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SHORT COMMUNICATION

Molecular biological identification of monoxenous trypanosomatids and *Leishmania* from antropophilic sand flies (Diptera: Psychodidae) in Southeast Brazil

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The application of new molecular techniques to the genomic study of leishmanial parasites will have a substantial impact on our understanding of the dynamics of specific transmission cycles of clinically important *Leishmania* species (such as *Leishmania braziliensis* and *Leishmania infantum*), particularly in the various regions of Brazil where they are endemic. In this study, we used a polymerase chain reaction (PCR) technique targeting the conserved region of minicircle kDNA, aiming to detect *Leishmania* DNA in a sample of 430 female sand flies identified as *Lutzomyia intermedia* (171), *Lutzomyia whitmani* (115), *Lutzomyia fischeri* (76), *Lutzomyia monticola* (31), *Lutzomyia migonei* (15), *Lutzomyia firmatoi* (8), *Lutzomyia pessoai* (8) and *Lutzomyia salesi* (6). Sand flies were collected in peridomiciliary dependencies within an endemic area of *L. braziliensis* cutaneous leishmaniasis in Southeast Brazil (Afonso Cláudio, Espírito Santo). Of the sand fly species examined, 18 of 76 *L. fischeri* were found to be naturally infected. After sequence analysis, *Leishmania* and monoxenous lower trypanosomatid parasites were determined to be associated with the insect infections. The results indicate that a positive PCR is not sufficient in itself to indicate *Leishmania* infection and that the false detection of leishmanial infection within suspected vectors of human leishmaniasis would overestimate the true transmission rate of this parasite.

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More than 350 different phlebotomine sand fly species (Diptera: Psychodidae) from the Americas are known, but less than 40 of these have been implicated as proven or suspected vectors of human leishmaniasis (Grimaldi and Tesh 1993). Hence, monitoring natural *Leishmania* infections in sand fly populations is an important epidemiological parameter for controlling transmission, the estimation of which depends on the reliable identification of infected sand flies. False detection of leishmanial infection within suspected vectors of human leishmaniasis, however, will overestimate the true epidemiologic risk of parasite transmission.

Several studies on natural infections of sand fly vectors with *Leishmania* and other trypanosomatids have been reported (Wallace and Hertig 1968; Arias et al. 1985; Naiff et al. 1989). Because direct methods to detect parasites in sand flies are limited, polymerase chain reaction (PCR)-based assays have been used for detecting *Leishmania*-infected sand fly vectors in many regions where the disease is endemic (Paiva et al. 2006; Kato et al. 2007; Savani et al. 2009). The reported performances of these tests, however, indicate that some probes appear limited in distinguishing between *Leishmania* and other trypanosomatids (Degrave et al. 1994). Further application of a combination of molecular approaches (such as RFLP, DNA hybridisation and sequencing) should help identify parasitic species in these microorganisms.

Cutaneous leishmaniasis caused by *Leishmania braziliensis* is widely found in the state of Espírito Santo, Brazil, and constitutes an important health problem (Falqueto et al. 2003). The transmission cycle of *L. braziliensis* occurs in areas situated at medium and lower elevations (50–750 m above sea level); the maintenance cycle seems to involve domestic animal reservoirs (canine and equine) and sand flies with peridomiciliary habitats *(Lutzomyia intermedia*, *Lutzomyia whitmani, Lutzomyia migonei* and *Lutzomyia fischeri)* (Ferreira et al. 2001). Here, we extend these studies, attempting to correlate the presence of infected sand flies with the occurrence of cutaneous leishmaniasis caused by *L. braziliensis* in humans. Between 2006 and 2008, a search for suspected vectors was conducted in an endemic rural area located in the municipality of Afonso Cláudio, Espírito Santo. Sand flies were collected periodically in the peridomiciliary dependencies in the study area situated at 20°16′08″S and 41°03′01″W. A sample of 430 female sand flies was morphologically identified using the criteria proposed by Young and Duncan (1994) and was composed of *L. intermedia* (171), *L. whitmani* (115), *L. migonei* (15), *L. fischeri* (76), *Lutzomyia monticola* (31), *Lutzomyia firmatoi* (8), *Lutzomyia pessoai* (8) and *Lutzomyia salesi* (6).

DNA from all the insects that were collected was extracted individually using the Wizard® SV Genomic DNA Purification System Kit (Promega, Madison, USA) and resuspended in 20 μL of hydration solution. Insect pools (n=10) were made by admixing 2 μL of individually extracted DNA, totalling 20 μL for each pool. PCR reactions targeting the conserved region of the minicircle kDNA (mkDNA) were performed using 3 μL of each pool, using the primers Forward: 5′-GGGGAGGGGCGTTCTGCGAA-3′ and Reverse: 5′-GGCCCACTATATTACACCAACCCC-3 (Michalsky et al 2007) in a final reaction volume of 20 μL. When an insect pool tested positive using PCR, each individual specimen of that pool was submitted to mkDNA PCR to evaluate the individual infection status using 2 μL of individual DNA in a final PCR reaction volume of 20 μL (Rocha et al. 2010). Negative pools were submitted to cytochrome-b PCR using the protocol described by Ishikawa et al. (1999), with minor modifications, to evaluate the quality of the extracted DNA. Male sand flies were used as negative controls for the extraction.

A 10-μL aliquot of the PCR reaction targeting the minicircle conserved region, performed with either the insect pools or individual specimens, was submitted to electrophoresis on a 6% polyacrylamide gel stained with ethidium bromide. All individual PCR reactions that produced a fragment of ~120 bp were digested using the restriction enzyme *Hae*III (Volpini et al. 2004), which was based upon previous studies that indicated the ability of this PCR-RFLP approach to distinguish between *L. braziliensis*, *L. amazonensis* and *L. chagasi* (Volpini et al. 2004; de Andrade et al. 2006). The patterns obtained were compared with those observed for the *Leishmania* reference strains, IOC/L566 (MHOM/BR/1975/M2903) *L. braziliensis*, IOC/L579 (MHOM/BR1974/PP75) *L. infantum* and IOC/L575 (IFLA/BR/1967/PH8) *L. amazonensis*.

Of the 76 *Lu. fischeri* females, 18 were PCR-positive, with one specimen yielding two fragments of ~80 and ~150 bp and the other 17 yielding the ~120-bp fragment. The latter 17 specimens were submitted to RFLP. Among those 17, one had a pattern similar to *L. braziliensis* and the other 16 had a pattern similar to *L. amazonensis*, according to previous studies (Volpini et al. 2004; de Andrade et al. 2006).

To confirm the identification of the parasites in the sand fly infections, the ~120-bp PCR fragments were purified from a 2.5% agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and sequenced using the BigDyeTM Terminator v3.0 Cycle Sequencing kit, using the BigDye reagent (1:4 dilution) in a final volume of 10 μL with 20–60 ng of the purified PCR products and 3.2 pmol of the forward and reverse primers. The purified products were sequenced in duplicate for each primer.

The sequences obtained (90 bp) were used in a global BLAST search of the NCBI database. The complete sequence of the specimens that produced a RFLP pattern compatible with *L. braziliensis* had 91% (4e-20) similarity to the deposited sequence of *L. braziliensis* minicircle kDNA (EU370880.1). The specimens that had PCR-RFLP patterns comparable with *L. (Leishmania)* produced polymorphic sequences 88% to 100% similar to *Herpetomonas samuelpessoai*. The sequence obtained in this study were deposited in the GenBank database under the accession number GU248530.

These results led us to test the specificity of the primers using five monoxenous trypanosomatid species (Santos et al. 2007): *Crithidia fasciculata* (CT-IOC 048 ATCC 11745), *Blastocrithidia culicis* (CT-IOC 041 ATCC

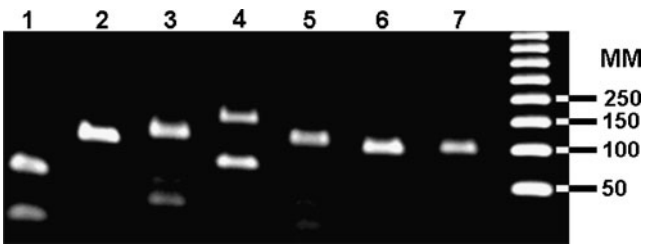

Fig. 1 Six percent acrylamide gel electrophoresis of non-digested (*lane 4*) and *Hae*III-digested (*lanes 1–3* and *5–7*) mkDNA-PCR products from different trypanosomatids. *1 Leishmania braziliensis, 2 L. amazonensis, 3 L. infantum*, *4 Blastocrithidia culicis*, *5 Crithidia fasciculata*, *6 Phytomonas serpens* and *7 Phytomonas* spp., *MM* molecular marker, 50-bp ladder

30257), *Herpetomonas muscarum muscarum* (CT-IOC 020 ATCC 30260), *Phytomonas serpens* (isolate 9 T, CT-IOC 189) and *Phytomonas sp*. (CT-IOC 083). Except for *Blastocrithidia* and *H. muscarum muscarum*, all of the samples tested showed a PCR product of ~120 bp. All PCR reactions showing the ~120 bp fragment were further characterised using PCR-RFLP using *Hae*III. After *Hae*III digestion, these monoxenous trypanosomatids had patterns similar to *L. amazonensis* (Fig. 1), i.e., there was no digestion by this enzyme (Volpini et al. 2004).

B. culicis showed two fragments after the PCR amplification: ~80 and ~150 bp (Fig. 1). The same pattern was seen in one of the sand fly-derived DNA samples, suggesting that this insect was likely infected by *Blastocrithidia* spp. Moreover, *H. muscarum muscarum* also showed two PCR fragments: ~20 and ~100 bp (data not shown).

For the first time, we reveal an association of *H. samuelpessoai* and *Blastocrithidia* spp. with *L. fischeri* using a combination of molecular approaches to discriminate between these microorganisms. Previous studies have reported *Lutzomyia* species infected with trypanosomatids other than *Leishmania,* including *Trypanosoma*, *Endotrypanum* spp., *Crithidia* spp., and *Leptomonas* spp. (Wallace and Hertig 1968; Arias et al. 1985; Naiff et al. 1989). The high index (16/76; 21%) of *H. samuelpessoai* infection associated with *L. fischeri* is probably related to the mechanism of transmission of monoxenous trypanosomatids. In most case, insects become infected by trypanosomatids through the ingestion of the organism, either directly or indirectly via faeces, animal blood, or plant sap (Wallace 1966). The flagellates may be carried throughout multiple insect stages, from larva to pupa to adult (Clark et al. 1964).

In conclusion, the results presented in this study reinforce the importance of knowing the species specificity of the primers employed in PCR approaches. Although PCR amplification of the conserved region of the minicircle kDNA (Degrave et al. 1994) is a highly sensitive tool for detecting *Leishmania*-infected sand flies (de Pita-Pereira et al. 2005; Carvalho et al. 2008), our findings question the specificity of this molecular marker. In general, PCR assays targeting the conserved region of kDNA minicircles for the diagnosis of *Leishmania* are complemented by other molecular approaches to confirm the etiological identification (de Pita-Pereira et al. 2005; Miranda et al. 2002). In this study, however, PCR-RFLP was not able to differentiate some *Leishmania* species from other trypanosomatids. Finally, a positive PCR result is not sufficient in itself to indicate *Leishmania* infection, demonstrating that taking the similarities among *Leishmania* and monoxenous trypanosomatids (Fernandes et al. 1993) into consideration is critical when interpreting such results. In addition to phlebotomines, the natural infection of other ectoparasites, such as mites and fleas (Coutinho et al. 2005; Coutinho and Linardi 2007), requires special attention during assay interpretation.

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