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Detection and identification of *Leishmania* species in field-captured phlebotomine sandflies based on mini-exon gene PCR

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Abstract

Leishmaniasis is one of the most diverse and complex of all vector-borne diseases. Because it involves several overlapping species and sandfly vectors, the disease has a complex ecology and epidemiology. Adequate therapy and follow-up depend on parasitological diagnosis, but classical methods present several constraints when identifying species. We describe a polymerase chain reaction (PCR) which uses primers designed from mini-exon repetitive sequences that are specific for subgenus *Leishmania Viannia* (PV), as well as sequences with specificity for genus (PG) that can distinguish between *Leishmania* species from other insect flagellates with minor differences in PCR products. For standardization, these PCR were tested in experimentally infected sandflies, and *Leishmania* infection in these insects was successfully confirmed. This methodology identified a 3.9% infection rate in field-captured phlebotomine sandflies from an endemic region in Brazil. Natural infection by *Leishmania* species was identified in three samples of *Lutzomyia longipalpis*, of which two were *Leishmania* (*L.*) *chagasi* and one *Leishmania* (*L.*) *amazonensis*. Irrespective of specific epidemiological conclusions, the method used in this study was able to identify *Leishmania* infections both in experimentally infected and field-captured phlebotomine sandflies, and could be a useful tool in epidemiological studies and strategic planning for the control of human leishmaniasis.

Keywords: Leishmaniasis; Vectors; Diagnosis of *Leishmania*; PCR; Mini-exon

1. Introduction

Leishmaniasis is a heteroxenous protozoan infection transmitted by phlebotomine sandflies and is highly prevalent in tropical regions such as Brazil. The infection has a broad spectrum of manifestations due to the variety of morphologically similar parasite species as well as the different host susceptibilities. This results in a spectrum of diseases, frequently occurring in the same area (Falqueto and Sessa, 1991). Treatment and prognosis need to be individualized, making parasite species-specific diagnosis essential (Grevelink and Lerner, 1996). Apart from minimal differences in *Leish-*

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mania distribution in the vector's digestive tract (Shaw and Lainson, 1987), the flagellate forms of this parasite are indistinguishable between species, making specific diagnosis in routine optical microscopic investigation impossible (Michalsky et al., 2002). The development of accurate methods for identifying the species of *Leishmania* in the insect vector is therefore crucial for epidemiological studies or control programs.

Molecular approaches have recently allowed diagnosis of infectious diseases based on polymerase chain reaction (PCR). This technique has permitted the identification of several parasite species (Oskam et al., 1996; Arez et al., 2000) and could be valuable in *Leishmania* detection in phlebotomine sandflies (Cabrera et al., 2002).

In this study, sequences inferred from mini-exon regions shared by all *Kinetoplastida* were used. The nuclear mini-exon genes consist of 200 copies in tandem separated into transcribed and nontranscribed genes. The transcribed region consists of a highly conserved exon and a moderately conserved intron among species of the same genera or subgenera. The nontranscribed region consists of a variable intergenic region among *Leishmania* species that is absent in vertebrate hosts and vectors (Fernandes et al., 1994).

The aim of this study was the standardization of a PCR methodology for identification of *Leishmania* species in sandfly vectors. For this purpose, protocols for phlebotomine sandfly storage and DNA extraction were evaluated for detecting both the infection and the infecting species in field-captured phlebotomine sandflies in order to evaluate the vector-associated transmission risk for humans and/or reservoirs.

A step-by-step approach was used to develop the PCR reaction. The first step was standardization using parasites from axenic cultures. This was followed by reaction with nucleic acids extracted from experimentally infected laboratory-reared sandflies fed on infected animals or by artificial membrane feeder. Finally, the PCR method was tested in field-trapped vector phlebotomines from areas in Mato Grosso do Sul, Brazil, that are endemic for tegumentary and visceral leishmaniasis.

2. Materials and methods

2.1. Parasites, sensitivity and specificity

Primers were assayed with the following strains of *Leishmania* and parasites: *Leishmania* (*L.*) *amazonensis* (IFLA/BR/67/PH8), *Leishmania* (*L.*) *chagasi* (MHOM/BR/1974/PP75(M2682)), *Leishmania* (*V.*) *braziliensis* (MHOM/BR/1975/M2903), *Leishmania* (*V.*) *guyanensis* (MHOM/BR/1975/M4147), *Leishmania* (*V.*) *peruviana* (MHOM/PE/M12715), *Endotrypanum shaudini* (GML 30), *Crithidia fasciculata* (ATCC 30267), *Trypanosoma cruzi* (Y) and *Plasmodium falciparum* (ADA). Sensitivity and specificity tests were carried out as described for *Viannia* subgenus primers (PV) in Paiva et al. (2004).

2.2. Phlebotomine sandfly colonies

Lutzomyia longipalpis ($n = 14$), *Lutzomyia almerioi* ($n = 04$) and *Nyssomyia intermedia* ($n = 26$) reared in the Entomology Laboratory of the Faculdade de Saúde Pública/USP were used for *in vivo* infection. The experimental membrane-feeding infections were carried out in the Medical Entomology Laboratory of the Centro de Pesquisas René Rachou, FIOCRUZ-MG, Belo Horizonte, using *Lu. longipalpis* ($n = 391$) reared in the same laboratory.

2.3. Phlebotomine sandfly storage and DNA extraction

Three groups of phlebotomine sandflies were stored in the following conditions until DNA extraction was carried out: the first group at 4 °C, the second in ethanol 70% and the third in isopropanol. A modified Triton X 100/DTT protocol was used for DNA extraction (Oskam et al., 1996).

Briefly, after adding 1ul of proteinase K (20 mg/ml) to the DNA sample and after 3 h of incubation at 60 °C, the DNA was precipitated by adding sodium acetate (3 M, pH 5.2) at final concentration of 0.3 M and two volumes of absolute ethanol. After overnight incubation at −20 °C, the DNA was washed with ethanol 70%, dried and resuspended in 20 μl of sterile water.

2.4. Mini-exon PCR assay

Repetitive mini-exon sequences described by Degrave, W. (accession nos. X69446, X69442 and L05000) were used to design the specific primers for *Leishmania* genus (PG) and for *Viannia* subgenus (PV) as previously described by Paiva et al. (2004). In this study, both PV and PG primers were used.

The following PG primers were used: forward (PG1) 5′-TTT ATT GGT ATG CGA AAC TTC C-3′ and reverse (PG2) 5′-GAA ACT GAT ACT TAT ATA GCG TTA G-3′. A 25 μl reaction mixture containing DNA, 1 μM of primers, 0.2 mM of dNTPs, 2.5% formamide, 1× Taq buffer, 2 mM of $MgCl_2$ and 0.5 U of Taq DNA poly-

merase was placed in a thermal cycler (Eppendorf Mastercycler gradient serial no. 5331) at 95 °C for 5 min for initial denaturation, followed by 35 cycles at 95 °C for 1 min, 50 °C for 30 s, 72 °C for 1 min and then 72 °C for 6 min for final extension. Products (10 μl each) were separated on 1.5% agarose gel electrophoresis.

2.5. "Gold standard" test using ribosomal DNA (rDNA)

In order to evaluate the quality of both storage and DNA extraction by different methods, ribosomal DNA primers (S4/S12) (Uliana et al., 1994) were used. These primers can detect positive samples by amplifying a 540 bp fragment corresponding to *Leishmania* sp.

2.6. Experimental infections

Female phlebotomine sandflies were fed on hamsters provided by the local animal colony and infected with the *Leishmania* strains described above. Hamster lesions caused by *L.* (*L.*) *amazonensis* were offered to *Lu. almerioi* and *N. intermedia*, while *Lu. longipalpis* fed on the dorsal regions of hamsters infected with *L.* (*L.*) *chagasi*.

Artificial infections were carried out following the Tesh and Modi (1984) protocol. Sandflies were allowed to feed through a chick skin membrane in an artificial feeding device containing heparinized mouse blood seeded with 2×10^7 amastigotes or with mouse blood with heat-inactivated serum seeded with 2×10^7 promastigotes. The sandflies were maintained at 25 °C with relative humidity >80%. They were provided with a solution of 50% sucrose plus 0.001% gentamicin and water *ad libitum* until dissection. Infection was confirmed by dissecting 10% of the fed insects after the 3rd or 4th day. The digestive tracts of female sandflies were dissected and examined for the presence of parasites at 600× magnification under optical microscope.

All females were stored in isopropanol or kept at 4 °C.

2.7. Field phlebotomine sandflies

Field-captured phlebotomine sandflies were obtained from Antônio João County, in the state of Mato Grosso do Sul, Brazil. Specimens were captured with CDC light traps (Sudia and Chamberlain, 1962) installed in a number of peridomicile and intradomicile areas in three districts: Aldeia Campestre, Aldeia Marangatú and Povoado Campestre. The specimens were then identified in Dourados Regional Entomology Laboratory/SES-MS under stereomicroscopy. The sandfly nomenclature and taxonomy used has been described elsewhere (Galati, 2003).

To investigate natural infection by flagellates, the females were also examined at 400× magnification under a bacteriological microscope. Following this, the insects were immersed in ethanol 70% and sent to the Protozoology Laboratory of the Instituto de Medicina Tropical for *Leishmania* identification by PCR.

2.8. Infection rate

Once dissected, pools of sandflies from the endemic areas that had been identified as the same species were separated, stored in tubes and sent to the laboratory. In order to minimize possible errors and quantify *Leishmania* infections/insect, a minimal infection rate (MR) was estimated using the formula MR = number of positive groups (pools) × 100/number of total insects.

3. Results

3.1. Specificity of mini-exon primers

Specific primers for detection of *Viannia* subgenus have been previously described and were used to amplify a 177 bp fragment (Paiva et al., 2004). Both primers (PG and PV) detected 0.15 pg of *Leishmania* DNA. Fig. 1 shows the expected length of amplified genus fragments: 230 bp for *L.* (*V.*) *braziliensis, L.* (*V.*) *guyanensis* and *L.* (*V.*) *peruviana*, 260 bp for *L.* (*L.*) *amazonensis,* and 360 bp for *L.* (*L.*) *chagasi*. As can be seen, these primers

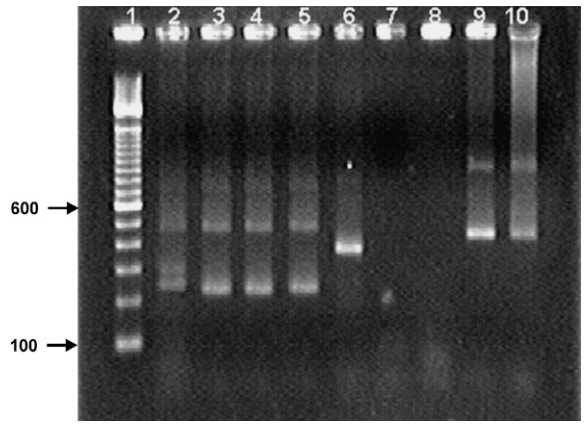

Fig. 1. PCR specificity using PG primers. Electrophoresis on 1.5% agarose gel. 1, MW (100 bp DNA ladder); 2, *L.* (*L.*) *amazonensis* (260 bp); 3, *L.* (*V.*) *braziliensis*; 4, *L.* (*V.*) *guyanensis*; 5, *L.* (*V.*) *peruviana* (230 bp); 6, *L.* (*L.*) *chagasi* (360 bp); 7, *T. cruzi*; 8, *P. falciparum*; 9, *C. fasciculata* (410 bp); 10, *Endotrypanum shaudini* (410 bp). Fragment lengths were calculated using a GDAS 1200 Labworks 4.0 UVP system.

did not amplify DNA fragments of *T. cruzi* or *P. falciparum*. A 410 bp fragment was amplified in the DNAs of the flagellates *C. fasciculata* and *E. shaudini*.

3.2. Phlebotomine sandfly storage

Although the three storage methods showed positive results for amplification of *Leishmania* DNA, storage in isopropanol proved to be the most practical method for carrying out field analysis of phlebotomine sandflies.

3.3. Experimental Leishmania infection—detection by dissection and PCR reactions

3.3.1. Experimental infection of phlebotomine sandflies fed on hamster infected with L. (L.) amazonensis and L. (L.) chagasi

Thirty female phlebotomines were fed on a hamster infected by *L.* (*L.*) *amazonensis*. Four of them were identified as *Lu. almerioi* and 26 as *N. intermedia*. rDNA (S4/S12) primers detected *Leishmania* DNA in only one *N. intermedia* female (Fig. 2) and did not amplify *Leishmania* DNA from any of the *Lu. longipalpis* ($n = 14$) females that fed on infected hamster with *L.* (*L.*) *chagasi* infection.

PG and PV primers were used, and the results were negative for insects that had been fed on hamster infected with both *L.* (*L.*) *amazonensis* and *L.* (*L.*) *chagasi* parasites. No flagellate forms were seen in microscopic observation, thus confirming PCR results.

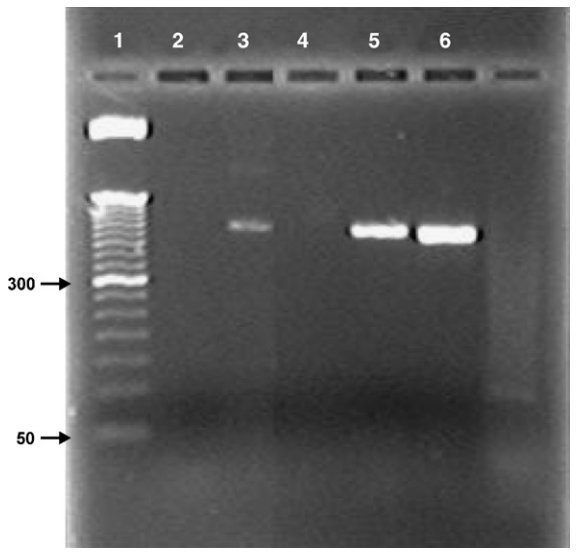

Fig. 2. PCR using S4/S12 primers with phlebotomines sandflies that fed on infected hamster with *L.* (*L.*) *amazonensis*. 1.5% agarose gel. 1, MW (50 bp DNA ladder); 2, *N. intermedia* (sample 1); 3, *N. intermedia* (sample 2); 4, negative control; 5, positive control *L.* (*V.*) *braziliensis*; 6, positive control *L.* (*L.*) *amazonensis*. Fragment lengths were calculated using a GDAS 1200 Labworks 4.0 UVP system.

Table 1
Identification under optical microscope of flagellates in phlebotomine sandflies (*Lu. longipalpis*) experimentally infected with promastigote and amastigote forms of *L.* (*V.*) *braziliensis*

	Promastigotes	Amastigotes
Number of insects	12	20
Positive	9	7
Positive (%)	75	35

3.3.2. Artificially infected phlebotomine sandflies

All the females belonging to *Lu. longipalpis* were fed artificially on membrane containing promastigote or amastigote forms from *L.* (*V.*) *braziliensis* culture. To confirm *Leishmania* infection, 10% of these females (12 of those fed on promastigotes and 20 of those fed on amastigotes) were dissected at 600× magnification under optical microscope. Table 1 shows the percentages of insects infected by promastigote and amastigote forms (75% and 35%, respectively).

PG primers detected infections in 44.4% of phlebotomine sandflies infected with the promastigote form and in 45.4% of those infected with the amastigote form, while *Viannia* subgenus primers (PV) were more sensitive ($P < 0.0001$, χ^2-test), detecting infection in 83.3% and 45.4% of phlebotomine sandflies infected with promastigote and amastigote forms, respectively (Table 2). S4/S12 primers detected 88.8% of the insects infected with the promastigote form and 50% of those infected with the amastigote form.

3.4. Field-captured phlebotomine sandflies

The majority of sandflies were identified as *Lu. longipalpis*. Only one specimen from each of the following species was identified: *N. whitmani*, *Evandromyia cortelezzii*, *Evandromyia lenti* and *Brumptomyia avellari*. The presence of the flagellate forms in some of the *Lu. longipalpis* specimens (1.24%) was confirmed by observation at 400× magnification under optical microscope.

Table 2
PCR results with PG and PV primers

Promastigotes	44.4% (8/18)	83.3 (15/18)
Amastigotes	45.4% (10/22)	45.4% (10/22)
Total (%)	45% (18/40)	62.5% (25/40)

Lu. longipalpis experimentally infected with *L.(V.) braziliensis*. Each sample contains an average of 10 phlebotomine sandflies.

Table 3
Field-captured phlebotomine sandflies (Dourados, MS) assayed by PCR with r DNA, PG and PV primers

Samples	rDNA	PG	PV	Insects *n*	Sandfly species	Location where captured
1	neg	neg	neg	11	*Lu. longipalpis*	Povoado Campestre Peri
2	neg	neg	neg	1	*N. whitmani*	Aldeia Campestre Peri
3	neg	neg	neg	1	*Lu. longipalpis*	Aldeia Campestre Intra
4	neg	neg	neg	1	*E. cortelezzi*	Povoado Campestre Intra
5	neg	neg	neg	1	*E. lenti*	Aldeia Campestre Peri
6	neg	neg	neg	1	*B. avelori*	Aldeia Campestre Intra
7	pos	neg	neg	3	*Lu. longipalpis*	Aldeia Campestre Peri
8	pos	pos[a]	neg	12	*Lu. longipalpis*	Povoado Campestre Peri
9	pos	pos[b]	neg	1	*Lu. longipalpis*	Povoado Campestre Intra
10	pos	pos[b]	neg	3	*Lu. longipalpis*	Aldeia Marangatú Peri
11a	pos	neg	neg	17	*Lu. longipalpis*	Povoado Campestre Peri
11b	pos	neg	neg	16	*Lu. longipalpis*	Povoado Campestre Peri
12	pos	neg	neg	2	*Lu. longipalpis*	Povoado Campestre Peri
13	pos	neg	neg	11	*Lu. longipalpis*	Povoado Campestre Peri
M.R.	10.4%	3.9%	0%			

The presence of the flagellates was detected in 1.24% (1/81) of these insects by dissection under optical microscope. M.R. = positive group number × 100/total insect number. neg: negative, pos: positive.
[a] Fragment length: 260 bp.
[b] Fragment length: 360 bp.

Trypanosomatid infection was detected by S4/S12 primers in 10.4% of *Lu. longipalpis* females, while PG primers detected infection in 3.9% of them (Table 3) by amplifying a 360 bp fragment (two samples) and a 260 bp fragment (one sample), corresponding to *L.* (*L.*) *chagasi* and *L.* (*L.*) *amazonensis*, respectively (Fig. 3). All samples were negative for *Viannia* subgenus when PV primers were used.

4. Discussion

As already described in several papers, leishmaniasis is a diverse and complex disease caused by many species of *Leishmania* (Bryceson, 1996). Correct identification of the parasite species in the vectors is therefore crucial for epidemiological studies and control measures. Identification of the parasites in the vector is frequently based on morphology, the localization of flagellate forms in the digestive tract or their growth in the in vitro culture.

It is clear that molecular techniques are more sensitive and have greater specificity than the dissection method. Polymerase chain reaction (PCR) methods are valuable in the identification of leishmaniasis parasites isolated from patients (Degrave et al., 1994).

Several PCR methodologies have been used to determine the infectivity of phlebotomine sandflies (Cabrera et al., 2002), but in general the diagnosis is laborious since a second PCR is required to identify the *Leishmania* species or the amplified fragments need to be sequenced. Diagnosis based on ITS and k DNA

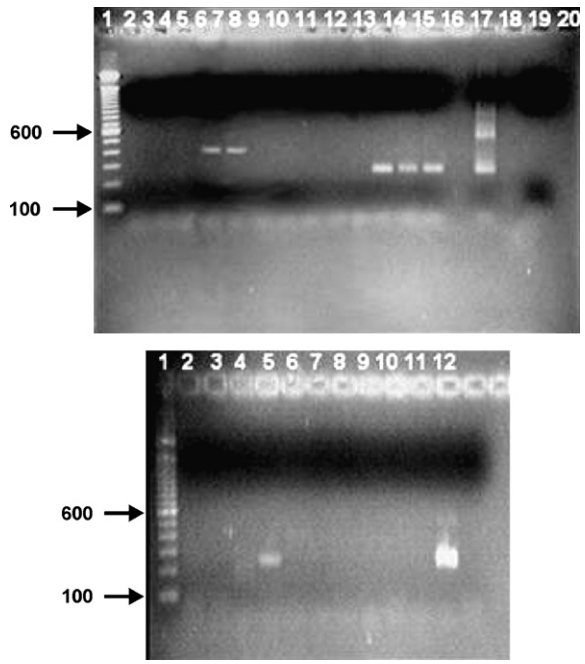

Fig. 3. PCR with PG primers using field-captured phlebotomine sandflies. Electrophoresis on 1.5% agarose gel: (**1**) 1, MW (100 bp DNA ladder); 2–9, field-captured phlebotomine sandflies; 17, *L.* (*V.*) *brasiliensis*; 19, negative control. (**2**) 1, MW (100 bp DNA ladder); 2–11, field-captured phlebotomine sandflies; 12, *L.* (*L.*) *amazonensis.* Fragment length was calculated using a GDAS 1200 Labworks 4.0 UVP system.

sequences followed by sequencing recently identified *P. papatasi* infected by *Leishmania* (*L.*) *major* in a tegumentary leishmaniasis focus in Iran (Parvisi et al., 2005).

This study describes a PCR method using primers inferred from mini-exon sequences to determine natural infection of phlebotomine sandfly species by *Leishmania* parasites. In order to increase the effectiveness of our fieldwork, storage of insects was standardized using three methods, all of which proved successful. For practical reasons, however, immersing captured phlebotomine sandflies in isopropanol proved to be the most suitable method.

To develop a PCR reaction, mini-exon sequences were used to design primers for *Viannia* subgenus (PV) (Paiva et al., 2004) and species of *Leishmania* parasites (PG). These primers amplify fragments of different lengths. The variation in mini-exon nontranscribed spacers allows them to be used to distinguish *Kinetoplastida* species (Marfurt et al., 2003). The primers were first assayed for their specificity and sensitivity (Paiva et al., 2004) and then tested on experimentally infected insects.

PG and PV primers were able to detect a minimum of 1.5 *Leishmania* parasites (Paiva et al., 2004), and similar results with the same sequences were shown by Harris et al. (1998) and Marfurt et al. (2003). PG primers were also able to identify species of *Leishmania* and separate other flagellates such as *Endotrypanum* sp. and *Crithidia* sp., which are morphologically undistinguishable under optical microscope, by the length of their fragments (Fernandes et al., 1993; Katakura et al., 2003). All fragments had their sequences compared and confirmed with others in Genebank.

Many experimental phlebotomine sandfly infections are carried out in infected animals as they are closer to natural conditions (Lainson et al., 1979). Infectivity rates, however, are low and may result in a false negative reaction (Tesh and Modi, 1984). In view of this, we chose to infect phlebotomine sandflies with an artificial feeder. This method proved to be both practical and efficient for experimental infection, allowing insects to be fed with both promastigote and amastigote forms.

PG primers were found to be less sensitive in detecting promastigotes than were PV primers. This difference could be due to the condition of the DNA, as the former require amplification of long fragments. The use of both PG and PV primers, however, could provide a more specific diagnosis, as they confirmed *L.* (*V.*) *braziliensis* infections in this study.

The low positivity of *L.* (*V.*) *braziliensis* amastigote infections detected was expected, because it is known that *Lu. longipalpis* is not the main vector of this *Leishmania* species.

Epidemiological studies depend on the correct identification of both the phlebotomine sandfly species and the infecting agent (Michalsky et al., 2002). This is usually done by dissection and microscopic observation of the digestive tract of the phlebotomine sandfly, although we believe that the infection rate based on this method is underestimated. Some authors have reported infection rates in several endemic areas of Brazil ranging from 0.8% to 0.06% (Azevedo and Rangel, 1991; Queiroz et al., 1994; Ryan et al., 1990; Miranda et al., 2002; Casanova et al., 1995; Rangel et al., 1985; Luz et al., 2000; Silva and Grunewald, 1999). The percentages of naturally infected phlebotomine sandflies in areas where tegumentary leishmaniasis is the predominant form of the infection were 0.47% for *N. whitmani*, 0.06% for *Migonemyia migonei* (Queiroz et al., 1994), 0.6% for *Pintomyia pessoai*, 0.8% for *Pintomyia misionensis* (Silva and Grunewald, 1999) and 0.24% for *Nyssomyia neivai* (Casanova et al., 1995).

Similar infection rates were observed in *Lu. longipalpis* captured in areas of visceral leishmaniasis in Colombia (0.59%) (Corredor et al., 1989) and Venezuela (0.28%) (Feliciangeli et al., 1999). The corresponding figures for Brazil ranged from 0.25% to 7.14% (Sherlock, 1996; Santos et al., 1998; Lainson et al., 1985).

In the present study, 81 phlebotomine sandflies captured in endemic indigenous areas of Mato Grosso do Sul where cases of leishmaniasis have been reported since 1998 and dogs have a suggestive visceral disease profile were assayed using PCR methods with PV and PG primers to determine the leishmaniasis infection rate.

Using the dissection method, we were able to detect flagellate infections in 1.24% of these insects. Although rDNA primers were more sensitive and able to detect infection by Trypanosomatidae (10.4%), PG and PV primers were able to identify the species of the etiologic agent, confirming an infection rate with *L.* (*L.*) *chagasi* and *L.* (*L.*) *amazonensis* of 3.9%. Several authors have also described higher PCR rates than those detected by dissection (Miranda et al., 2002; Rodriguez et al., 1999).

Diagnosis based on PCR is also important to determine the vectorial capacity in areas where many species of phlebotomine sandflies coexist. In India, PCR using mini-exon regions identified *Leishmania* (*L.*) *donovani* infections in *P. argentipes*, *P. papatasi* and *Sergentomyia babu* (Mukherjee et al., 1997). In Peru, *Leishmania* (*Viannia*) was only found in *Lutzomyia ayacuchensis*, although *Lu. noguchii* was present in a sympatric form (Caceres et al., 2004). *Lu. ayacuchensis* was also identified as the vector of *L.* (*L.*) *mexicana* in some regions of Ecuador (Kato et al., 2005).

In Brazil, the main vector of *L.* (*L.*) *chagasi* is *Lu. longipalpis* (Lainson et al., 1985; Sherlock, 1994), except in Mato Grosso do Sul, where *L. cruzi* may also be a visceral leishmaniasis vector (Santos et al., 1998). The finding of *L.* (*L.*) *chagasi* infections in *Lu. longipalpis* captured in a visceral leishmaniasis focus in Mato Grosso do Sul confirmed previous reports of their vectorial capacity.

The presence of *L.* (*L.*) *amazonensis* infection in only one pool of insects captured in these areas needs to be confirmed by increasing the number of captured insects. Similar results, however, were confirmed in other areas of Mato Grosso do Sul by Savani, E. (2004) (personal communication) using rDNA primers.

In experimental infections, *Lu. longipalpis* can be infected by *L.* (*L.*) *chagasi*, *L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis* (Da Silva et al., 1990). *L.* (*L.*) *amazonensis* has also been shown to be transmitted by *Lu. longipalpis* bites after blood-feeding on infected hamsters (Sherlock, 1996).

The other sandfly species were negative for the presence of *Leishmania* parasites. This could be attributed to the reduced number of insects available for analysis in each species or to the fact that these insects cannot be incriminated as vectors for the species of *Leishmania* found in this region.

We have standardized PV and PG primers and shown their effectiveness in detecting infection in experimentally infected or field-captured phlebotomine sandflies. The fact that this PCR methodology can identify *Leishmania* species and their sandfly vectors makes it a useful tool for field studies and an invaluable aid in epidemiological investigations.

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