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EVALUATION OF SPECIFIC PRIMERS FOR SPECIES IDENTIFICATION OF *Leishmania (V.) braziliensis*

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ABSTRACT

Leishmaniases are infectious diseases with different clinical forms and prognoses, making accurate species identification particularly important. We evaluated the performance of LBF1/LBR1 *L. (V.) braziliensis* specific primers by PCR and compared the results with *Leishmania* spp. identified by monoclonal antibodies (mAbs). Of 29 *L. (V.) braziliensis* identified by mAbs, 16 (53.3%) were detected; and 7 (63.6%) of 11 unidentified *Leishmania* spp. showed the 536 bp band. 87.7% of serodeme III *Leishmania* isolates were identified by these primers. These results indicate a poor correlation between the two identification methods used, and also suggest the existence of genetic variability among *L. (V.) braziliensis* isolates from the northwest region of Paraná state.

KEY WORDS: Polymerase chain reaction; *Leishmania braziliensis*; diagnosis.

RESUMO

Avaliação de primers específicos para identificação de espécies de *Leishmania (V.) braziliensis*

Leishmanioses são doenças infecciosas com diferentes formas clínicas e prognóstico, portanto a identificação da espécie é importante. Nós avaliamos o desempenho dos iniciadores LBF1/LBR1 específicos para *L. (V.) braziliensis* por PCR e comparamos com resultados de *Leishmania* spp identificadas por anticorpos monoclonais. Das 29 *L. (V.) braziliensis* identificadas por anticorpos monoclonais, 16 (53,3%) foram detectadas e 7 (63,6%) das 11 *Leishmania* spp não identificadas

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apresentaram a banda de 536 pb. Estes iniciadores identificaram 87,7% de *Leishmania* do serodema III. Estes iniciadores indicam uma pequena correlação entre os dois métodos usados e também sugerem a existência de uma variabilidade genética entre isolados de *L. (V.) braziliensis* da região noroeste do estado do Paraná.

DESCRITORES: Reação em cadeia da polimerase; *Leishmania braziliensis;* diagnóstico.

INTRODUCTION

Leishmaniases are vector-transmitted infectious diseases caused by protozoa of the genus *Leishmania,* and constitute a global public health problem, especially in tropical and subtropical countries. The variety of clinical manifestations of these diseases is related to the existence of different *Leishmania* species and also to the host's immune response (WHO, 2010). The identification of the parasite is of clinical and epidemiological importance, because, in addition to confirming the diagnosis of infection, accurate identification assists in the choice of treatment, as the manifestation of the disease and sensitivity to drugs can vary depending on the infecting species. Identification also contributes to the development of control measures in areas where malaria is endemic, and to epidemiological studies, allowing determination of the geographical distribution of the species (Grimaldi & Tesh, 1993; Degrave et al., 1994).

In Brazil, six species of *Leishmania* belonging to the subgenera *Viannia* and *Leishmania* have been reported as etiological agents of American cutaneous leishmaniasis; among them *L. (V.) braziliensis* and *L. (L.) amazonensis*. The former causes cutaneous and mucosal ulcers that may recur after treatment, and these ulcers are more aggressive than lesions caused by *L. (L.) amazonensis* (Passos et al., 1999). These parasites are morphologically indistinguishable but can be differentiated by isoenzyme analysis (Figueira et al., 2008), molecular methods (Degrave et al., 1994), and monoclonal antibodies (Shaw et al., 1986).

Molecular methods for the detection and differentiation of *Leishmania* spp. include conventional PCR (Rodgers et al., 1990; Lopez et al., 1993; Degrave et al., 1994; Marcussi et al., 2008), RAPD (Noyes et al., 1996) and SSR-PCR (simple sequence repeat anchored-PCR) (Volpini et al., 2001) and quantitative PCR methods such as RT-qPCR (Weirather et al., 2011) among others.

Conventional PCR, an economical technique, made possible the study of specific primers that identify genus, 13A/13B (Rodgers et al., 1990); subgenus, MP3H/MP1L for *L. (Viannia)* (Lopez et al, 1993), and species LBF1/LBR1 for *L. (V.) braziliensis* (Marcussi et al., 2008) of *Leishmania* spp. Monoclonal antibodies (mAbs) even though not the gold standard technique to identify *Leishmania* species, they have a better discriminatory capacity for distinguishing populations of parasites within the same species (Cupolillo et al., 1993).

Thus, this study evaluated the performance of LBF1/LBR1 *L. (V.) braziliensis* specific polymerase chain reaction (PCR) primers (Marcussi et

al., 2008) in the identification of *Leishmania* spp. isolates and their capacity to discriminate among serodemes.

MATERIALS AND METHODS

For this evaluation, two groups of *Leishmania* isolates were used. The first group contained 29 samples of *L.* (*V.*) *braziliensis* isolated from dogs and humans, 28 of which were previously identified by their reactivity profile with monoclonal antibodies (serodeme). The second group contained 11 samples of *Leishmania* spp. isolated from humans and not yet identified to species level. *L.* (*V.*) *braziliensis* (MHOM/BR/1975/M2903) was used as a reference strain. (All isolates were from the cryopreservation bank of the Laboratório de Leishmanioses, Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Paraná, Brazil).

Promastigote forms were cultured until the exponential growth phase in 199 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA), 2 mM L-glutamine (Sigma-Aldrich, USA), 1% sterile female human urine, and antibiotics (100 U/mL penicillin G, 100 µg/mL streptomycin) at 25°C, washed with PBS (phosphate-buffered saline) and stored at -20° C until use.

For DNA extraction, 300 µL of GT solution (guanidine isothiocyanate-phenol) was added to the parasite pellet and homogenized by inversion for 1 min. Then, 50 µL of cold chloroform was added and the tube was gently shaken by inversion and centrifuged (9300 g for 10 min). The supernatant was transferred to another tube containing 300 µL of cold absolute ethanol, and the tube was shaken by inversion for 1 min, then centrifuged (9300 g for 15 min). The pellet was washed twice with cold absolute ethanol (9300 g for 10 min). The sediment was dried in a drying bath (Bioplus IT-2002) at 95°C. The sediment was dissolved with 50 µL of TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0), after incubation on a rotary shaker for 6 h at room temperature, and stored at -18° C until use. The DNA samples were quantified with Quant kit dsDNA BR Assay on a QubitTM fluorometer (Invitrogen, USA).

The PCR was performed with LBF1 (5'-AAATTCGCGTTTTTTGGCC TCCCCG-3') and LBR1 (5'-GCATAAACTAGAGACGGAACAGAG-3') PCR primers, which amplify a fragment of 536 base pairs (bp) of the kDNA minicircle of *L. (V.) braziliensis* (Marcussi et al., 2008) in a final volume of 25 µL containing 1 µM of each primer (Invitrogen, Brazil), 1 U of Taq DNA polymerase (Invitrogen, USA), 1.5 mM magnesium chloride, 1 X enzyme buffer, and 4 µL (200 ng) of the DNA sample. The amplification reaction was performed in a thermocycler (Biometra PC, Germany) with initial denaturation at 94°C for 5 min, followed by 26 cycles of 1 min and 30 s at 94°C, 1 min and 30 s at 58°C, and 2 min at 72°C. After the cycles were complete, the samples were heated for 10 min at 72°C for final extension and stored at 4°C.

After amplification, 10 µL of each PCR product was analyzed on 1.2% agarose gels containing ethidium bromide (0.25 mg/mL) to reveal the bands under

an ultraviolet light transilluminator (MacroVue UV-20, Hoefer, USA). A 100 bp molecular weight marker (Invitrogen, Brazil) was used. The proportions of positives and confidence intervals were calculated by the Mid-P Exact Test using OpenEpi version 2.2.1 software.

RESULTS

DNA (200 ng) from each *Leishmania* isolate was used for the analysis. All isolates that tested negative were again subjected to PCR amplification, using 400 ng of DNA. *L. (V.) braziliensis* M2903 strain showed the 536 bp band and was used as a positive control in further amplifications. Figure 1 shows a representative agarose gel of PCR products produced using LBF1/LBR1 primers.

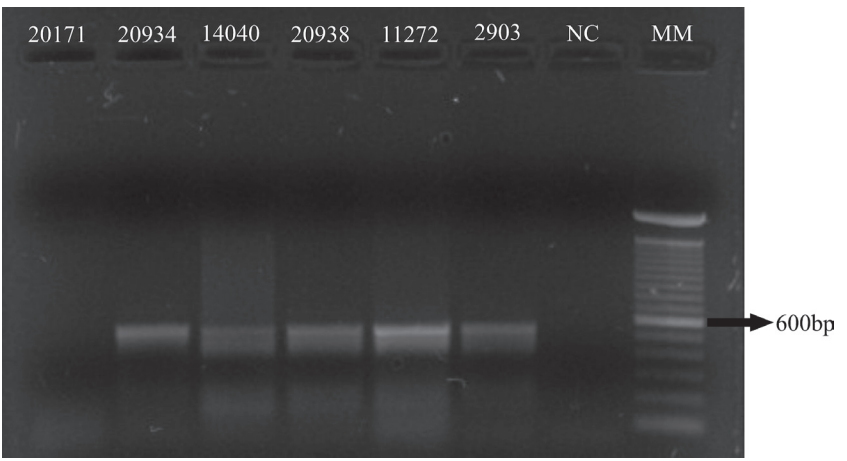

Figure 1. Representative agarose gel of PCR products obtained from *Leishmania (Viannia) braziliensis* promastigotes with LBF1/LBR1 specific primers. Lanes: 20171 (MCAN/BR/2001/M20171); 20934 (MHOM/BR/2002/M20934); 14040 (MHOM/BR/1992/M14040); 20938 (MHOM/BR/2002/M20938); 11272 (MHOM/BR/1987/M11272); 2903 (MHOM/BR/1975/M2903) as positive control; NC, water as negative control; and MM, a 100 bp molecular marker.

The performance of LBF1/LBR1 in the present work confirmed the results obtained from previous studies (Marcussi et al., 2008). Furthermore some other isolates from dogs, humans (not yet identified) and *L. (V.) braziliensis* already identified by species-specific monoclonal antibodies, a once gold standard assay for the identification of *Leishmania* spp, were also tested with some interesting results.

PCR analysis of the isolates showed that 15 of the 29 previously identified *L. (V.) braziliensis* showed the 536 bp band, confirming the identification. Including the M2903 strain, there were 16 positive PCR results for *L. (V.) braziliensis* (53.3%; 95% CI, 35.59-70.46%), (Table 1). Also, 7 of the 11 *Leishmania* spp. strains showed the 536 bp band identifying them as *L. (V.) braziliensis* (63.6%, 95% CI, 33.64-87.22%), (Table 1). None of the isolates from dogs was positive by this technique.

Table 1. Results of PCR using LBF1/LBR1 primers on *Leishmania (V.) braziliensis* and *Leishmania* spp isolates.

	PCR LBF1/LBR1 Positive samples % (95% CI)
L. (V.) braziliensis (n=30)	16 53.33% (35.59 – 70.46%)
Leishmania spp. (n=11)	7 63.63 % (33.64 – 87.22%)

Among the isolates previously identified by monoclonal antibodies as *L. (V.) braziliensis* belonging to serodemes I (n=12), II (n=7), III (n=7) and VII (n=2), this pair of PCR primers showed a higher detection rate for serodeme III (87.71%; 95% CI, 46.98-99.29%), (Table 2).

Table 2. Results of PCR using LBF1/LBR1 primers among serodemes of *Leishmania (V.) braziliensis.*

L. (V.) braziliensis	LBF1/LBR1 Positive PCR % (95%CI)
Serodeme I (n=12)	6 50.00% (23.38 – 76.62%)
Serodeme II (n=7)	2 28.57% (5.10 – 66.98%)
Serodeme III (n=7)	6 87.71% (46.98 – 99.29%)
Serodeme VII (n=2)	1 50.00% (2.50 – 97.50%)

DISCUSSION

Because the different *Leishmania* species are generally considered to be indistinguishable by their morphology, other criteria are used for their identification. According to Lainson & Shaw (1987), the biological methods used to identify *Leishmania* species include biochemical, immunological, biological development in the host/culture medium, and geographical distribution of parasites. Commonly used non-biological methods are restriction fragment length polymorphism (RFLP) and isoenzyme characterization.

Reactivity of the parasite with monoclonal antibodies (mAb) has been used in a wide variety of studies to identify *Leishmania*. Shaw et al. (1986) defined 7 different *L. (V.) braziliensis* serodemes, based on mAb reactivity to promastigotes. However, this presents some procedural problems, difficulties in interpretation of data, and the necessity to obtain large amounts of live parasites.

The kinetoplast (kDNA), an organelle unique to the kinetoplastids, is known to contain approximately 10,000 small circular DNAs, known as kDNA minicircles, which are between 600 and 800 bp, in members of the genus *Leishmania*. The abundance and other characteristics of these molecules have made them the target for a number of PCR-based techniques (Rodrigues et al., 2002).

Marcussi et al. (2008) described the LBF1/LBR1 pair of primers that amplify a 536 bp fragment of kDNA minicircles from *L. (V.) braziliensis.* Even though these primers are not very sensitive (50 ng/mL DNA), which impedes their use as diagnostic primers, they are highly specific, and do not show the 536 bp DNA fragment with *L. (V.) panamensis*, *L. (V.) guyanensis*, *L. (V.) naiffi*, *L. (V.) lainsoni*, *L. (V.) shawi*, *L. (L.) chagasi*, *L. (L.) amazonensis*, *L (L.) major* and *T. cruzi.* This specificity makes them promising primers for species identification of *L. (V.) braziliensis*.

According to the results of this study, 15 of the 29 strains previously identified as *L. (V.) braziliensis* and 7 of 11 strains of *Leishmania* spp. showed a 536-bp band. Despite the high specificity demonstrated for the LBF1/LBR1 primers (Marcussi et al., 2008) and the expectations for the primers used in epidemiological studies, the results of the molecular and biological methods were not in agreement, although the PCR positivity was higher for serodeme III. This lack of correlation between molecular and biological methods may be due to high intraspecific variability (Cupolillo et al., 1995) of the members of the *Leishmania (Viannia*) complex, which constitute a biologically diverse group of microorganisms (Cupolillo et al., 1997; Cupolillo et al., 1998).

Analyses of kDNA of species of *L. braziliensis* complex isolates in Rio de Janeiro and Minas Gerais generally give very different results from isolates in northern Brazil (Amazonas, Pará, and Amapá), and are grouped in three different schizodemes, demonstrating high heterogeneity in parasites of this complex (Lainson & Shaw, 1987). Grimaldi & McMahon-Pratt (1991) reported polymorphism in populations of *L. (V.) braziliensis* and *L. (V.) guyanensis* based on reactivity patterns with species-specific monoclonal antibodies grouping them into serodemes.

Brito et al. (2009) also found conflicting results, in which five isolates of *L. (V.) braziliensis* identified by reactivity with monoclonal antibodies were identified as *L. (V.) shawi* by multilocus enzyme electrophoresis (MLEE) and PCR-RFLP. According to the authors, these data indicate the existence of polymorphism in the population of *L. (V.) shawi* circulating in Pernambuco, and that this population expresses epitopes similar to *L. (V.) braziliensis*. This genetic variability can also be

present among isolates of *L. (V.) braziliensis* from Paraná, and probably the target for the primer pair used in the present study is a less-conserved region.

The results demonstrated poor correlation between the biological methods that are commonly used in the identification of *L. (V.) braziliensis,* and PCR using specific primers such as LBF1/LBR1, which may be due either to the low sensitivity of the assay described by Marcussi et al. (2008), or to a possible genetic variability within the species *L. (V.) braziliensis* in northwest Paraná.

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