

Considerations of potential vectors and animal reservoirs in an emerging cutaneous leishmaniasis area in *São Domingos* ranch, *Paraná State* in Southeastern Brazil

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ABSTRACT

The aim of this study was to better understand the dynamics of *Leishmania* sand flies and reservoirs in *São Domingos* ranch, Araçongas municipality, *Paraná State*, an anthropic environment in an endemic area of cutaneous leishmaniasis (CL). Sand flies were collected in wild animal burrows, residences and in the forest, with *Falcão* light trap (FA), Shannon trap (SH) and quadrangular pyramidal trap (QP). The search for *Leishmania* was made on sand flies, biological samples of wild rodents and dogs using PCR and culture; while parasite direct search (DS) was carried out on animal skin lesions; infection of gold hamsters; and indirect immunofluorescence (IIF) test in dog blood samples. Eighty eight (88) sand flies were collected with FA traps and 526 sand flies using the SH trap, with a predominance of *Pintomyia fischeri*. Six hundred and one (601) specimens of *Brumptomyia brumpti* were collected in armadillo burrows, with the QP trap. Seventeen (17) wild rodents were captured, six of them had skin lesions with characteristics of *Leishmania* infection. Even though no positive test was found for *Leishmania*, epidemiological surveillance should be maintained, remembering that the human buildings are situated only 50 m from the forest. Considering the species of wild animals and sandflies found in *São Domingos*, the negative test found do not exclude the existence of the *Leishmania* transmission cycle in this preservation area.

KEYWORDS: Cutaneous leishmaniasis. Hosts. Vectors. Epidemiology.

INTRODUCTION

The distribution of leishmaniasis in 98 countries shows its importance in world public health¹. In Latin America, the incidence of leishmaniasis, especially cutaneous leishmaniasis (CL), has been increasing and in Brazil, 635,399 cases all over the States were reported from 1990 to 2013². During this period, in the Southern region of the country, there were 13,889 (2.2%) cases, with 94.9% in *Paraná State*, in 316,399 municipalities².

In South, Central and North America, the evolutionary cycle of several *Leishmania* species in natural environments comprises wild terrestrial and arboreal mammals (Rodentia, Edentata, Marsupialia and Primates) and sand flies from several species³⁻⁶. The cohabitation of different species of sand flies, reservoirs and parasites in most distinct environments comprises a complex ecological scenario that makes it difficult to understand the epidemiology of leishmaniasis. Out of approximately 900 phlebotomine sand flies species identified in the world, 500 are in America with roughly half in Brazil⁶, but only 30 of them exhibit vectorial importance⁷⁻¹⁰.

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Received: 11 January 2017

Accepted: 5 June 2017

The vertebrate vector/host relationship is a determinant factor in the *Leishmania* cycle due to the need of the vector to seek in mammal animals, the food (blood) that allows it to reproduce, suggesting that the sources of food and ecological factors determine the geographical distribution of vectors, hosts, and parasites¹¹.

In areas of old colonization associated with remaining modified forests, there is evidence of sand fly adaptation and *Leishmania* wild reservoirs, providing the protozoa cycle formation of this genus in the peridomestic and rural areas and urban center outskirts¹²⁻¹⁴. This fact explains the endemic CL persistence in these areas. The detection of several sand fly species¹⁵⁻¹⁷, wild animals¹⁷⁻²⁰ and domestic animals (dogs, cats, and horses)^{13,14,21,22} infected by *Leishmania* in anthropic environments, explains partially, CL persistence in these environments.

The coexistence of human populations, reservoirs and *Leishmania* vectors in anthropic areas requires investigations to verify the implications of changes in CL epidemiology in endemic areas with the same characteristics. As the anthropic process in the natural environment is constant in *Paraná State* investigations in endemic areas of old colonization can contribute to the design of the most appropriate procedures for the control of this parasitosis.

Membrive *et al.*¹⁴ verified 41 human CL cases in rural locations of the Araongas municipality, from 1999 to 2008. In eight of the studied locations, only human cases occurred while in six of the locations, both human and canine cases occurred¹⁴. The *São Domingos* ranch was one of the studied rural locations; it is an area that suffered successive alterations, and where four human CL cases occurred in 2007 and five dogs had canine CL in 2008. Even though no human case occurred in the last few years, those facts led to the development of this study in *São Domingos* ranch, aiming a better understanding the dynamics of *Leishmania*, sand flies and reservoirs in an anthropic environment in an endemic area of CL.

MATERIALS AND METHODS

Characteristics of the Araongas municipality and description of the study area

The study was conducted in the *São Domingos* ranch, located at 23° 29' 50.17"S and 51° 27' 47.50" W, Araongas municipality, Northern central *Paraná State*, Southern Brazil. It has a subtropical climate, with a maximum temperature of 32 °C and a minimum of 8 °C and lowered plateau of plain and little-dissected tops¹⁴. *São Domingos* ranch is a permanent preservation area with approximately 2.1 ha, rocky soil, sharp slope and visibly degraded primary

forest. The human buildings are situated 50 m away from the forest. The residents cultivate soybeans, corn, wheat, tomatoes and work with poultry farming.

Sand fly collection and identification

For the collection of sand flies, *Falcão* light traps (FA)²³, Shannon light traps (SH)²⁴ and a quadrangular pyramidal trap (QP) were used (Figure 1).



Figure 1 - Quadrangular pyramidal trap (QP), especially developed to collect these insects in wild animal burrows

FA traps: These traps operate overnight without human presence, and its installation is easy in diversified environments (domiciles, domestic animal shelters, forests and others). Sand flies were collected with one FA trap installed on the porch of a residence and another in the dog shelter, from 6:00 pm to 8:00 am, four times a week, in December 2014 (224 hours) and February 2015 (224 h). Two FA traps were installed inside the forest, from 6:00 pm to 8:00 am once a week, from March to May and from August to November 2015, totaling 392 h of collection per trap.

SH trap: Sand flies were captured inside the forest with this trap, next to a bamboo bush, from 6:00 pm to 12:00 pm, once a month, in March, April, May, August, September and October 2015, totaling 36 h of captures. The SH trap attracts sand fly species with some degree of anthrophilia due to human presence during insects collection.

QP trap: The homemade QP trap, especially designed to collect insects in wild animal burrows, was constructed as follows: (i) a quadrangular structure in the base (40×40 cm) and a rectangular form in the top (20×30 cm), joined by iron bars with 70 cm of length; (ii) this structure was covered with white cloth and has two opening sides that allow the hand passage for handling the Castro-type suction tube²⁵ for the sand fly collection that would emerge from

inside the burrow into the trap; (iii) the bottom of the trap is hollow and the top is closed with transparent glass for insect viewing. This trap was placed over the entrance of the wild animal burrows. To promote the insect collection with the QP trap, a small tree branch was introduced inside the burrow, and circular movements were carried out. Due to the lack of clarity inside the forest, the collections were made during the day and a flashlight was needed to visualize the sand flies inside the QP. In each burrow, at the end of the collection, the suction tube was labeled with the ecotope number, sealed with nylon mesh for ventilation, packed in a thermal box with ice and transported to the laboratory, where the sand flies were dissected and identified.

The area where the sand flies were collected with the QP was delimited and divided into 40 sectors, each with 500 m², to facilitate the finding of natural ecotopes (wild animal burrows, especially armadillos), which were numbered in wooden stakes and georeferenced for their monitoring. Two hundred and six (206) wild animal burrows were found. In each of them, a collection period was carried out from 8:00 am to 12:00 am, three days a week, during the months of September and October, 2014. Given that the sand flies were captured in only six burrows (4, 24, 108, 109, 145, and 158) after this period, the collections were maintained only in these six burrows, one day in the first week of each month, from 8:00 am to 12:00 am, from November 2014 to October 2015.

Photographic record

The six burrows where the sand flies were collected were monitored with two digital cameras (Bushnell® USA) for ten consecutive days, in February and March 2016, to verify the presence of armadillos and other mammals. Each burrow was monitored for 240 h.

The collected sand flies were kept alive until their processing in the laboratory. The male sand flies were preserved in 70% ethanol and subsequently stained and identified. The females were kept alive for *Leishmania* search by dissection and identification of the species according to the genitalia morphology²⁶. The flagellates detected in the sand fly digestive tract were cultivated in 199 medium (Invitrogen), containing 10% inactivated fetal bovine serum and antibiotics for *Leishmania* isolation. The cultures were incubated at 27 °C and observed for four weeks to check eventual parasite growth.

The female sand flies in which no flagellates were found, but containing blood in their digestive tract, were conserved in tubes with isopropanol, each with 1 to 14 specimens of the same species and from the same ecotope, for subsequent *Leishmania* DNA detection by PCR.

The insects were processed at the Laboratory of Medical Entomology of the *Secretaria Municipal de Saúde* of Arapongas. Then, it was followed by nomenclature⁶ and abbreviations²⁷ of sand flies. The nomenclature of the species follows Galati⁶, and the abbreviations of the genera follow Marcondes²⁷.

Capture and collection of biological material from wild mammals

Wild rodents were captured in bamboo bushes, with four traps of the type “live rat traps” (Havahart, USA) in the month of November 2015. A total of 720 h of capture per trap was carried out, with an inspection performed every 48 h.

The captured rodents were anesthetized with ketamine and examined for the presence of suspected CL lesions²⁶. The suspected rodents were euthanized for the collection of biological materials (blood, skin, spleen and liver) for culture, and preparation of slides for microscopy, PCR and hamster infection. The rodents without suspected lesions were returned to their original environment after the capture period.

For *Leishmania* research by PCR, specific primers targeting the parasite kDNA were used. Liver, spleen and skin fragments intended for PCR were kept in STE buffer (NaCl 0.1 M; Tris 10 mM; pH 8.0; Na₂EDTA.2H₂O 1 mM; pH 8.0), and preserved at 30 °C until DNA extraction.

Collection of dog biological material

A sample of blood (5 mL) was collected from 10 brachial vein of the dog to perform the indirect immunofluorescence test (IIF). Serum samples were stored at -20 °C. The IIF for leishmaniasis was performed using *L. (V.) braziliensis* promastigotes- and anti-dog immunoglobulin G conjugated to fluorescein (Sigma), considering titles > 1:40 significant.

The biopsy was performed to collect tissue fragments of the dogs with cutaneous lesions suggestive of CL, after lesion asepsis and topical 1% xylocaine inoculation, for parasite direct search (DS) and parasite isolation in gold hamster (*Mesocricetus auratus*). For the DS, fragments were smeared on slides, stained using the Giemsa method and examined under an optical microscope.

Leishmania detection in sand flies by multiplex PCR- and in biological samples from wild mammals by conventional PCR

DNA extraction of sand flies was done according to Oliveira *et al.*²⁸. The DNA extraction of wild animals'

biopsy samples was performed by kit Puregene® (Gentra - USA), according to the manufacturer's instructions. For multiplex PCR, two pairs of primers were used: A1 and A2, which amplify a fragment of 110-120 bp of the conserved region of DNA from the minicircle of the kinetoplast (kDNA) of the genus *Leishmania*²⁹ and 5Llac and 3Llac, which amplify a fragment of 220 bp from the *IVS6* gene region of the cacophony in insects of the genus *Lutzomyia*³⁰. The PCR reaction mixture (final volume 25 µL) was composed of 0.5 mM of each of the primers (Invitrogen), 0.24mM dNTP (Invitrogen), 1U Taq DNA Polymerase (Invitrogen), 1.5 mM MgCl₂, 1 × enzyme buffer, and 2 µL DNA template. The amplification was carried out in a G96G & G96GEN cyler (Biosystems) at 95 °C for 5 min for initial denaturation, followed by 35 cycles, each divided into three stages, of denaturation (30 sec at 95 °C), annealing (30 sec at 55 °C), and polymerization (30 sec at 72 °C). After this, the extension was continued for a further 10 min at 72 °C, and the tubes were then kept at 4 °C until analysis. This PCR reaction mixture and amplification were performed according to Santos BA et al. (unpublished data).

Two conventional PCR of biological material were performed from wild animals samples. For the first PCR, primers A1 and A2²⁹ were used. The second round of amplification was performed with the primers MP3H and MP1L, which amplify a fragment of 70 bp from the conserved region of the kinetoplast minicircle (kDNA) of the subgenus *Leishmania* (*Viannia*)³¹.

In both PCR assays, DNA of *L. (V.) braziliensis* was used as the positive control while sterile water was used as the negative control.

Ethical aspects

The captures and procedures with wild animals were

performed with the license approved by the *Environmental Institute of Paraná* (IAP) (protocol N° 06/14). All procedures with hamsters were performed according to the protocols approved by the Ethics Committee on Animal Use from the *State University of Maringá* (CEUA-EMU) (protocol N° 083/14).

Statistical analyses

The proportion and G tests were analyzed by the BioEstat software version 5.3, and the Mid-P exact test was performed by using the OpenEpi software version 3.1, while the level of statistical significance was set at p < 0.05.

RESULTS

Five sand flies were collected with FA traps in the porch of the residence (Table 1), and none in the dog shelter. In the forest, 83 sand flies were collected from the species *Pintomyia pessoai* (Barreto & Coutinho), *Pintomyia fischeri* (Pinto), *Nyssomyia whitmani* (Antunes & Coutinho), *Migonemyia migonei* (France), *Expapillata firmatoi* (Barreto, Martins & Pellegrino), and *Brumptomyia brumpti* (Larrousse); 21 (25.3%) of them were male and 63 (74.7%), female; from the six collected species, 52 (62.6%) were *Pi. fischeri* (Table 1). There was no difference in the number of sand flies collected per hour in relation to sex and environment (p=0.529). All females collected with FA trap were submitted to dissection for the search of flagellate forms. Flagellate forms were found only in one specimen of *Mi. migonei* collected in the forest. An aliquot of its digestive tract (0.1 mL) was subjected to multiplex PCR, with a negative result, and another aliquot (0.5 mL) was inoculated into hamster's hand paws but did not result in *Leishmania* isolation.

Table 1 - Sand flies collected with FA trap in São Domingos ranch, Arapongas municipality, Paraná State, Brazil

Species/Ecotope/Sex	Porch of the residence ¹			Forest ²		
	Male	Female	Total	Male	Female	Total
<i>Brumptomyia brumpti</i>	0	0	0	0	4	4
<i>Migonemyia migonei</i>	0	1	1	0	3	3
<i>Pintomyia fischeri</i>	0	4	4	11	41	52
<i>Nyssomyia whitmani</i>	0	0	0	6	6	12
<i>Expapillata firmatoi</i>	0	0	0	0	2	2
<i>Pintomyia pessoai</i>	0	0	0	4	6	10
Total	0	5	5	21	62	83
%		100.0	100.0	25.3	74.7	100.0

1. Operating time of the FA trap in the porch of the residence: 448 hours. 2. Two FA traps were installed inside the forest. Operating time of each FA trap in the forest: 392 hours.

By using the SH trap, 526 sand flies of eight species were collected: *Pi. pessoai*, *Nyssomyia neivai* (Pinto), *Pi. fischeri*, *Ny. whitmani*, *Mi. migonei*, *Psathyromyia Shannoni* (Dyar), *Pintomyia monticola* (Costa Lima), and *Br. Brumpti*; 105 (20.0%) of them were male and 421 (80.0%) female (Table 2). The proportion of female was significantly higher than that of male ($p < 0.001$). Of the total collected, 384 (73.0%) were *Pi. fischeri*; and from these, 81 (21.1%) were male and 303 (78.9%) female (Table 2). There was no difference in the species distribution and sex of sand flies ($p=0.536$).

Table 2 - Sand flies collected with SH trap in São Domingos ranch, Arapongas municipality, Paraná State, Brazil

Species/Sex	Male	Female	Total
<i>Brumptomyia brumpti</i>	0	2	2
<i>Pintomyia monticola</i>	0	2	2
<i>Migonemyia migonei</i>	3	23	26
<i>Pintomyia fischeri</i>	81	303	384
<i>Nyssomyia whitmani</i>	20	76	96
<i>Migonemyia neivai</i>	0	5	5
<i>Psathyromyia shannoni</i>	1	7	8
<i>Pintomyia pessoai</i>	0	3	3
Total	105	421	526

Operating time of the SH trap: 36 hours.

During the captures with SH trap, it was observed that *Pi. fischeri* specimens did not land on the trap walls, but on the bamboos, close to where the trap was installed, at the height of three to four meters, from 7:00 pm to 9:00 pm. After this hour, when the wind started blowing, these sand flies landed about one meter from the ground, always on the bamboos, where they were collected.

Table 4 - Results of test for *Leishmania* search in wild rodents and domestic dogs, carried out in the São Domingos ranch, Arapongas municipality, Paraná State, Brazil

Animals	CA (n)	AL (n)	Organ culture*			PCR*	IH*	DS*	IIF*
			Spleen	Liver	Skin				
Rodents									
<i>Akodon</i> sp.	6	2	0/2	0/2	0/2	0/4	0/2	0/2	0/0
<i>Oligoryzomys</i> spp.	4	2	0/2	0/2	0/2	0/4	0/2	0/2	0/0
<i>Nectomys</i> spp.	4	1	0/1	0/1	0/1	0/2	0/1	0/1	0/0
<i>Oryzomys</i> spp.	3	1	0/1	0/1	0/1	0/2	0/1	0/1	0/0
Dogs									
<i>Canis familiaris</i>	10	1	0/0	0/0	0/1	0/0	0/1	1/1	0/10
Total	27	7	0/6	0/6	0/7	0/6	0/7	1/7	0/10

CA=Captured animals; AL=Animals with lesion; IIF=Indirect immunofluorescence test; PCR=Polymerase chain reaction performed with a fragment of spleen and liver of each rodent with lesion; DS=parasite direct search performed in lesions; IH=Infection in hamster; *Positive or negative result/number of samples.

Six hundred and one (601) specimens of *Br. brumpti* were collected with the QP trap in six of the 206 wild animal burrows, of which 77.2% were male and 22.8% female (Table 3). All females (137) were dissected for the search of flagellate forms in the digestive tract and the salivary gland; none of them showed flagellate forms. The multiplex PCR for detection of *Leishmania* DNA was performed with 52 females of *Br. Brumpti* (nine pools), which contained blood in the digestive tract. All pools contained the 220 bp fragment from the *IVS6* gene region of the cacophony of the sand flies, but none contained the 110-120 bp fragment from the conserved region of the kinetoplast minicircle (kDNA) of the genus *Leishmania*. The digital cameras (Bushnell) revealed that the burrows were inhabited by armadillos *Dasyus novemcinctus*.

Table 3 - Sand flies of the species *Br. brumpti* collected with QP trap in six of 206 wild animal burrows found in the São Domingos ranch, Arapongas municipality, Paraná State, Brazil, from September 2014 to October 2015

Burrow number	Male	Female	Total
Burrow 4	42	7	49
Burrow 24	74	24	98
Burrow 108	132	47	179
Burrow 109	89	14	103
Burrow 119	73	28	101
Burrow 124	54	17	71
Total	464	137	601
%	77.2	22.8	100.0

Using the “live rat traps”, 17 rodents were captured, from the species *Akodon* spp., *Oligoryzomys* spp., *Nectomys* spp., and *Oryzomys* spp. (Table 4). Two rodents *Akodon* spp. and two *Oligoryzomys* spp. had ear lesions; one specimen of

Oryzomys spp. had an unpigmented area in the tail, and the other had a nodule in the tail. One specimen of *Nectomys spp.* had a snout lesion and an unpigmented area in the tail. The culture of spleen, liver, and fragment of skin lesion; PCR for detection of *Leishmania* DNA in liver and spleen of rodents; infection in hamster, and DS of lesions were negative (Table 4).

The IIF results of the ten dogs was negative (Table 4). One of the ten dogs had ear lesions suggestive of CL, of which samples were collected to perform DS, culture in 199 medium and infection in hamster. Amastigotes were found in DS, but culture and infection in hamsters were negative (Table 4).

DISCUSSION

In the *São Domingos* ranch, the collected species of sand flies were *Br. brumpti*, *Ex. firmatoi*, *Mi. migonei*, *Ny. neivai*, *Ny. whitmani*, *Pi. fischeri*, *Pi. monticola*, *Pi. pessoai*, and *Ps. shannoni*, which have already been reported in the *Paraná State*^{16,32-34}. The species *Mi. migonei*, *Ny. whitmani*, *Ny. neivai* and *Mi. migonei* have already been found to be naturally infected by *Leishmania* spp. in *Paraná State*^{28,35} and other Brazilian States^{15,16,36-44}. Despite the low levels of sand flies collected when compared to studies carried out in Northern *Paraná State*^{16,26-29}, the importance of this study lies in the observations that could be made, as in the same locality wild animals where captured, possibly representing *Leishmania* reservoirs and sand flies as well as dogs were examined.

The species of *Pi. fischeri* predominated in the collection with FA traps in the forest, constituting 62.6% from the total, and 86.5% of this species were female. Close to the same bamboos where these two traps were placed, 73.0% of the sand flies collected with SH were female *Pi. fischeri*. These data alongside with evidence that the natural breeding sites of *Pi. fischeri* occur on the ground, at the base of trees^{45,46} allow us to consider the possibility that this species is associated with bamboo plantations, where they find shelter and conditions for procreation, with high humidity and organic matter in decomposition. Lainson *et al.*⁴⁷ suggested that *Pi. fischeri* is an example of sand fly that can adapt to environments modified by human action and is still able to transmit *L.(V.) braziliensis* to wild animals in secondary forests with fragments still preserved. Furthermore, it is possible that this sand fly species and the wild rodents occupy the same habitat given that the traps for the rodents were installed in the same bamboos.

However, only four sand flies were collected in the porch of the residence; this fact should not be disregarded given that *Pi. fischeri* has already been found to be infected by *Leishmania*⁴⁴. Furthermore, it is highly anthropophilic, adapts

well to domestic animal shelters and invade the domiciles to suck human blood^{26,48}. For this reason, the possibility of the sand fly population increment, and the proximity of the forest with possible reservoirs of *Leishmania*, the actions to control sand fly populations should always be carried out, under the risk of CL cases increment.

In South America, especially in Brazil, sand flies of the genus *Brumptomyia*, rather than the *Dasypodidae*⁴⁹, have been commonly collected in animal burrows, indicating that there is a close association of this sand fly genus with armadillos. The armadillos (*Dasybus novemcinctus*) have already been identified as *Leishmania naiffi* hosts^{25,50}. All *Br. brumpti* collected in armadillo burrows, in *São Domingos* ranch, were negative by dissection for the search of flagellate forms and also by PCR for *Leishmania* search.

Researchers have described wild rodents as reservoirs of *Leishmania*¹⁷. Barros *et al.*⁵¹ captured 257 wild mammals from three orders (Rodentia, Marsupialia and Lagomorfa), five families and 15 species, but isolated *Leishmania* from only two rodents; and Gomes *et al.*⁵² captured 36 rodents in an endemic area for CL, all with negative results. The small number of rodents captured in the forest show that these animals populations are low in the surveyed locality. This factor also limits the understanding of the role of wild rodents in the *Leishmania* transmission cycle. Besides that, the isolation rate of *Leishmania* in wild animals is very low and requires the capture of a large number of animals^{51,52}. Despite the negative results of the PCR for *Leishmania* search in six of the 17 wild rodents captured, the skin lesions had characteristics of *Leishmania* infection.

In November 2014, part of the studied property was deforested and occupied by greenhouses devoted to fruit and vegetables cultivation, close to the remaining forest, where synthetic chemicals were used in pests management. This procedure may have affected the sand fly population, explaining the low number of insects collected.

Even if the IIF performed with dog blood samples were negative, and no positive test was found for *Leishmania*, epidemiological surveillance should be maintained with periodic examinations of domestic animals, remembering that the human buildings are situated only 50 m from the forest. Considering the species of wild animals and sandflies found in *São Domingos*, the negative test found do not exclude the existence of the *Leishmania* transmission cycle in this preservation area.

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