Natural *Leishmania* sp. reservoirs and phlebotomine sandfly food source identification in Ibitipoca State Park, Minas Gerais, Brazil

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Leishmania *spp are distributed throughout the world and different species are associated with varying degrees of disease severity. However, leishmaniasis is thought to be confined to areas of the world where its insect vectors, sandflies, are present. Phlebotomine sandflies obtain blood meals from a variety of wild and domestic animals and sometimes from humans. These vectors transmit* Leishmania *spp, the aetiological agent of leishmaniasis. Identification of sandfly blood meals has generally been performed using serological methods, although a few studies have used molecular procedures in artificially fed insects. In this study, cytochrome b gene (*cytB*) polymerase chain reaction (PCR) was performed in DNA samples isolated from 38 engorged* Psychodopygus lloydi *and the expected 359 bp fragment was identified from all of the samples. The amplified product was digested using restriction enzymes and analysed for restriction fragment length polymorphisms (RFLPs). We identified food sources for 23 females; 34.8% yielded a primate-specific banding profile and 26.1% and 39.1% showed banding patterns specific to birds or mixed restriction profiles (rodent/marsupial, human/bird, rodent/marsupial/human), respectively. The food sources of 15 flies could not be identified. Two female* P. lloydi *were determined to be infected by* Leishmania *using internal transcribed spacer 1 and heat shock protein 70 kDa PCR-RFLP. The two female sandflies, both of which fed on rodents/marsupials, were further characterised as infected with* Leishmania (Viannia) braziliensis. *These results constitute an important step towards applying methodologies based on* cytB *amplification as a tool for identifying the food sources of female sandflies.*

Key words: phlebotomines - food source identification - *cytB* PCR - *Leishmania* natural infection

Sandflies are natural vectors of various pathogens, including *Leishmania* spp. These insects are distributed throughout most regions of the world and present significant biodiversity in the neotropics, where their density is dependent on weather conditions.

Assessment of the presence of *Leishmania* in different sandfly species is critical to understanding the eco-epidemiology of leishmaniasis. Some sandfly species feed exclusively on specific vertebrates; however, others are opportunistic and have been shown to feed on various hosts, including species that can serve as *Leishmania* reservoirs (Tesh et al. 1971, Christensen et al. 1982, Marassá et al. 2006).

The genus *Psychodopygus* Mangabeira, 1941 includes species of medical and veterinary importance, several of which are involved in the transmission of *Leishmania braziliensis* or have been reported to show anthropophilic behaviours (Ward 1977, Lainson & Shaw 1979, Gil et al. 2003). This genus is restricted to jungle areas and, with few exceptions, is rarely associated with humans (Carvalho et al. 2006). In a study conducted in the Parque do Sabiá complex, municipality of Uberlândia, state of Minas Gerais (MG), Brazil, *Psychodopygus davisi* (Root, 1934) was predominant in urban areas (Rodrigues et al. 2011). Souza et al. (2009) have also reported the presence of *P. davisi* and *Psychodopygus ayrozai* in peridomiciles in areas of autochthonous cases of canine visceral leishmaniasis and American cutaneous leishmaniasis in Angra dos Reis, state of Rio de Janeiro (RJ), Brazil.

Identification of an animal reservoir in a vector-borne disease transmission cycle is critical for the establishment of an efficient control strategy. Furthermore, identification of the blood meals of haematophagous insects provides information on host-feeding preferences or host-feeding patterns under natural conditions, which may provide indirect data regarding reservoir potential (Haouas et al. 2007). Furthermore, blood meal studies have contributed to our understanding of the life cycles of both vectors and transmitted pathogens and to the establishment of control strategies for these arthropods.

The study described herein evaluated bio-ecological features associated with female sandfly food sources and the detection of *Leishmania* sp. in sandflies collected at Ibitipoca State Park, in MG.

MATERIALS AND METHODS

Study area - Ibitipoca State Park is the fourth most visited protected area in Brazil and the most visited area in MG. It is located between the coordinates 21º40' 21º43'S and 43º52' 43º54'W in southeastern Brazil, with altitudes ranging from 1.050-1.784 m above sea level (Rodela 1998, Neto et al. 2007). The climate is classified as humid mesothermal, with dry winters and mild summers.

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The park covers an area of 1.488 ha. It represents an area of important geological (characterised by quartzite cave formations) and biological heritage and serves as a home to endangered native flora and fauna. The average annual temperature in the region ranges from 18-20ºC, with minima and maxima ranging from 21.5-36ºC in summer and 2-14.5ºC in winter (Dias et al. 2002).

Fly collection methods - Sandflies were collected monthly at a small farm equipped with a chicken coop, cattle pen and pigsty located at the boundary of the state park with a pasture area bordering a small forest and in Ibitipoca State Park using light traps (model HP, HP Biomédica, Sabará, MG, Brazil) (Carvalho et al. 2011). Females were identified by transferring the guts of the sandflies to a drop of buffered saline on a microscope slide using a pair of mounted entomological pins. A coverslip was then placed over the drop and the thorax, spermathecae and cibarium were examined under an optical microscope. We identified sandfly species according to morphological characters using the taxonomic key of Galati (2003).

A license for the collection of biological material was obtained from the State Institute of Forestry (169/08) and the Brazilian Institute of Environment and Renewable Natural Resources (15237-1). Samples from the farm were collected after a consent form was signed by the owner.

Identification of female sandfly food sources - Engorged female sandflies were killed by freezing to completely arrest the digestion process and the insects were subsequently placed in 6% dimethyl sulfoxide and stored at -70ºC until use. The identification of female food sources was performed via polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the cytochrome b gene (*cytB*), which is present in different vertebrate hosts.

DNA was extracted from captured engorged female flies using the Gentra Puregene Tissue kit (Qiagen, Valencia, CA). A 359-bp fragment of the *cytB* gene was amplified using previously described primers (Steuber et al. 2005). The primers used were the *cytB* forward primer, 5'-CCATCCAACATCTCAGCATGATGAAA-3' and the *cytB* reverse primer, 5'-GCCCCTCAGAATGATATTTGTCCTCA-3'. Amplification reactions were prepared in 1x buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 mM forward and reverse primers, 1 U Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 5 µL of DNA template in a final volume of 50 µL. PCR was performed in an automated thermal cycler with the following cycling conditions: 35 cycles of 95ºC for 30 s, 58ºC for 30 s and 72ºC for 1 min, followed by a final extension step at 72ºC for 6 min. DNA samples extracted from the blood of different vertebrate hosts provided by the Leishmaniasis Laboratory of the René Rachou Institute were used as positive controls for all PCR reactions.

Samples from which the specific 359-bp product was amplified were subjected to RFLP analysis via digestion with various enzymes (*Taq*I, *Hae*III and *Mwo*I). Digestion reactions were prepared in a final volume of 15 µL containing 1 U of enzyme, 1x enzyme buffer and 12.5 µL of PCR product. The mixtures were incubated at the appropriate temperature for the respective enzyme for 2 h. Restriction patterns were analysed after separation of the obtained fragments by electrophoresis in a 2% agarose gel and were compared with the digestion patterns of PCR products from the DNA extracted from the blood of different vertebrate hosts. The PCR-RFLP patterns were visualised by staining the gels with ethidium bromide.

Verification of natural Leishmania infection - Detection of *Leishmania* in the female flies was performed by ITS1 (internal transcribed spacer 1) and heat shock protein 70 kDa (*hsp70*) PCR-RFLP (Schönian et al. 2003, Garcia et al. 2004). The 350-bp ITS1 fragment was amplified using the primers LITSR 5'-CTGGATCATTTTCCGATG-3´ and L5.8S 5'-TGATACCACTTATCGCACTT-3'. The primers HSP70 forward 5'-GACGGTGCCTGCCTACTTCAA-3' and reverse 5'-CCGCCCATGCTCTGGTACATC-3' were used to extend a 1,300-bp region of the *hsp70* gene. RFLP analysis was subsequently performed with *Hae*III by comparing the obtained restriction profiles to patterns obtained using PCR products from the following reference strains: *Leishmania amazonensis* (FLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903), *Leishmania infantum* (MHOM/BR/74/PP75) and *Leishmania guyanensis* (MHOM/BR/75/M4147).

RESULTS

PCR amplification of *cytB* was performed using DNA extracted from 38 engorged *Psychodopygus lloydi* females. The expected 359 bp *cytB* PCR product was obtained for all of the samples tested. Negative controls consisting of the reagent mixture amplified in parallel with the samples were free of amplicons (Fig. 1). RFLP was performed by digesting the obtained PCR products with *Taq*I and *Mwo*I, which produced the expected banding pattern associated with each of the following vertebrate hosts: marsupials, rodents, chickens, cattle and humans. We were unable to conclusively identify the blood meal source of the females using the enzymes *Taq*I and *Mwo*I (Fig. 2).

The PCR-RFLP profiles of *cytB* digested with the *Hae*III endonuclease were indistinguishable from the

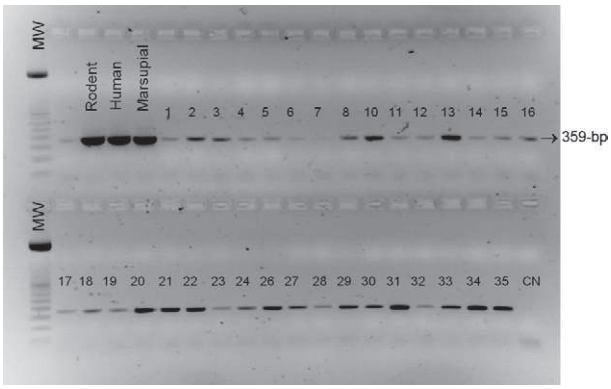

Fig. 1: cytochrome b gene polymerase chain reaction of DNA samples isolated from 38 engorged *Psychodopygus lloydi*. Molecular weigh (MW): 100-bp; 35: DNA samples taken from engorged flies collected from Ibitipoca State Park, state of Mina Gerais, Brazil.

pattern observed for human *cytB* obtained from eight *P. lloydi* females (34.8%) and avian *cytB* from six *P. lloydi* females (26.1%). Additionally, digestion with *Hae*III resulted in characteristic banding patterns associated with more than one vertebrate host (mixed profile) (Fig. 3). Mixed profiles were observed in nine samples (39.1%), suggesting that these females may have fed on multiple hosts. The mixed profiles corresponded to rodent/marsupial (n = 4), human/chicken (n = 2) and rodent/marsupial/human (n = 1) combinations as well as other combinations with various undefined host banding patterns (n = 2). We were unable to identify the food sources of 15 engorged female flies.

The *hsp70* and ITS1 PCR-RFLP analyses performed using DNA samples isolated from two female *P. lloydi* were positive for *L.* (*V.*) *braziliensis* (Fig. 4). The analysis of the food sources of these females showed that both had fed on rodents and/or marsupials.

DISCUSSION

In Brazil, leishmaniasis is characterised by complex and specific epidemiological profiles for each transmission focus. There are several species of *Leishmania* with the potential to cause different clinical manifestations that can be transmitted by various vectors and they are maintained by various vertebrate reservoirs. Therefore, it has become increasingly important to study the association between vectors and vertebrate hosts as a means of improving disease control and intervention strategies.

PCR-RFLP is a useful molecular tool that can be employed for the detection and identification of *Leishmania* sp. in vertebrate host samples or phlebotomine vectors (Minodier et al. 1997, Muller et al. 2003, Pita-Pereira et al. 2005, Paiva et al. 2007). The main advantages of this method are its simplicity and practicality and that it provides accurate identification of blood sources. Moreover, the effectiveness of this technique is independent of the stage of an infection and its location within the insect's digestive tract (Perez et al. 1994). Haematophagous dipterans feed on blood, which is necessary for ovum development and egg production is directly proportional to the quantity of blood ingested (Ready 1979, Lehane 2005). Several factors stimulate sandfly feeding behaviours and, similar to other haematophagous insects searching for an appropriate host, are mediated by characteristics intrinsic to these insects and by environmental factors (Lehane 2005). Stimuli that attract sandflies include temperature and body odour (Brazil & Brazil 2003).

With respect to sandfly food preferences, some species of Neotropical sandflies can feed on a variety of warm-blooded animals. Many species of phlebotomines captured using human bait also feed on other vertebrates. *Lutzomyia longipalpis* (Lutz & Neiva 1912) provides the best known example of food source eclecticism, as this species exhibits the ability to feed on humans, dogs, birds and other animals (Oliveira et al. 2008). Other species with similar feeding habits include *Nyssomyia intermedia* (Lutz & Neiva 1912), *Migonemyia migonei* (França 1920) and *Pintomyia fischeri* (Pinto 1926), which in anthropic environments, have been shown to adapt to animal shelters and to invade homes to feed on humans (Brazil et al. 1991, Gomes et al. 1995, Andrade Filho et al. 2007).

In the present study, blood meal identification in phlebotomine females was performed using *cytB* PCR-RFLP. This method was employed due to its practicality and rapidity. However, PCR-RFLP results are occasionally inconclusive due to poor amplification of *cytB* products, the presence of very similar banding profiles between different host species or incomplete digestion. Furthermore, the specificity of this type of assay is sometimes low, especially with respect to identifying groups of closely related vertebrates, such as primates. It is im-

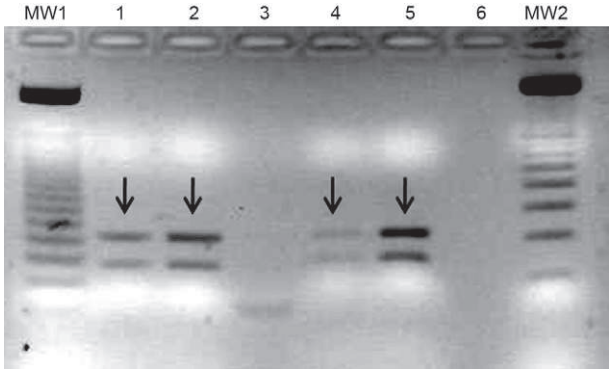

Fig. 2: polymerase chain reaction-restriction fragment length polymorphism using *Taq*I digestion of cytochrome b gene (*cyt*B) amplified from DNA isolated from six engorged *Psychodopygus lloydi*. Molecular weigh (MW)1: 100-bp; 2: 50-bp; 1-6: DNA samples taken from engorged flies captured in Ibitipoca State Park, state of Minas Gerais, Brazil. The arrows represent the rodent, marsupial or cattle *cyt*B restriction profiles.

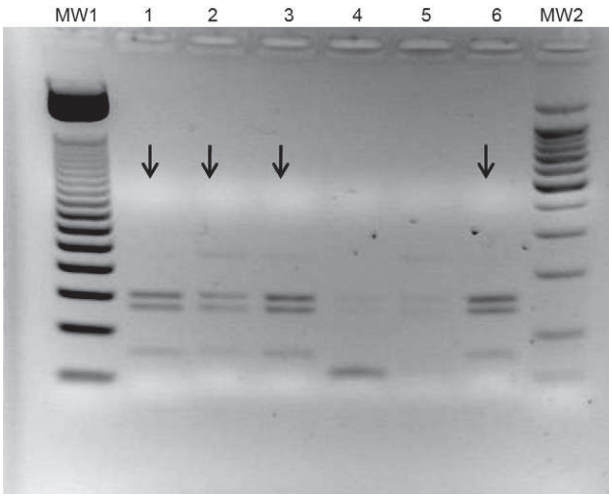

Fig. 3: polymerase chain reaction-restriction fragment length polymorphism using *Hae*III digestion of cytochrome b gene amplified DNA from six engorged *Psychodopygus lloydi*. Molecular weigh (MW)1: 50-bp; 2: 100-bp; 1-6: DNA samples taken from engorged flies captured in Ibitipoca State Park, state of Minas Gerais, Brazil. Arrows represent the mixed restriction profile containing bands representing various hosts (chicken or chicken/human).

portant to clarify that although certain blood meals were identified as human in this study, they could have been from another primate. Because the primers used are not specific to human *cytB*, the banding patterns could not be conclusively determined to be human, although this is most likely the case. In such instances, it is advisable to use a more accurate method, such as sequencing of *cytB*.

In this study, the food source could not be identified for 15 sandflies. These samples could be further subjected to DNA sequence analysis, which can distinguish a vertebrate species blood source. However, the amount of available extracted DNA was not sufficient for these experiments. Despite the inability to achieve species identification, it is important to identify the vertebrate groups on which the phlebotomines fed. This information can aid in better describing the epidemiological context of a leishmaniasis transmission focus.

The observation that eight *P. lloydi* females fed on humans and presented mixed feeding profiles (human/chicken or rodent/marsupial/human), combined with the finding that these flies were naturally infected with *L. braziliensis,* demonstrates that this species exhibits anthropophilic behaviour in addition to the ability to feed on a variety of host species. Although finding *Leishmania*-positive engorged flies is not sufficient to establish this sandfly species as a disease vector, these results suggest that *P. lloydi* may be a potential vector associated with *Leishmania* sp. transmission and may cause human cases of American cutaneous leishmaniasis in this region. However, further studies using non-fed flies are needed to evaluate *Leishmania* infection of *P. lloydi* and its vectorial competence.

Seven species of *Psychodopygus* have been reported in MG, including *P. lloydi* (Andrade & Dantas-Torres 2010).

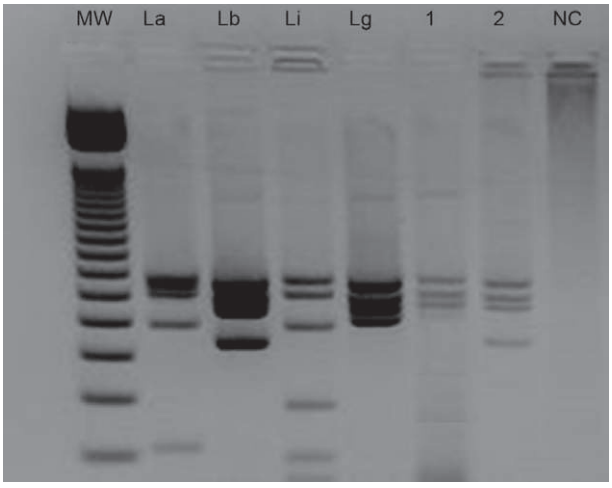

Fig. 4: polymerase chain reaction-restriction fragment length polymorphism using *Hae*III digestion of the heat shock protein 70 kDa (*hsp70*). Molecular weigh (MW): 50-bp; La: strain pattern *Leishmania amazonensis* (IFLA/BR/67/PH8); Lb: strain pattern *Leishmania braziliensis* (MHOM/BR/75/M2903); Lg: strain pattern *Leishmania guyanensis* (MHOM/BR/75/M4147); Li: strain pattern *Leishmania infantum* (MHOM/BR/74/PP75); 1, 2: samples amplified from DNA extracted from *Psychodopygus lloydi* females; NC: negative control.

This species has also been reported in RJ, São Paulo (SP) and Paraná (Andrade Filho et al. 1997, Santos et al. 2007).

A previous study demonstrated that out of four species considered to be potential vectors of *Leishmania* sp. associated with cutaneous leishmaniasis in Itatiaia National Park (Afonso et al. 2007), three belonged to the genus *Psychodopygus,* while in Parque Estadual do Alto Ribeira, SP, *P. ayrozai* (Barretto & Coutinho 1940) and *Psychodopygus geniculatus* (Mangabeira 1941) were the most abundant anthropophilic species identified (Galati et al. 2010). Observations of the sandfly fauna of Além Paraíba, MG, demonstrated infection of *Psychodopygus hirsuta* with a parasite of the *L. braziliensis* complex (Rangel et al. 1985).

Although there have been no reported cases of leishmaniasis in the region near Ibitipoca State Park, two *P. lloydi* specimens were found to be naturally infected with *L. braziliensis and* both were identified as exhibiting a mixed feeding profile (rodent/marsupial). Several studies have demonstrated the potential for rodents and marsupials to serve as *L. braziliensis* reservoirs in Brazil (Brandão-Filho et al. 2003, Schallig et al. 2007). Therefore, the finding that two *P. lloydi* specimens were infected with *L. braziliensis*, combined with a mixed feeding profile (rodent/marsupial), confirms previous observations and validates the methodology used to draw the conclusions made in this study, which increases the reliability of these results.

Only seven species of phlebotomines have been collected in Ibitipoca State Park, with the predominant species being *P. lloydi*, corresponding to 85% of the sandflies captured (Carvalho et al. 2006). The identification of two *P. lloydi* females infected with *L. braziliensis* suggests that this insect maintains *L. braziliensis* infections in a sylvatic cycle in Ibitipoca State Park for the following reasons: (i) both individuals were engorged with the blood of rodents/marsupials, (ii) this sandfly species was the most abundant sandfly species in the park, (iii) there are no data on the vector competence of *P. lloydi* and (iv) there have been no reported human cases of cutaneous leishmaniasis in the study area.

These results constitute an important step towards validating the use of *cytB* PCR as a tool for identifying the food sources of naturally feeding female sandflies. The development of more sensitive methodologies for this purpose is critical because current approaches designed to determine food sources are unreliable and insensitive, including serological methods (Gomez et al. 1998, Agrela et al. 2002, Bongiorno et al. 2003, Svobodová et al. 2003, Marassá et al. 2006, Rossi et al. 2008) and precipitin tests (Javadian et al. 1977, Morrison et al. 1993, Nery et al. 2004, Afonso et al. 2005). The sensitivity of *cytB* PCR was very high in the present study, as the expected fragment was observed for all of the samples tested. In contrast, the specificity was poor because nearly 40% of the *cytB* PCR-RFLP assays did not identify the source of the blood meal. The potential reasons for this large number of unidentified feeding sources include post-PCR obstacles related to the RFLP technique, which has limitations for the analysis of banding profiles. To improve the resolution of this assay, additional tools, such as primers designed

to amplify *cyt*B gene fragments from different species of birds and mammals and sequencing of genes such as *cytB* or PNOC (prepronociceptin), may be used in studies addressing the feeding behaviours of these insects. The data presented in this report suggest that the described method for the characterisation of sandfly blood meals was promising and effective for identifying the origin of *cytB* amplified from blood collected from the intestines of female sandflies. This information not only contributes to our understanding of the biology of these insects, but can also be used to design better vector control strategies to mitigate the incidence of leishmaniasis in endemic areas.

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