggest that the genus is highly variable (Boité et al., 2012; Kuhls et al., 2013). This variation directly affects public health in numerous countries, particularly Brazil, where many species coexist in particular regions and cause different clinical forms of leishmaniasis with distinct treatment responses, making diagnosis based on geographical location highly challenging (Grimaldi and Tesh, 1993; Cupolillo et al., 2003).

Molecular methods have been used previously for the identification and characterization of the causative agents of leishmaniasis, mainly in studies of genetic variability. Due to the specificity of these techniques, detection and genetic characterization of the disease can be performed simultaneously (Hulton et al., 1991; Harris et al., 1996).

The methods used include enterobacterial repetitive intergenic consensus (ERIC) sequence-based polymerase chain reaction (PCR). ERIC-PCR enables the characterization, comparison, and identification of variations in the genomes of homologous bacterial strains, and facilitates epidemiological research (Versalovic et al., 1991; Dorneles et al., 2014; Carvalho et al., 2016). Furthermore, it has high discriminatory power for differentiating closely related species and shows good reproducibility compared to other methods (Fendri et al., 2013; Carvalho et al., 2016).

Thus, the goal of this study was to standardize the ERIC-PCR approach for the epidemiological identification of *Leishmania* strains using genomic DNA and kDNA. We estimated the genomic differences between four *Leishmania* species and established their kDNA signatures. This is the first study to employ nested PCR with ERIC-PCR of the kinetoplast region.

MATERIAL AND METHODS

Leishmania DNA

We used genomic DNA isolated from pure cultures of four *Leishmania* species (*L. braziliensis*, *L. chagasi*, *L. guyanensis*, and *L. amazonensis*) for characterization. DNA samples (100 ng/μL) were provided by the René Rachou Research Center (Centro de Pesquisas René Rachou), a FIOCRUZ branch in Belo Horizonte, State of Minas Gerais, Brazil. Each DNA sample used was quantified by 1.0-1.5% agarose gel electrophoresis and employed in PCR and ERIC-PCR.

ERIC-PCR

Characterization of the genetic profiles of and clonal relationships between isolates of *L. braziliensis*, *L. chagasi*, *L. guyanensis*, and *L. amazonensis* was performed by genomic DNA polymorphism analysis. We used previously described PCR primers targeting conserved regions of the ERIC sequence, as follows: ERIC 1, 5'-TGT AAG CTC CTG GGG ATT AAC-3'; and ERIC 2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (Duan et al., 2009). Reactions were performed in a 50-μL mix containing 1X *Taq* buffer from a KAPA PCR kit (KAPA Biosystems, Wilmington, MA, USA), 2.5 mM $MgCl_2$, 1 μM deoxynucleotides, 0.5 U Kappa *Taq* polymerase (KAPA Biosystems, Wilmington, MA, USA), 1 μM each primer, and 10 μL *Leishmania* DNA. Amplification conditions were as follows: one initial cycle of denaturation at

95°C for 1 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min, before a final extension at 72°C for 7 min. Amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide and photodocumented using the Gel Logic 212 Pro system (CARESTREAM, New Haven, CT, USA).

Amplification profiles were visually analyzed by four observers and converted into a binary data matrix according to the presence or absence of DNA fragments. To assess the genetic relationships between isolates, the matrix was subjected to multivariate cluster analysis by the complete linkage method to calculate Euclidean distances and generate a dendrogram using the statistical program Minitab version 16 (Minitab Inc., State College, PA, USA). The *Escherichia coli* BL21(DE3+) strain was used as an outgroup.

kDNA PCR

We used the kDNA F (5'-CCA GTT TCC CGC CCC G-3') and kDNA R (5'-GGG GTT GGT GGT GTA AAA TAG-3') primers to amplify a 780-bp sequence in a conserved region of *Leishmania* minicircle kDNA (kinetoplast mitochondrial DNA) by PCR. The reactions were performed in a 25-µL mix containing 10X buffer, 2 mM $MgCl_2$, 1 µM deoxynucleotides, 1 U *Taq* polymerase, 1.0 µM each primer, and 5 µL *Leishmania* DNA. Cycling conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, prior to a final extension at 72°C for 30 min. PCR products were visualized on a 1% agarose gel stained with ethidium bromide and photodocumented as above.

kDNA purification (kDNA PCR)

Following kDNA PCR and visualization of the products by 1% agarose gel electrophoresis, the 780-bp amplified fragment for each *Leishmania* strain was extracted from the gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

Nested PCR (kDNA ERIC-PCR)

To determine kDNA signatures, the purified 780-bp fragments amplified by kDNA PCR as described above were used in ERIC-PCR. The ERIC 1 and ERIC 2 primers mentioned above, complementary to conserved ERIC sequences, were employed. The 50-µL reactions comprised 1X KAPA PCR kit *Taq* buffer (KAPA Biosystems, Wilmington, MA, USA), 2.5 mM $MgCl_2$, 1 µM deoxynucleotides, 0.5 U KAPA *Taq* polymerase (KAPA Biosystems), 1 µM each primer, and 5 µL purified *Leishmania* DNA. The following amplification conditions were used: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 92°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 1 min, before a final extension for 16 min. The amplified products were visualized on a 1.5% agarose gel stained with ethidium bromide and photodocumented as outlined above.

Amplification profiles were visually analyzed by four observers and converted into binary data according to the presence or absence of DNA fragments. Genetic relationships between isolates were estimated by subjecting the resulting matrix to multivariate complete-linkage cluster analysis to calculate Euclidean distances and generate a dendrogram in Minitab version 16, as for the genomic DNA ERIC-PCR.

RESULTS

In the present study, molecular characterization of four *Leishmania* strains isolated from pure cultures was performed to determine genomic differences and similarities and kDNA signatures.

ERIC-PCR electrophoretic profiles were generated by amplifying genomic DNA from the four *Leishmania* species using the ERIC 1 and ERIC 2 primers. Polymorphic DNA banding patterns of good intensity were observed, revealing a degree of genetic variation. DNA fragments ranged in size from 100 to 2000 bp, and although high genetic variance was observed, some profiles obtained from different strains shared certain bands in common (Figure 1).

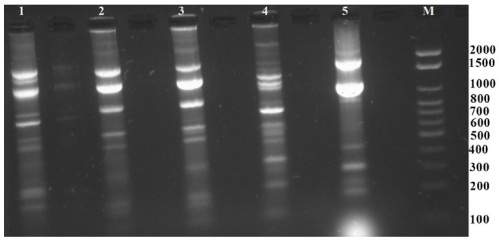

Figure 1. Enterobacterial repetitive intergenic consensus sequence-based polymerase chain reaction to compare the genetic profiles of different *Leishmania* species standard strains. *Lane 1 = Leishmania braziliensis*; *lane 2 = Leishmania guyanensis*; *lane 3 = Leishmania amazonenses*; *lane 4 = Leishmania chagasi*; *lane 5 = Escherichia coli* BL21(DE3+) (outgroup). *Lane M* = 100-bp DNA molecular weight marker (Norgen Biotek, Thorold, Canada); lengths (bp) are indicated to the right of the 1.5% agarose gel.

By examining the distinct fragments generated, we constructed a binary matrix from the electrophoretic profiles based on the presence or absence of DNA bands, which were analyzed in sets and used to construct a dendrogram. Similarity analysis allowed classification of the *Leishmania* strains into a single cluster (I). Within this, *L. guyanensis* and *L. amazonensis* (IA 2.1 and IA 2.2) formed a group with a similarity of 61.0%, with which *L. braziliensis* clustered with 42.0% similarity (IA1 and IA2). *L. chagasi* was associated with the group comprising these three species based on a similarity of 28.0% (IA and IB). *E. coli* BL21(DE3+) (group II) was used as an outgroup in the analysis; as expected, no genetic similarity with the *Leishmania* strains in group I was observed (Figure 2).

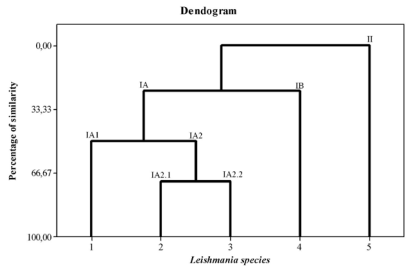

Figure 2. Dendrogram of the genetic relationships between *Leishmania* species standard strains obtained by enterobacterial repetitive intergenic consensus sequence-based polymerase chain reaction. *Leishmania* strains were clustered together into group I, with *Escherichia coli* BL21(DE3+) forming group II, according to percentage similarity. 1 = *Leishmania braziliensis*. 2 = *Leishmania guyanensis.* 3 = *Leishmania amazonensis*. 4 = *Leishmania chagasi*. 5 = *Escherichia coli* BL21(DE3+) (outgroup).

To characterize kDNA profiles, PCR was carried out using kDNA F and kDNA R primers. Satisfactory amplification was observed, almost all the products of which corresponded to kDNA, and the bands were suitable for extraction and purification (Figure 3).

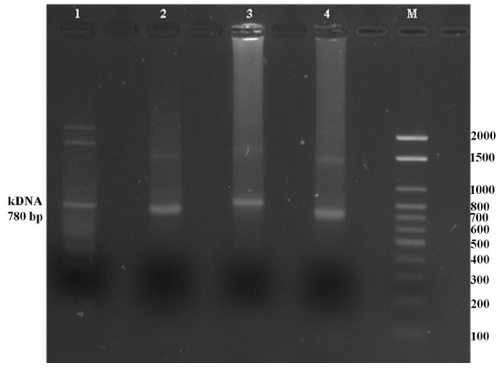

Figure 3. Kinetoplast DNA (kDNA) polymerase chain reaction to analyze kDNA profiles. *Lane 1 = Leishmania braziliensis*; *lane 2 = Leishmania guyanensis*; *lane 3 = Leishmania amazonenses*; *lane 4 = Leishmania chagasi*. *Lane M* = 100-bp DNA molecular weight marker (Norgen Biotek); lengths (bp) are indicated to the right of the 1% agarose gel.

The kDNA signatures consisted of a pattern of fragments ranging from 100 to 600 bp. A comparison of the size (in bp) and number of the major bands in each kDNA profile revealed similarities and differences between the *Leishmania* strains (Figure 4).

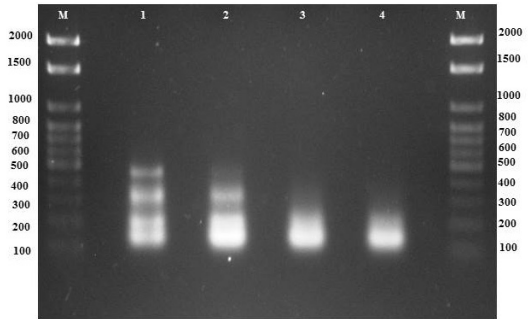

Figure 4. Kinetoplast DNA enterobacterial repetitive intergenic consensus sequence-based polymerase chain reaction to compare the genetic profiles of different *Leishmania* species standard strains. *Lane M* = 100-bp DNA molecular weight marker (Norgen Biotek); lengths (bp) are indicated to the left and right of the 1.5% agarose gel. *Lane 1 = Leishmania braziliensis*; *lane 2 = Leishmania guyanensis*; *lane 3 = Leishmania chagasi*; *lane 4 = Leishmania amazonensis*.

The main bands in the kDNA signatures of the different *Leishmania* species were analyzed and converted into a binary data matrix according to their presence or absence (Figure 4), to construct a dendrogram. The *Leishmania* strains were categorized into two groups (I and II). Group I consisted of *L. braziliensis* and *L. guyanensis*, with 26.0% similarity (IA and IB), and group II of *L. amazonensis* and *L. chagasi*, which exhibited 100% similarity (IIA and IIB; Figure 5).

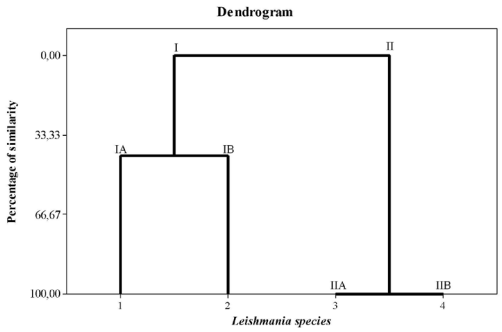

Figure 5. Dendrogram of the genetic relationships between *Leishmania* species standard strains obtained by kinetoplast DNA enterobacterial repetitive intergenic consensus sequence-based polymerase chain reaction. *Leishmania* strains were distributed into two groups (I and II) according to percentage similarity. 1 = *Leishmania braziliensis*. 2 = *Leishmania guyanensis*. 3 = *Leishmania amazonensis*. 4 = *Leishmania chagasi*.

DISCUSSION

Molecular methods have been used previously to identify and characterize the causative agents of leishmaniasis, mainly in studies of genetic variability. Given the specificity of such techniques, the detection and genetic characterization of this disease can be achieved simultaneously (Hulton et al., 1991; Harris et al., 1996; da Silva et al., 2004).

Madruga et al. (2002) demonstrated the use of ERIC-PCR in the characterization of a protozoan parasite. The authors conducted a molecular epidemiological study to identify the genetic diversity present among *Babesia bigemina* protozoa isolated from five states in Brazil. Genetic analysis was performed by random amplification of polymorphic DNA (RAPD), repetitive extragenic palindromic elements (REP)-PCR, and ERIC-PCR, which revealed polymorphisms among the isolates. The RAPD results were reproducible, in that the amplified polymorphic fragments were consistently detected, with the exception of certain weaker bands that were not always generated in reactions using different DNA preparations. The study also demonstrated that both REP- and ERIC-PCR can be used for genetic analysis of protozoan parasites, although the latter has greater discriminatory power than the former.

The goal of the present study was to standardize the ERIC-PCR method for epidemiological identification of *Leishmania* strains using genomic DNA and kDNA. We estimated the genomic differences between various *Leishmania* species and determined their kDNA signatures. This is the first study to use nested PCR with ERIC-PCR of the kinetoplast region.

The pattern of polymorphic DNA fragments resulting from ERIC-PCR revealed some degree of genetic variation between the *Leishmania* strains (Figure 1). The profiles observed were similar to those recorded in the literature for different *Leishmania* species, demonstrating the genetic variability of these organisms. The ERIC-PCR products described by Leal (2010) ranged in size from 100 to 1700 bp for *Leishmania* reference samples (FIOCRUZ) and isolates from 11 patients with visceral leishmaniasis. Yazidi et al. (2015) used RAPD-PCR to determine the genetic variability of 39 *Leishmania major* isolates from five endemic foci. Their analysis revealed a high degree of polymorphism (43%) among isolates, and showed that 99% of the observed variation was within, rather than between, each region studied.

Although in the present study, fragment sizes ranging from 100 to 2000 bp were generated, some strains shared certain DNA bands (Figure 1), as reflected in the dendrogram,

in which species were grouped by similarity (Figure 2).

Species in the *Leishmania* genus are morphologically similar, with slight differences. Current molecular techniques enable the evaluation of the intrinsic characteristics of these parasites. The development of these methods has resulted in important advances in our knowledge of *Leishmania* genotypes and the possibility of characterizing them on an individual level (Cupolillo et al., 1998; Schriefer et al., 2004).

L. guyanensis, *L. amazonensis*, and *L. braziliensis* were grouped in our dendrogram with high similarity values, ranging from 61.0 to 42.0% (Figure 2). This can be explained by the results of previous studies, in which these species have all been identified as belonging to the *Viannia* subgroup and causing cutaneous leishmaniasis (Cupolillo et al., 1998; Schriefer et al., 2004). The differences and similarities found also reveal a possible association between *Leishmania* genetic profiles and the reservoir type and geographical origin of the corresponding species. According to Gomes et al. (1995), the diversity of *Leishmania* species in the *Viannia* subgroup is less pronounced among populations found in nearby regions. Genotypic polymorphisms correlate with the geographic origin of isolates for Old World (Schönian et al., 2000; Toledo et al., 2002) and New World *Leishmania* species (Gomes et al., 1995; Cupolillo et al., 2003).

L. guyanensis is predominantly present in the states of Amazonas and Acre. (Gontijo and de Carvalho, 2003; Silveira et al., 2004). *L. amazonensis* can be found in various regions of Brazil (Barral et al., 1993), as can *L. braziliensis*, although at a low frequency in Amazonas (Bittencourt and Barral, 1991; Gontijo and de Carvalho, 2003; Carvalho et al., 2015).

L. chagasi exhibited the lowest similarity to the other three species (28.0%) in the genomic DNA dendrogram, perhaps as it belongs to the *Leishmania* subgroup and causes visceral leishmaniasis. Despite these differences in subgenus and the type of leishmaniasis caused, estimation of genetic distance revealed some similarity when the species were grouped together (group I). Moreover, they belong to the same genus and may occur concurrently in the same geographic regions (Lessa et al., 2007; Ministério da Saúde, 2007; Lainson, 2010). *E. coli* BL21(DE3+) (group II) was used as an outgroup. As expected, this isolate showed no genetic similarity to the *Leishmania* strains in group I (Figure 2).

Previous studies have indicated that genetic variations between *Leishmania* isolates correlate with clinical manifestations of the disease and its response to anti-leishmanial treatments. Oryan et al. (2013) conducted kDNA sequencing to identify heterogeneity among isolates of *L. major*, which causes cutaneous leishmaniasis, and its relationship with clinical features of this disease in humans. Nested PCR revealed a high degree of polymorphism, whereas sequencing analysis suggested associations between the genetic heterogeneity of the parasite, its geographical origin, and clinical manifestations of leishmaniasis in humans. Patients with the same clinical characteristics and geographical origin were infected with phylogenetically neighboring strains.

van Thiel et al. (2011) observed differences between the clinical conditions and therapeutic responses of Dutch military personnel infected with *L. major* from southern and northern Afghanistan. The clinical presentation of cutaneous leishmaniasis was milder in the south, and study subjects infected in this region of the country appeared to respond better to leishmanicidal treatment. Molecular analysis of the isolates indicated that these differences may have had a genetic basis. The severity of leishmaniasis is influenced by the pathogenicity of the parasite species or strain and the host immune response (Garcia et al., 2005). These findings may also explain why the similarity-based groupings in the present study correlated with clinical manifestations.

Cutaneous leishmaniasis exhibits several clinical forms. The localized cutaneous form is characterized by primary involvement of the skin. Single or multiple (up to 200) ulcerative lesions commonly result. All *Leishmania* species related to cutaneous involvement can cause this form, although their prevalence varies between geographic regions (Ministério da Saúde, 2009).

The disseminated cutaneous form is relatively rare, being observed in up to 2% of cases. It is characterized by the appearance of multiple acneiform papular lesions on various body parts, particularly the face and torso. The species recognized as causative agents include *L. amazonensis* and *L. braziliensis* (Barral et al., 1993; Barral et al., 1995; Gontijo and de Carvalho, 2003). The recidiva cutis form is marked by nodular lesions of late onset and long duration. Initially appearing in isolated instances, they then become confluent around or within the scar of previous *Leishmania* injuries. In Brazil, this form is caused by *L. amazonensis* and *L. braziliensis* (Bittencourt and Barral, 1991; Barral et al., 1993; Gontijo and de Carvalho, 2003).

The diffuse cutaneous form, an unusual type with aggressive clinical manifestations, is present in Brazil and caused by *L. amazonensis*. It first presents as a single lesion, evolving slowly into several non-ulcerated nodules and plaques covering large areas of the skin (Barral et al., 1993). Mucosal leishmaniasis is a latent, chronic form resulting in metastatic lesions of mucous membranes. It is principally caused by *L. braziliensis*, but other species such as *L. amazonensis* and *L. guyanensis* have also been implicated (Barral et al., 1993; Gontijo and de Carvalho, 2003; Silveira et al., 2004).

Therefore, the variety and extent of the clinical manifestations characteristic of *Leishmania* infection, and in particular, variations in pathogenicity, may derive from the genetic diversity of both host and parasite.

In this study, following extraction and purification of the 780-bp kDNA fragments, ERIC-PCR analysis was carried out. The electrophoretic pattern comprised bands ranging from 100 to 600 bp (Figure 4), and considerably fewer DNA fragments were generated compared to ERIC-PCR of genomic DNA (Figure 1). Our results are consistent with those of previous studies, as the kinetoplast corresponds to 15-35% of the total cellular DNA and multiplies independently of the genomic DNA. kDNA is made up of thousands of minicircles forming a complex network with maxicircles of circular and compact DNA molecules (Simpson et al., 1987). Considering the detection of distinct bands in the electrophoretic profiles, the binary matrix of present and absent DNA fragments changed significantly because of the reduction in polymorphic bands resulting from kDNA amplification.

We compared the size (in bp) and number of the principal DNA fragments of each kDNA signature generated by ERIC-PCR. It was possible to depict the relative similarities and differences between the patterns obtained from each *Leishmania* strain using a dendrogram (Figure 5).

The genetic distance observed between *L. braziliensis* and *L. guyanensis* was larger in the ERIC-PCR-based dendrogram created using kDNA than in that obtained with genomic DNA, which grouped them with a similarity of 42.0% (IA1 and IA2). After kDNA ERIC-PCR and construction of a new dendrogram, the above species were included in group I with 26.0% similarity (IA and IB). However, *L. amazonensis* and *L. chagasi* demonstrated 100% similarity (IIA and IIB), and were clustered in a second group (Figure 5), owing to the decreased number of polymorphic DNA fragments. However, the 100% similarity observed between *L. amazonensis* and *L. chagasi* was unexpected, as the former belongs to the *Viannia* subspecies and causes cutaneous leishmaniasis, whereas the latter is a member of the *Leishmania* subgroup and causes the visceral form. This result may be due to the small number of DNA bands in the electrophoretic profiles kDNA ERIC-PCR, since the amplified

fragment kDNA is too small to generate a higher band profile. Comparing the ERIC-PCR electrophoretic profiles and dendrograms, those based on genomic DNA gave better results than those generated using the kinetoplast region. Genomic DNA amplified by ERIC-PCR yielded products that ranged in size from 100 to 2000 bp, whereas kDNA signatures consisted of 100- to 600-bp fragments. The kDNA ERIC-PCR involved multiple amplification reactions of kinetoplast sequences only. Thus, few binding regions were available for the ERIC primers to initiate DNA amplification, explaining the limited variation of the bands generated by nested PCR (Simpson et al., 1987). Therefore, the dendrogram obtained from these nested PCR profiles contained different groupings from that based on ERIC-PCR of genomic DNA, with decreased genetic distance between strains because of the few DNA fragments analyzed. A greater number of fragments results in improved discriminatory power. In keeping with the present study, Alvarenga et al. (2012) demonstrated a certain degree of polymorphism among kinetoplast genetic signatures. Low-stringency single specific primer (LSSP)-PCR of kDNA hypervariable regions previously amplified from *Leishmania infantum* isolated from humans revealed genetic signatures consisting of 93- to 619-bp DNA fragments and high intraspecific variation. The heterogeneity among *L. infantum* kinetoplast profiles obtained by LSSP-PCR may reflect not only the high genetic diversity frequently observed in *Leishmania* strains, but also the substantial intraspecific variation between kDNA molecules within the Trypanosomatidae family.

CONCLUSION

Our results show that for epidemiological identification of *Leishmania* strains, the ERIC-PCR method is more effective when genomic DNA, rather than kDNA, is used. Epidemiological studies could be conducted to more precisely determine which strains are present in specific regions, to obtain an accurate assessment of the leishmaniasis types affecting different populations, and the appropriate treatments.

The polymorphic DNA patterns resulting from ERIC-PCR revealed a degree of genetic variation between the *Leishmania* strains. Based on this information, we hypothesized which differences were caused by mutations; however, DNA sequencing is required to verify these predictions. The kDNA ERIC-PCR amplification products resulted in a dendrogram showing a similarity of 100% between *L. amazonensis* and *L. chagasi*. This may have been due to the limitations of the electrophoretic profiles generated by this technique, or because only four strains were used. Thus, it is necessary to include additional strains to validate the ERIC-PCR method for genomic DNA and determine their genome sequences to enable the epidemiological identification of *Leishmania* species.

Conflicts of interest

The authors declare no conflict of interest.

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