An investigation of *Leishmania* spp. in Long-eared opossums (*Didelphis* spp.) from urban and peri-urban areas in Bauru (Sao Paulo, Brazil)

Maria Emília Bodini Santiago a, Karina Reinaldo Fattori b, Danísio Prado Munarí, Aparecida de Fátima Michelind, Valeria Marçal Felix Lima b

a Mestre - Pós-graduação em Ciência Animal, Universidade Estadual Paulista – Faculdade de Odontologia de Araçatuba, Araçatuba- SP, Brasil.

b Departamento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Odontologia- Curso de Medicina Veterinária- Araçatuba Campus, SP, Brasil.

c Departmento de Ciências Exatas - Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal - SP, Brasil.

d Programa de Microbiologia – Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal - SP, Brasil.

Address for correspondence: Faculdade de Medicina Veterinária - UNESP, Rua Clóvis Pestana, 793 Araçatuba/SP CEP 16050-400, Brasil.

Fone: (0XX)18-3636-1422 E-mail: vmflima@fmva.unesp.br
Abstract

Blood and bone marrow samples were taken from 112 Long-eared opossums (Didelphis spp.), collected between March 2005 and February 2006, from urban and peri-urban areas of Bauru, São Paulo State, Brazil, to evaluate the hypothesis that these animals might constitute a reservoir for Leishmaniasis. Anti-Leishmania ssp. antibodies were screened in the serum samples using an Enzyme-Linked Immuno-sorbent Assay (ELISA) and the Polymerase Chain Reaction (PCR). PCR was performed on fragments of DNA samples from Leishmania spp. using primers 13A and 13B, and showed a positive outcome in 91.6% of the 112 samples tested. Of the 107 samples analyzed by ELISA, 71% were positive. Evidence of epidemiological risk factors such as a circulating parasite and freely moving vectors suggests that Long-eared opossums may participate in the transmission cycle of Leishmaniasis in Bauru.

Keywords: Leishmania ssp., Didelphis ssp., ELISA, PCR
1. Introduction

Leishmaniasis, a disease caused by a digenetic parasite, can appear in different clinical forms, depending on the *Leishmania* species involved (Gontijo & Carvalho, 2003). In Brazil, the dermotrophic form of the disease is caused by six different *Leishmania* species of the *Leishmania vianna* complex (Basano & Camargo, 2004). The visceral form is caused by *Leishmania chagasi* of the *Leishmania donovani* complex (Gontijo & Mello, 2004). American cutaneous leishmaniasis was registered for the first time in the city of Bauru, Sao Paulo State, Brazil, in 1908, during deforestation for the construction of the Brazilian Northwest Railway (Genaro, 2003). At the time, the disease was known as ‘Bauru ulcer’.

In 2002, the first cases of the visceral form of Leishmaniasis were registered in dogs, and the first human cases were reported in 2003. Although the local government implemented control measures, the occurrence of human cases has increased: 65 cases and 4 deaths were registered during 2006. One control measure, euthanasia, was performed in the case of 4,452 dogs of which 27% exhibited a positive diagnosis for Leishmaniasis, confirmed by laboratorial analysis. Fifty-five percent exhibited classical symptoms of visceral leishmaniasis while 18% suffered from other ailments (Gomieri, 2006 unpublished data).

Bauru possesses areas of forest fragments that are undergoing severe anthropic pressures, which leads wild animals to occupy its urban areas. When such animals enter the urban environment and encounter abundant and easy food sources, they incorporate such areas into their habitat. Bauru also has many species of *Lutzomyia*. 
The marsupial opossums, *Didelphis* spp. (Linnaeus, 1758), known in Brazil as the “gambá” or “mucura”, are animals very well adapted to various environments, and are considered examples of evolutionary success (Legey et al, 1999). These opossums are found frequently in forested areas around Bauru and also within the urban environment, habitating the roofs of houses, hospitals and industrial buildings.

In Brazil, *Didelphis* spp. are found naturally infected by *Leishmania* spp. in Manaus, Amazonas State (Arias & Naiff, 1981), in Barra de Guarituba, Rio de Janeiro State (Cabrera et al, 2003) and in Amaraji, Pernambuco State (Brandao-Filho et al, 2003). However, although Leishmaniasis now occurs in São Paulo State, no investigation has evaluated *Didelphis* spp. as a possible reservoir of the disease in this important state.

Through the identification and knowledge of the biology of natural reservoirs, more efficient measures can be taken to control Leishmaniasis. The present study thus aims to evaluate species of the genus *Didelphis* as a possible reservoir of *Leishmania* spp. in urban and peri-urban areas of Bauru.

2. Material and methods

2.1. Study area

Bauru city is located in the center-west region of Sao Paulo State (22° 18’ 53" S; 03’ 38" W) and has a total area of 702 km² with an urban perimeter of 120 km² (17% of total area). The naturally preserved vegetation around Bauru consists of cerrado, cerradão and secondary forest, totaling 3,771 hectares, which represents 14% of the municipal area. Pluviometric indices vary from 33
to 286 mm per year, and the mean yearly temperature is 23.6 °C
(www.bauru.sp.gov.br).

2.2. Opossum capture and sample collection

Opossums were captured using a variety of different manners: with the help of specifically designed wooden traps (73 x 28 x 35 cm); by manual capture; and by the voluntary delivery of animals by the Fire Brigade and the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA). Between March 2005 and February 2006, traps baited with bananas, bread and minced meat were set in the evening in forested areas, in streets, houses and trees, and near public garbage cans, being checked before dawn.

Prior to sample collection, captured animals were examined for the presence of wounds that might suggest Leishmaniasis. These individuals then received a microchip implant to avoid duplicating sample collection. Samples were taken after anesthesia by Ketamine (30 mg/kg) and Xylazine (2 mg/kg); 1.0 ml blood was collected from the caudal vein, and 100 µl of bone marrow was collected from the femur under local anesthesia (Lidocaine 2%), using a 40 x 12 needle and a 20-ml syringe. The material was transferred to sterile, 1.5-ml Eppendorf® tubes containing EDTA and was held at -20 °C until processing. After recovery, each animal was returned to the place of capture or to a forest fragment.

Capture was performed with permission from IBAMA (license #113/2005) and was approved by the Ethics Committee of the Universidade Estadual Paulista, Araçatuba campus.
2.3 Parasite isolation and detection of amastigotes

Approximately 10 µl of bone marrow was transferred to tubes containing Blood Based Agar (BBA) culture medium, (Difco B45) and the same amount of RPMI culture medium, and cultured in a BOD incubator at 26 °C. Diff quick-stained bone marrow smears were examined microscopically for the presence of amastigotes.

2.4 Indirect enzyme-linked immunosorbent assay

The ELISA assay was based on the protocol provided by Lima et al, (2003). Micro-plates (Greiner-bio One Microloan 600, Germany) were coated with 20 µg/ml total antigen from *Leishmania chagasi* in 100 µl buffer (0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plates were then washed in washing buffer (PBS + 0.05% Tween 20®) and blocked for 1 h at room temperature with 150 µl PBS containing 10% fetal bovine serum. After a further wash, 100 µl of opossum serum sample diluted in PBS containing 10% fetal bovine serum and 0.05% Tween 20® (concentration 1:200) were added and the plates were incubated for 1.5 h at room temperature. After incubation, the plates were washed again, and 100 µl of anti-IGG (produced in sheep and diluted 1: 100 in PBS + 0.05% Tween 20®) were added and incubated for 30 min. The washing process was repeated and 100 µl peroxidase-conjugated protein A (Sigma, St. Louis, MO), diluted 1: 1000 in the same solution were added. After 30 min at 37 °C, the plates were washed again and the substrate added (o-phenylenediamine and H2O2 in phosphate-citrate buffer). The reaction was stopped by the addition of 50 µl 1N HCl. Absorbance was measured at 490 nm using an automatic reader (Spectra Count, Packard®). The samples were
analyzed in duplicate and a blank well (PBS + 0.05% Tween 20® solution) was included in all plates.

Owing to the lack of reactivity of the opossum serum to proteins A and G, an anti-igG opossum antibody was produced. This antibody was isolated from the pooled sera of previously immunized sheep after precipitation with sodium sulfate, purification by ion exchange DEAE chromatography and dialysis (Laboratório de Zoonoses e Doenças Transmitidas por Vetores, PMSP).

2.5 Fucose-mannose ligand-ELISA

Isolation and chemical characterization of FML obtained from stationary growth phase promastigotes of *L. donovani* Sudan (LD IS/MHOM/SD/00-strain IS) was performed as previously described (Palatnik et al, 1989). The FML (80 µg/mL) was solubilized in carbonate buffer (pH 9.6) and used to coat flat-bottom in 96-well plates (Cornig Incorporated, USA). The plate was incubated for one hour at room temperature and so overnight at 4°C. The plates were then washed in washing buffer (PBS + 0.05% Tween 20®) and blocked for 1 h at room temperature, with 100µl PBS containing 5% skimmed milk. After a further wash, 50 µl of opossum’s serum samples diluted in PBS containing 5% skimmed milk and 0.05% Tween 20® (1:50) were added and the plates were incubated for 1 h at 37°C. After incubation, the plates were wash again and 50 µl of anti-igG, produced in sheep and diluted 1:100 in PBS containing 5% skimmed milk and 0.05% Tween 20®, concentration 1:100 and incubated for 1 h at 37°C. The washing process was repeated and 50 µl peroxidase-conjugated protein A (Sigma, St. Louis, MO) diluted 1:000 in the same solution was added. After 1 h at 37°C, the plates were washed again and the substrate added (o-
phenylenediamine and H$_2$O$_2$ in phosphate-citrate buffer). The reaction was stopped by the addition of 50µl 1N HCl. Absorbance was measured at 490nm using an automatic reader (Spectra Count, Packard®). The samples were analysed in duplicate a blank well (PBS + 5% skimmed milk + 0.05% Tween 20®PBS 20® solution) was included in all plates. The cut-off point was established using sera from negative opossum, employing the mean value plus 2 sd obtained for the group as a reference.

2.6 PCR protocols

DNA obtained from the bone marrow aspirate was extracted after lysing by freezing and thawing three times, and washing in 1x SSC buffer solution (NaCl 3 M, sodium citrate 0.3 M, pH 7.0). For digestion, 300 µl of lysing solution were added (10% SDS in 0.2 M sodium acetate) together with 20 µg/ml proteinase K. Samples were incubated at 56 °C for 2 h and the DNA was extracted using the phenol/chloroform/isoamyl alcohol method (25:24:1) according to Sambrook, (1989). After extraction, DNA was re-suspended in 50 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and incubated for 3 min at 60 °C. The material was held at -20 °C until use. The 13A (3’-GTG GGG GAG GGG CGT TCT-5’) and 13b (3’-ATT TTA CAC CAA CCC CCA GTT-5’) primers were used (Rodgers, 1990) to amplify a 120-bp fragment of cDNA from the base of the *Leishmania* spp. mini-circle. The primers 13A and 13B were previously shown to be specific in *Leishmania* species (Reale et al, 1999). The polymerase chain reaction was undertaken in a 60-µl volume containing 30 pmol of each indicator (Invitrogen®), 0.2 mM DNTPs (Invitrogen®), 1.5 mM MgCl$_2$ (Invitrogen®), 5 U Taq DNA Polymerase (Invitrogen®), 50 nM buffer
solution, milliQ water and 100-700 ng DNA. Amplification was performed in an Eppendorf® Mastercycler Thermocycler gradient with initial heating to 95 °C for 5 min, followed by 33 cycles at 95-57-72 °C for 1.5 min, 1.5 min and 2 min respectively. Extension was carried out at 72 °C for 10 min and the final product was kept at -20 °C until analysis. For each reaction, a negative control (no DNA), a positive control with DNA extracted from a *Leishmania chagasi* promastigote culture (MHOM/BR00/MER02) and two molecular weights were added.

The amplified product was analyzed by electrophoresis in 2% agarose gel followed by ethidium bromide staining and visualization under UV light.

2.6 Statistical analysis

McNemar’s non-parametric test and the Kappa coefficient were used to compare the positive indices furnished by the two diagnostic methods used, ELISA and PCR.

3. Results

3.1 Captured animals

Of the 112 opossums captured, 102 were *Didelphis albiventris* and 10 were *Didelphis aurita*. On clinical examination, no animal showed signs of injury that might suggest Leishmaniasis.

3.2 Parasite isolation and detection of amastigotes
Flagellated parasite forms were found in 18.8% (21/112) of the cultures while 16.1% (18/112) of the smears contained amastigote forms.

3.3 Indirect ELISA and PCR

A cut-off value of 0.349 was established using an ROC curve (MedCalc, version 9.0.1.1) from the analysis of 107 serum samples. The ELISA assays demonstrated that 29% (31/107) of the sera were negative, and 71% (76/107) were positive for *Leishmania* antibodies.

A comparative analysis of the 107 paired serum samples tested by ELISA and PCR showed that 67.3% were positive and 4.7% were negative by both tests; 24.3% were positive by PCR alone, and 3.7% by ELISA alone (Figure 1). Analysis of the ELISA findings, considering the PCR finding as a gold test, gave 73.5% sensibility and 66.7% specificity (Table 1).

Of the bone marrow samples tested, 91.6% (103/112) were positive and 8% (9/112) were negative. Figure 2 illustrates some typical sample amplifications.

3.4 Fucose-mannose ligand-ELISA

The positive samples in both PCR and indirect ELISA crude antigen were submitted to Fucose-mannose ligand-ELISA and 25% were seropositive (figure 3).

3.5 Statistics
The McNemar test indicated a significant difference between the positivity values obtained by the ELISA and PCR methods. The Kappa value (0.195) was considered poor.

4. Discussion

Wild animals constitute important reservoirs of Leishmaniasis (Jacobson et al, 2003), and many mammalian species, especially rodents, edentates, carnivores, primates and marsupials carry *Leishmania* spp. (Shaw & Lainson, 1987; Grimaldi & Tesh, 1993).

The opossum *Didelphis* spp. can harbor both the viscerotrophic and the dermatrophic strains of *Leishmania* (Franco, 1990). Experimental infection of *D. marsupialis* with *L. chagasi* reveals that this species can present the disease at a sub-clinical level (Travi et al, 1998), no animal showed signs of injury that might suggest Leishmaniasis, but 71% of the animals in this study were *Leishmania*-seropositive by ELISA, and 91.6% by PCR.

The ELISA test is usually employed in epidemiological studies and is also used to complement the diagnosis of various diseases, visceral Leishmaniasis among them (Camargo-Neves et al., 2006). The use of ELISA to detect anti-*Leishmania* spp. antibodies in the opossum has not been performed before. Previous investigations employing different sorological tests have found 29% positivity in Barra de Guaratiba, Rio de Janeiro State, using an indirect immunofluorescent assay (Cabrera et al, 2003), and a 61.9% infection rate in animals from Manaus, Amazonas State, using Arias and Naiff's culture (1981) method. The percentage positivities for the Bauru samples analyzed here are similar to those reported for Manaus.
Although the ELISA method can be useful to identify animals with a positive profile, there are certain limitations to the technique such as cross-reactivity between *Leishmania* spp. and *Trypanosoma* (Rosario et al, 2005; Rami et al, 2005; Nunes et al, 2006) and between *Leishmania* spp. and *Babesia* (Rosario et al, 2005), creating false positives. Opossums of the genus *Didelphis* can serve as reservoirs for *Trypanosoma* (Deane et al., 1984; in Legey et al., 1999; Bar et al., 1999; Grisard et al., 2000; Ruiz-Pena & Cruz-Reyes, 2002; Gurgel-Gonçalves et al, 2004) and *Babesia* (Herrera & Urdaneta-Morales, 1991). Simultaneous infection of *Didelphis marsupialis* by *T. cruzi* and *L. chagasi* has been demonstrated (Travi et al, 1994). A further limitation to the ELISA technique in *Didelphis* seen here was the lack of reactivity by IgG to peroxidase-conjugated proteins A and G, an inconvenience that required the production of an opossum anti-serum in sheep.

The fact that conventional serological techniques use crude antigens, considerably limits their specificity. To overcome this problem the antibody response in leishmaniasis has been studied using others antigens. The fucose-mannose ligand (FML) is a surface glycoprotein complex isolated from *L. donovani* promastigotas that has a sensibility of 100% and a specificity of 96% in the diagnosis in human kala-azar Brazilian patients infected by *L. chagasi* and sensibility and specificity of 100% in dogs with canine visceral leishmaniasis (Cabrera et al, 1999) in addition, no cross reaction was observed in patients infected with other Leishmania species, nor in those with Chagas disease (Palatnik- de Souza et al, 1995). The FML-ELISA showed 25% of seropositivity suggesting that other than viscerotrophic Leishmania species could be shelter by opossum.
The percentage of animals from Bauru showing a positive profile (91.6%) is much greater than that reported by other investigators like Zulueta et al (1999) who found 7% positivity by PCR testing in blood, liver and spleen samples from opossums in Venezuela. Telleria et al (1999) found no opossums with a positive profile in Cajuata (Bolivia) while Brandão-Filho et al (2003) reported 13.3% positivity using spleen samples in Amaraji, Pernambuco State. The high positivity found in the present study may derive from the analysis of bone marrow rather than blood. Mello and Teixeira (1984) note that greater quantities of amastigotes can be found in bone marrow and spleen tissues.

Our comparison of the two analytical methods revealed samples positive by PCR but negative by ELISA. Such findings may be a consequence of the small number of parasites present in the tissues analyzed, since a positive correlation between antibody titers in the serum and number of parasites has been reported in dogs (Reis et al, 2006). The PCR reaction can be positive when the parasite load is low. However, antibody production is stimulated only by a higher antigenic load. The positivity seen in samples by ELISA with corresponding negative results by PCR may be explained by the cross-reactivity of the antibodies to other antigens, not evaluated in the samples used.

Several studies in human and dogs had been showed that the PCR detect higher positivity rates for all of the other diagnostic methods studies, anti-Leishmania serological testing, culture and microscopic examination of lesion biopsy specimens (Riera et al, 2005, Rodrigues et al, 2002, Oliveira et al, 2005) similar to our finding.
The high incidence of infection seen in opossums suggests that these species participate in the transmission cycle of Leishmaniasis. This hypothesis is corroborated by a study demonstrating the feeding preference of the vector *L. evansi* for *Didelphis marsupialis* when compared with the squirrel *Sciurus granatensis*, and the rodents *Heteromys anomalus* and *Zygodontomys brevicaudata* (Adler et al., 2003). The high incidence of infection in opossums from an urban area suggests that these species may provide a natural bridge between the wild and domestic cycles of the disease owing to their co-existence with dogs.

Considering the various relevant factors such as the presence of elevated numbers of *Lutzomyia* spp. in the city; the synatropic nature of the opossum; the presence of these animals in the urban environment in Bauru since 1999 (Santiago, 2006 unpublished data); the sharing of food by dogs and opossums; and the fact that the appearance of the disease in the dog precedes that in humans (Neves et al, 2001; Zapata, 2002), the hypothesis that the migration of opossums into the urban environment of Bauru has influenced the occurrence of visceral leishmaniasis in this city seems likely.

In conclusion, this study reveals high levels of infection by *Leishmania* spp. in *Didelphis* spp., suggesting that opossums may participate in the epidemiological cycle of Leishmaniasis in Bauru, São Paulo, Brazil.

Acknowledgments

This work was financed by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). We thank IBAMA and the 12° Grupamento de Bombeiros de Bauru.
6. References


Santa Catarina and Arvoredo Islands, Southern Brazil. Mem. Inst. Oswaldo Cruz 95, 795-800.


Polymerase chain reaction (PCR) is highly sensitive for diagnosis of mucosal leishmaniasis. Acta Trop. 94, 55-59.


Travi, B.L., Jaramillo, C., Montoya, J., Segura, I., Zea, A., Gonçalves, A., 1994. *Didelphis marsupialis*, na important reservoir of *Trypanosoma* (Schizotrypanum)


Figura 1: PCR amplification of *Leishmania* spp in DNA extracted from bone marrow aspirate of opossum, 2% agarose gel electrophoresis stained with ethidium bromide. MW 2000pb-100pb (Low Mass, Invitrogen); (-) negative control without DNA (Millq water); (+): positive control DNA extracted from culture *L. (L.)* *chagasi* (MHOM/BR00/ MER02 – Fiocruz BA, Brazil); 1 to 12 DNA extracted from bone marrow.

Figura 2: Serum reactivity against total antigen from *Leishmania (L.)* *chagasi* of opossum from an endemic area for leishmaniasis. The results represent absorbance
values at serum dilution of 1:100. The horizontal line represents the cut-off value (0.349).

Figura 3: Fucose-mannose ligand (FML)-ELISA reactivity with sera of opossum from an endemic area for leishmaniasis, opossum infected by *Leishmania* spp. The results represent absorbance values at serum dilution of 1:50. The horizontal line represents the cut-off value (0.083).