A comparison of molecular markers to detect *Lutzomyia longipalpis* naturally infected with *Leishmania (Leishmania) infantum*

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The aim of the present study was to detect natural infection by Leishmania (Leishmania) infantum *in* Lutzomyia longipalpis *captured in Barcarena, state of Pará, Brazil, through the use of three primer sets. With this approach, it is unnecessary to previously dissect the sandfly specimens. DNA of 280* Lu. longipalpis *female specimens were extracted from the whole insects. PCR primers for kinetoplast minicircle DNA (kDNA), the mini-exon gene and the small subunit ribosomal RNA (SSU-rRNA) gene of* Leishmania *were used, generating fragments of 400 bp, 780 bp and 603 bp, respectively. Infection by the parasite was found with the kDNA primer in 8.6% of the cases, with the mini-exon gene primer in 7.1% of the cases and with the SSU-rRNA gene primer in 5.3% of the cases. These data show the importance of polymerase chain reaction as a tool for investigating the molecular epidemiology of visceral leishmaniasis by estimating the risk of disease transmission in endemic areas, with the kDNA primer representing the most reliable marker for the parasite.*

Key words: visceral leishmaniasis - *Leishmania (Leishmania) infantum* - *Lutzomyia longipalpis*

Leishmaniasis is a group of diseases caused by various species of the protozoan *Leishmania* and showing a wide range of clinical manifestations. Leishmaniasis represents a serious threat to public health in tropical and subtropical regions of several countries (Alvar et al. 2012) and is considered one of the most important neglected parasitic diseases (WHO 2010). Approximately two million new cases per year are estimated, with 350 million people at risk of contracting the disease.

Visceral leishmaniasis (VL) is a systemic and chronic disease that is fatal in cases that do not receive suitable treatment (Freitas-Junior et al. 2012). Brazil is one of the six countries that together include more than 90% of all recorded VL cases worldwide (Alvar at al. 2012); in this case, the disease is caused by *Leishmania (Leishmania) infantum* [syn. *Leishmania (Leishmania) chagasi*] (Kuhls et al. 2011). The sandfly *Lutzomyia cruzi* can be infected by *L. (L.) infantum* (Santos et al. 2003, Missawa et al. 2011), but the sandfly *Lutzomyia longipalpis* is considered the main vector (Lainson & Rangel 2005).

The Brazilian VL control program is based on treating human cases, controlling vectors and reservoirs and euthanising seropositive infected dogs (MS/SVS/DVE 2006). One useful tool for epidemiological studies of leishmaniasis is the determination of the infection rate of parasites in sandflies (Kato et al. 2007).

Several methods have been applied to detect infection in sandflies. Classical methods consist of searching for promastigotes *in loco* and isolating the parasite after the dissection of the digestive tract of the insect in a culture medium, which requires experience (Fernandes et al. 1994, Perez et al. 2007).

Molecular diagnostic assays, such as the polymerase chain reaction (PCR) assay, have been used for the identification and characterisation of *Leishmania* in vectors, especially in epidemiological field studies when a large number of samples need to be handled (Rodríguez 1999, Miranda et al. 2002). Different targets derived from nuclear and kinetoplast parasite DNA have been used to detect *Leishmania* spp in naturally infected phlebotomines. These targets include the rRNA gene, the mini-exon-derived RNA gene, repeated genome sequences, glucose-6-phosphate dehydrogenase and the kinetoplast minicircle DNA (kDNA) minicircle (de Bruijin & Barker 1992, Castilho et al. 2003, Paiva et al. 2006).

Because several markers are available to detect different genic regions of *Leishmania*, it is important to determine and compare their efficiency in identifying the parasite in epidemiological surveys. The present study aims to detect natural infections by *L. (L.) infantum* in *Lu. longipalpis* captured in Barcarena, state of Pará (PA), Brazil, and to compare the PCR amplification rate of three markers: kDNA, the mini-exon gene and the 18S small subunit ribosomal RNA (SSU-rRNA) gene, with no previous dissection of the phlebotomine.

MATERIALS AND METHODS

Study area - The investigation was performed in northern Brazil in Santana do Cafezal (0º3'38.38"S 47º39'3.36"W), 7 km from the municipality of Barcarena (Fig. 1). According to the Köppen climate classification categories, Barcarena has a warm humid equatorial climate corresponding to the Amazon type of climate.

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The average annual temperature is approximately 27ºC. Abundant rainfall (> 2,500 mm/year) occurs more intensely in the first six months of the year (SEPOF-PA 2009). Approximately 80% of the population of Santana do Cafezal lives in wooden houses, with the remainder living in brick houses, next to vegetation and with domestic animal shelters nearby. The town has electricity, but has no basic sanitation and untreated water is commonly consumed.

Sandfly collections and species identification - Sandflies were captured between November 2003-February 2004, using CDC light traps placed overnight in peridomicile areas. The selection of the houses was based on previous entomological data as well as on the prevalence and incidence of human and canine cases of VL. The taxonomic identification of each specimen was determined according to Young and Duran (1994). In all, 280 female *Lu. longipalpis* were selected for molecular analysis.

DNA extraction - Total genomic DNA was extracted from sandflies following the Ready et al. (1997) method. Sandflies were macerated individually with a sterile tip in 1.5 mL tubes containing 100 µL of grinding solution [0.1 M Tris-HCl pH 7.5, 0.6 M NaCl, 0.1 M ethylenediamine tetraacetic acid (EDTA), 20 x spermine/spermidine mL, 10% sucrose], 10 µL of lysis buffer (10% sodium dodecyl sulfate, 10% sucrose, 17 µL diethylpyrocarbonate) and 30 µL of 8 M potassium acetate for protein precipitation. The DNA was precipitated with 96% ethanol and resuspended in 20 µL of Tris-EDTA solution (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

PCR assays - As a positive control for DNA extraction and to guarantee that all sandflies were correctly identified as *Lu. longipalpis*, all sandflies were amplified by PCR using the primers Lu1 (5'-TGAGCTTGACTCTAGTTTGGCAC-3') and Lu2 (5'-AGATGTACCGCCCCAGTCAAA-3') that amplify a specific fragment for the 28S rRNA gene of *Lu. longipalpis*. The PCR, performed according to Cabrera et al. (2002), amplified a fragment of approximately 370 bp. For the identification of *L. (L.) infantum*, all positive samples were amplified with different primer pairs: D1/D2 for kDNA, S1629/S1630 for the mini-exon gene and R221/R332 for 18S SSU-rRNA gene. These primer pairs amplify fragments of 780 bp, 400 bp and 603 bp, respectively. PCR assays were performed as shown in Table I.

Sensitivity of PCR for different molecular targets - To analyse the amplification capacity of the three targets (kDNA, SSU-rRNA and mini-exon), serial dilutions (1 fg to 100 ng) of *L. (L.) infantum* DNA (MCER/BR/1996/M15677), extracted from culture, were tested singly and in the presence of sandfly DNA [the same serial dilutions of *L. (L.) infantum* DNA were added to each uninfected *Lu. longipalpis* DNA preparation (30 ng/µL) in triplicate assays]. The specificity test was performed to check the possibility of nonspecific fragments and consisted of amplifying *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* DNA with the same PCR conditions used to amplify *L. (L.) infantum* DNA in *Lu. longipalpis* with kDNA, SSU-rRNA and mini-exon primers.

The PCR products were separated by horizontal electrophoresis on 1% agarose gel containing ethidium bromide (0.5 µg/mL) for 1 h at 100 V. The amplification products were visualised under ultraviolet light.

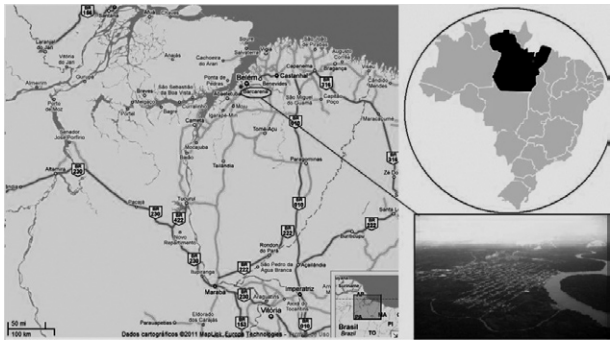

Fig. 1: map of the state of Pará and the municipality of Barcarena.

TABLE I

Genes and primer sequences used for the detection of *Leishmania (Leishmania) infantum*

Gene	Primer sequence (5'-3')	Fragment (bp)	Reference
kDNA	D1/D2 CCAGTTTCCCGCCCCG GGGGTTGGTGGTGTAAAATAG	780	Smyth et al. (1992)
Mini-exon	S1629/S1630 GGAATTCAATAWAGTACAGAAACTG GGGAAGCTTCTGTACTWTATTGGTA	400	Degrave et al. (1994), Fernandes et al. (1994)
SSU-rRNA	R221/R332 GGTTCCTTTCCTTGATTTAGC GGCCGGTAAAGGCCGAATAG	603	Van Eyes et al. (1992)

kDNA: kinetoplast DNA; SSU-rRNA: small subunit ribosomal RNA.

RESULTS

In the initial step, the effectiveness of genomic DNA extraction of sandflies was confirmed by the presence of a 370 bp band (fragment of 28S rRNA of *Lu. longipalpis*) in all infected and non-infected sandfly samples (Fig. 2). The specificity test using primers D1/D2, S1629/S1630 and R221/R332 for different *Leishmania* strains [*L. (V.) braziliensis*, *L. (L.) amazonensis* and *L. (L.) infantum*] showed that all primer pairs are highly specific.

The sensitivity assay using serial dilutions of *L. (L.) infantum* DNA derived from culture showed an amplification of up to 10 pg for kDNA, 100 pg for the mini-exon and 10 ng for SSU-rRNA (Fig. 3). However, if *L. (L.) infantum* DNA derived from culture was mixed with sandfly genomic DNA (1:1), a significant decrease was observed in the sensitivity of two pairs of primers: the primer D1/D2 was able to detect 1 ng of DNA, and R221/R332 was only able to detect up to 52.6 ng. The mini-exon primer showed no change in the ability to detect *L. (L.) infantum*, amplifying up to 100 pg even in the presence of *Lu. longipalpis* DNA (Fig. 4).

The PCR results for the *Lu. longipalpis* samples from Santana do Cafezal showed an infection rate of 8.6% using the D1/D2 primer, with the DNA from 24 of 280 female sandflies amplifying a fragment of 780 bp. In contrast, the DNA from 20 sandflies amplified a fragment of 400 bp with the S1629/S1630 primer and the DNA from only 15 sandflies amplified a fragment of 603 bp with the R221/R332 primer, resulting in infection rates of 7.1% and 5.3%, respectively (Fig. 5, Table II). When the PCR tests were combined, the following results were observed: 15/280 (5.4%) infected sandflies were detected using the three markers, 20/280 (7.1%) were detected using the 400 bp and 780 bp fragments, 15/280 (5.4%) were detected using the 400 bp and 603 bp fragments and 15/280 (5.4%) were detected using the 603 bp and 780 bp fragments.

DISCUSSION

In this study, we evaluated the applicability of three PCR markers to the detection of *Leishmania* within sandflies, with no previous dissection of the phlebotomine. The primers used for the detection of the parasite with PCR for the mini-exon gene (S1629/S1630), the 18S rRNA gene (R221/R332) and kDNA (D1/D2) are well known and have been used in several studies to diagnose *Leishmania* infection in clinical specimens as well as in the identification of culture isolates, material from animals and infected sandflies (Aransay et al. 2000, Quinnell et al. 2001, Kaouech et al. 2008).

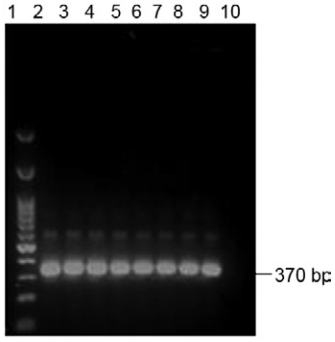

Fig. 2: polymerase chain reaction electrophoresis with amplified DNA of *Lutzomyia longipalpis* using primer for 28S rRNA gene. Lane 1: 50 bp DNA Ladder (Uniscience); 2-5: uninfected *Lu. longipalpis*; 6-9: infected *Lu. longipalpis*; 10: negative control.

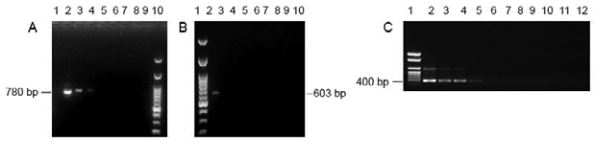

Fig. 4: polymerase chain reaction electrophoresis to evaluate the primer sensitivity using serial dilutions of *Leishmania infantum* DNA with *Lutzomyia longipalpis* DNA. A: primer D1/D2 [kinetoplast DNA (kDNA)] [Lane 1: negative control; 2-9 *L. (L.) infantum* DNA dilutions, respectively, of 52.6 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/µL and 10 fg/µL; 10: 100 bp DNA Ladder (Kasvi)]; B: primer R221/R332 [small subunit ribosomal RNA (SSU rRNA)] [Lane 1: 100 bp DNA Ladder (Kasvi); 2-9 *L. (L.) infantum* DNA dilutions, respectively, of 52.6 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/µL and 10 fg/µL; 10: negative control]; C: primer S1629/S1630 (mini-exon) [Lane 1: 100 bp øX174/Hae III (Invitrogen); 2-11: *L. (L.) infantum* DNA dilutions, respectively, of 100 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/µL, 10 fg/µL and 1 fg/µL; 12: negative control].

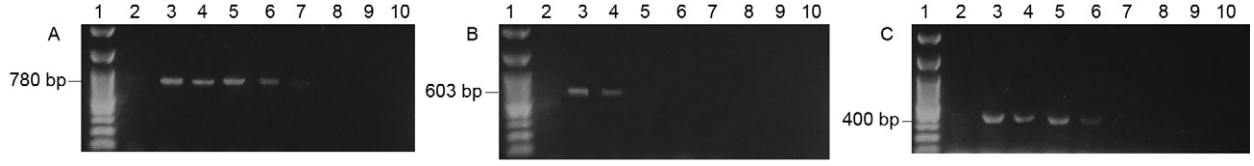

Fig. 3: polymerase chain reaction electrophoresis to evaluate the primer sensitivity using serial dilutions of *Leishmania infantum* DNA. A: primer D1/D2 [kinetoplast DNA (kDNA)] [Lane 1: 100 bp DNA Ladder (Kasvi); 2: negative control; 3-10: *L. (L.) infantum* DNA dilutions, respectively, of 100 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/µL and 10 fg/µL]; B: primer R221/R332 [small subunit ribosomal RNA (SSU rRNA)] [Lane 1: 100 bp DNA Ladder (Kasvi); 2: negative control; 3-10: *L. (L.) infantum* DNA dilutions, respectively, of 100 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/µL and 10 fg/µL]; C: primer S1629/S1630 (mini-exon) [Lane 1: 100 bp DNA Ladder (Invitrogen); 2: negative control; 3-10: *L. (L.) infantum* DNA dilutions, respectively, of 100 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/µL, 10 fg/µL and 1 fg/µL].

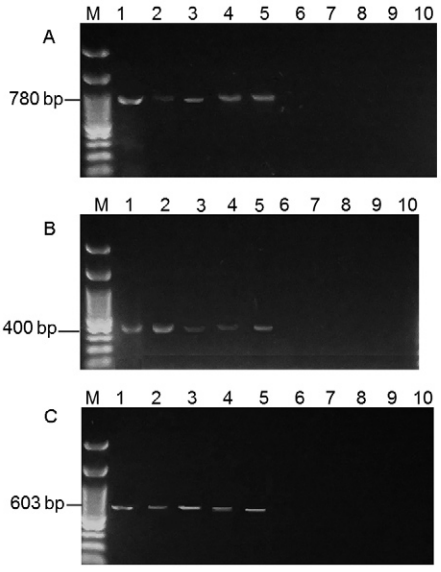

Fig. 5: detection of *Leishmania (Leishmania) infantum* in *Lutzomyia longipalpis* with kinetoplast DNA (kDNA), mini-exon and small subunit ribosomal RNA (SSU rRNA) targets. A: primer D1/D2 (kDNA) {Lane 1: positive control [*L. (L.) infantum* DNA from culture]; 2-5: *Lu. longipalpis* infected; 6-9: *Lu. longipalpis* non-infected; 10: negative control}; B: primer R221/R332 (SSU rRNA) {Lane 1: positive control [*L. (L.) infantum* DNA from culture]; 2-5: *Lu. longipalpis* infected; 6-9: *Lu. longipalpis* non-infected; 10: negative control}; C: primer S1629/S1630 (mini-exon) {Lane 1: positive control [*L. (L.) infantum* DNA from culture]; 2-5: *Lu. longipalpis* infected; 6-9: *Lu. longipalpis* non-infected; 10: negative control}.

TABLE II

Infection natural rate to mini-exon, kinetoplast DNA (kDNA) and small subunit ribosomal RNA (SSU-rRNA) genes

Targets	PCR+	PCR-	Infection rate (%)
kDNA	24	256	8.6
Mini-exon	20	260	7.1
SSU-rRNA	15	265	5.3

The marker for kDNA clearly showed higher sensitivity when tested on culture samples (10 pg). In contrast, primers R211/R332 and S1629/S1630 were only able to detect up to 100 pg DNA. These results agree with previous studies that showed that the kDNA marker is able to detect as little as 1 fg of DNA (de Bruijin & Barker 1992, Smyth et al. 1992).

Despite its low sensitivity in comparison with kDNA, the primer for the mini-exon gene has the advantage that in a single assay, it is possible to distinguish infection caused by *L. (V.) braziliensis*, *L. (L.) amazonensis* and *L. (L.) infantum* based only on the size of the fragment produced by PCR. The low sensitivity of the mini-exon gene primer is due to the number of DNA copies (Degrave et al. 1994) found inside the parasite.

When we tested the material extracted from sandflies captured in Santana do Cafezal, we observed that the frequency of infection detected in sandflies was 8.6% with the use of the primer D1/D2, a much higher rate than that found from the use of the S1629/S1630 and R221/R332 primers, whose sensitivity was 83.3% and 63%, respectively. This difference may be related to the low number of parasites in each cell and the influence of possible inhibitors on the amplification process. All infections detected by the 400 bp (n = 20) and 603 bp (n = 15) fragments were also identified by the 780 bp fragment, with no false positive samples for these markers.

Paiva et al. (2006) found a rate of 3.9% of natural infection by *L. (L.) infantum* in the municipality of Antônio João, state of Mato Grosso do Sul, using the primer for the mini-exon gene. In a similar study in the same state, Silva et al. (2008) found an infection rate of 1.9% for VL in Campo Grande. Soares et al. (2010) observed rates of 1.25% for an old colonisation and 0.25% for a recent colonisation on São Luís Island (state of Maranhão) using primers for kDNA. Independent of the primer used, the infection rate observed in the present study, 8.6% for the kDNA gene, was greater than those reported from these previous studies in Brazil. Cimerman and Cimerman (2003) and Missawa et al. (2010) state that the infection ratio of *Leishmania* is usually low, even in endemic areas, with average values below 3%. According to Cimerman and Cimerman (2003), transmission depends on the presence of high densities of *Lu. longipalpis*, as observed during outbreaks of the disease.

However, an infection rate of 19% (4/21) has been detected in Belo Horizonte (state of Minas Gerais) (Saraiva et al. 2010) based on the use of PCR for kDNA. The high rate observed might result from three factors: (i) the majority of samples were collected from the peridomicile, where several cases of VL occurred an year before the sampling, (ii) environmental conditions were favourable for the development of vectors, e.g., the high prevalence of canine infection, which is the most important source of infection for phlebotomines, and (iii) the molecular methods are sensitive and specific compared with traditional techniques of parasite visualisation.

de Oliveira et al. (2011) dissected 1,451 *Lu. longipalpis* females to evaluate the natural infection rate in Barcarena and found no infected flies. In contrast, Saraiva et al. (2010) found that the number of positive results based on the primer for kDNA was 500% greater than the value based on the intestinal dissection and microscopy of sandflies. This finding supports the reliability of kDNA in vectorial studies. Several authors, e.g., Degrave et al. (1994), Rodríguez et al. (1999) and Miranda et al. (2002), have also stated that molecular techniques are more sensitive and have greater specificity than the dissection method. The choice of techniques to investigate infection in sandflies is important because the different techniques can produce differing estimates of the true rates of natural infection.

Although research on individual samples may be more laborious, especially in large quantities, the great advantage over pooled samples is that a more realistic understanding of the frequency of infected sandflies oc-

curring in peridomicile areas can be achieved, especially in areas where new cases are beginning to emerge in dogs and humans. The improved understanding that results from research on individual samples is informative for assessing the risk to the population. If the number of captured sandflies is high, the investigation should undoubtedly use pools of specimens and evaluate the minimum infection rate (MR = nº of positive groups x 100/ total number of species).

These data show the importance of PCR as a tool for investigating the molecular epidemiology of VL and estimating the risk of disease transmission in endemic areas, with the primer for kDNA showing greater reliability as a marker for the parasite.

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