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Major Article

Phlebotomine sandfly fauna and natural *Leishmania* infection rates in a rural area of Cerrado (tropical savannah) in Nova Mutum, State of Mato Grosso in Brazil

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ABSTRACT

Introduction: American cutaneous leishmaniasis (ACL) has been reported in every municipality of the State of Mato Grosso, Brazil, but the transmission epidemiology remains poorly understood. Our study was developed in a rural area of the Nova Mutum municipality where four autochthonous cases of ACL were reported in 2009. Our aims were to describe the local phlebotomine sandfly fauna and to investigate the infection rates and infecting *Leishmania* species in the captured sandflies. **Methods**: Entomological captures were performed bimonthly at 10 fixed sites close to the edge of a forested area between June 2011 and April 2012. **Results**: A total of 3,743 phlebotomine sandflies belonging to 31 distinct species were captured. Approximately 75% of the specimens were females. The most abundant species (45.4%) was *Lutzomyia antunesi*, which was consistently captured at every site. Species that are epidemiologically important for ACL, such as *L. flaviscutellata*, *L. whitmani* and *L. umbratilis,* were also captured. *L. antunesi* and *L. ubiquitalis* were naturally infected by *Leishmania braziliensis* or *Le. guyanensis,* with minimum infection rates of 0.88% and 6.67%, respectively. Surprisingly, *L. antunesi* was infected by *Le. infantum* (synonym *chagasi*). **Conclusions**: The natural infection of *L. antunesi* and *L. ubiquitalis* by *Leishmania* sp. suggests that these species might play a role in the zoonotic cycle of ACL in Nova Mutum. The presence of *Le. infantum* in *L. antunesi* suggests that there may be a risk of an outbreak of visceral leishmaniasis (VL) in Nova Mutum.

Keywords: Phlebotomine sandfly. *Leishmania.* American cutaneous leishmaniasis. Nova Mutum. *Lutzomyia*.

INTRODUCTION

Leishmaniases are among the most prevalent infectious diseases caused by parasites in the world and display a wide distribution in the Americas, Africa, India, Asia and Mediterranean Europe[1,2]. Leishmaniases occur in approximately 90 different countries, currently infecting 14 million people with an increase of 2 million new cases per year. It is estimated that 350 million people are at risk for these diseases[2], which are caused by protozoa that belong to the *Leishmania* genus. Transmission occurs through the bite from infected phlebotomine sandflies (Diptera, Psychodidae). Several mammal species may act as natural reservoirs or hosts for leishmaniases. Humans and some domestic animals, including dogs and horses, are considered to be accidental hosts[3].

Human cases of American cutaneous leishmaniasis (ACL) have been reported in every Brazilian State[4]. In Mato Grosso (MT), ACL is endemic with 8,000 reported cases between 2009 and 2011 (MS/SINAN/SES/MT, 2011). Since 2005, autochthonous cases of ACL have been reported from every municipality of the state[5].

Lutzomyia whitmani, a phlebotomine sandfly species that is widely distributed in Brazil, is the main species associated with ACL transmission in MT[4]. This fly can be found in various MT biomes, such as *Cerrado* (tropical savannah), the Amazonian rainforest and *Pantanal* (tropical wetland). The phlebotomine sandfly fauna in these biomes is widely diversified and consists of species such as *Lutzomyia flaviscutellata, L. intermedia, L. migonei, L. umbratilis, L. wellcomei* and *L. whitmani*[5,6] all of them involved in the transmission epidemiology of ACL.

The identification of potential vector species for ACL and the natural rate of *Leishmania* infection are of fundamental importance for understanding the medical entomology and epidemiology of ACL, particularly in endemic regions. Applying molecular biology techniques, such as the polymerase chain

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reaction (PCR), enables the identification of genetic material of *Leishmania* in the total DNA extracted from phlebotomine sandfly macerates, even in minimal amounts. The main advantages of PCR are its sensitivity and specificity, regardless of the number, location or stage of the infecting *Leishmania* in the digestive tract of the sandfly[7]. In the last decade, PCR has been widely used in studies of vector competence of phlebotomine sandflies, even in areas with low rates of *Leishmania* infection[8-11]. Although MT is considered endemic for ACL, natural infection investigations on ACL vectors are rare there. Only three studies on natural infection by *Leishmania* have been reported in the state, two of which are related to visceral leishmaniasis (VL) vectors[12,13], and a third investigated a single *L. umbratilis* specimen that was infected by *Le. braziliensis*[14].

The goal of present study was to survey the phlebotomine sandfly in a rural area of the State of Mato Grosso in qualitative and quantitative terms, as well as to determine the natural rate of *Leishmania* sp. infection in the phlebotomine sandfly females captured there. The area under study is located in the municipality of Nova Mutum, where four autochthonous ACL cases were reported in 2009.

METHODS

Area under study

Nova Mutum (13º05'04"S, 56º05'16"W) is a 9,572.69km^2 municipality that is located in the mid-Northern area of the Brazilian State of Mato Grosso **(Figure 1)**. Our study was developed in a rural area of *Cerrado* (tropical savannah) that has an area of 16,000 hectares, and is located at 42km from the city center of Nova Mutum. The location has a native forest that consists of small to large trees **(Figure 2)** and has an ample amount of decaying vegetal organic matter. Various animal species can be found there, including monkeys, wild pigs, snakes, rats, scouts and armadillos. The area attracts people who engage in activities that put them in close contact with nature, such as swimming and fishing.

Phlebotomine sandfly capture and identification

Six entomological captures were performed bimonthly for three consecutive nights between June 2011 and April 2012. Ten Center for Disease Control (CDC) light traps were set five feet from the ground and 100m from one another at a transect of approximately 1,000 meters between the edge and the interior of the forest. The capture sites were plotted by GPS. The captured phlebotomine sandflies were packed in insulated containers and taken to the laboratory for adequate assembling and identification, as described by Young and Duncan[15]. The specimens were incorporated into the collection of the Entomology laboratory from the Health Department of Mato Grosso. The phlebotomine female sandflies were identified by dissecting the last three segments to allow for the visualization of the abdominal spermathecae and by head dissection to allow for the examination of specific taxonomic characteristics. Damaged specimens were identified at the genus level only. One to 10 specimens of phlebotomine female sandflies were pooled according to species, date and site of capture, and were then stored in 6% dimethyl sulfoxide (DMSO) at -20ºC until use.

FIGURE 1 - Geographical localization of the municipality of Nova Mutum (in red) in the State of Mato Grosso, Brazil. The capital of Mato Grosso, Cuiabá, is indicated by a solid blue circle.

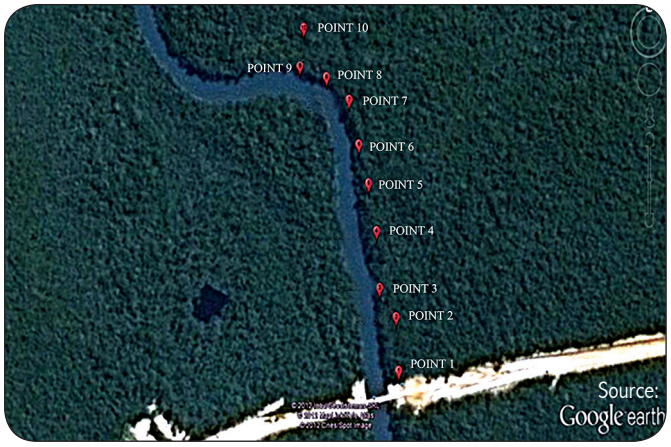

FIGURE 2 - Aerial view of the area under study in Nova Mutum, State of Mato Grosso, Brasil. The sites of entomological captures are indicated by red balloons.

Lutzomyia constitutive gene (cacophony)

The adequacy of DNA extraction from the phlebotomine sandflies was checked by polymerase chain reaction (PCR) with specific primers to the IVS6 region of the *Lutzomyia* genus (cacophony), as described by Lins[16].

Detection of *Leishmania* DNA in phlebotomine sandflies

Pooled samples of phlebotomine female sandflies were submitted to total DNA extraction using a commercial kit (GE HealthCare, Uppsala, Sweden). The presence of *Leishmania* DNA was tested by nested PCR (LnPCR) with primers that were directed at the small subunit ribosomal ribonucleic acid (SSUrRNA) gene[17,18]. The first amplification step was performed using R221 and R332 primers that are specific to the Kinetoplastida order but not exclusively to the *Leishmania* genus.

The PCR products were then tested using a new amplification step with R233 and R333 primers[18]. All amplifications were performed with the Illustra PuRe Taq Ready-To-Go PCR Beads kit (GE Healthcare, Uppsala, Sweden), and the products were analyzed using electrophoresis in agarose gels. DNA from *Le. braziliensis* (M2903 strain) and sterile distilled water were used as the positive and negative controls, respectively.

Identification of the *Leishmania* species in phlebotomine sandflies

The amplified bands from the PCR steps were extracted from the gels using the QIAquick extraction kit (QIAGEN, Hilden, Germany) and submitted for DNA sequencing using an ABI3130 analyzer (Applied Biosystems Inc., Foster City, California, USA). The sequences were analyzed using the Blast Nucleotide Standard software (blast.ncbi.nlm.nih.gov).

Minimum rates of *Leishmania* infection in phlebotomine sandflies

The minimum rates of *Leishmania* infection in the captured phlebotomine sandflies were calculated by dividing the number of positive pools of each sandfly species by the number of specimens of that species in that pool and then multiplying by 100[19].

RESULTS

A total of 3,743 phlebotomine sandfly specimens were captured in Nova Mutum. Of the captured specimens, 1,008 (26.9%) were males (M) and 2,735 (73.1%) were females (F), which resulted in an overall M/F ratio of 0.4% **(Table 1)**. The phlebotomine sandfly fauna consisted of 31 different species. The predominant species was *Lutzomyia antunesi* (Coutinho, 1939), which accounted for approximately 45% of the total captured specimens. *L. flaviscutellata* (Mangabeira, 1942), *L. whitmani* (Antunes & Coutinho, 1939) and *L. umbratilis* (Ward & Fraiha, 1977), all known ACL vectors, were also captured at the following percentages: 6.49%, 0.19% and 0.03%, respectively **(Table 1)**.

A total of 2,419 sandflies females were dissected and pooled for molecular analysis. Then, 293 pooled samples were obtained, which were distributed by species as follows: 196 of *L. antunesi,* 71 of *L. flaviscutellata,* eight of *L. yuilli yuilli,* 16 of *L. ubiquitalis,* one of *L. umbratilis* and one of *L. whitmani*. After LnPCR, the 353bp DNA fragment of the *Leishmania* genus was observed in 13 of the pooled samples (11 from *L. antunesi* and two from *L. ubiquitalis* [**Figure 3**]). No amplification products were detected in the remaining samples. The minimum rates of *Leishmania* infection were 0.88% and 6.67% for *L. ubiquitalis* and *L. antunesi,* respectively. The adequacy of the *Lutzomyia* DNA extraction was confirmed by the amplification of the cacophony gene in all 13 positive *Leishmania* samples (data not shown).

After the DNA sequencing and nucleotide alignment of the 353bp amplicons from the *Leishmania*-containing phlebotomine samples **(Figure 3)**, the infecting *Leishmania* species were identified as follows: *Le. braziliensis* (GQ332355) or *Le. guyanensis* (GQ332358) in six pooled *L. antunesi* samples (numbered 190, 224, 233, 246, 256 and 275) and in two pooled *L. ubiquitalis* samples (numbered 264 and 265); *Le. infantum* (GQ 332359) was identified in one pooled sample of *L. antunesi.* Poor DNA sequencing results were obtained for the four remaining samples.

TABLE 1 - Phlebotomine sandfly species captured with CDC light traps in a rural area of Nova Mutum, State of Mato Grosso, Brazil. Period: June 2011 to April 2012.

	Female		Male		Total	
Species	n	%	n	%	n	%
B. brumpti	14	0.51	6	0.60	20	0.53
L. antunesi	1,265	46.25	436	43.26	1,701	45.45
L. aragaoi	4	0.15	-	-	4	0.11
L. ayrozai	33	1.21	-	-	33	0.88
L. begonae	28	1.02	-	-	28	0.75
L. bourrouli	6	0.22	1	0.10	7	0.19
L. chagasi	15	0.55	-	-	15	0.40
L. claustrei	14	0.51	20	1.98	34	0.91
L. complexa	1	0.04	4	0.40	5	0.13
L. dasypodogeton	12	0.44	6	0.60	18	0.48
L. davisi	3	0.11	2	0.20	5	0.13
L. flaviscutellata	146	5.34	97	9.62	243	6.49
L. furcata	37	1.35	1	0.10	38	1.02
L. hermanlenti	9	0.33	9	0.89	18	0.48
L. lenti	1	0.04	-	-	1	0.03
L. llanosmartinsi	46	1.68	2	0.20	48	1.28
L. longipennis	9	0.33	6	0.60	15	0.40
L. octavioi	-	-	5	0.50	5	0.13
L. punctigeniculata	2	0.07	-	-	2	0.05
L. runoides	2	0.07	2	0.20	4	0.11
L. sallesi	-	-	3	0.30	3	0.08
L. saulensis	677	24.75	93	9.23	770	20.57
L. shannoni	34	1.24	-	-	34	0.91
L. shawi	1	0.04	-	-	1	0.03
L. sordellii	16	0.58	3	0.30	19	0.51
L. spp.	16	0.58	9	0.89	25	0.67
L. ubiquitalis	43	1.57	83	8.23	126	3.37
L. umbratilis	1	0.04	-	-	1	0.03
L. walkeri	282	10.31	212	21.03	494	13.20
L. whitmani	1	0.04	6	0.60	7	0.19
L. yuilli yuilli	17	0.62	2	0.20	19	0.51
Total	2,735	100.0	1,008	100.0	3,743	100.0

B.: *Brumptomyia*; *L.*: *Lutzomyia*; CDC: Center for Disease Control.

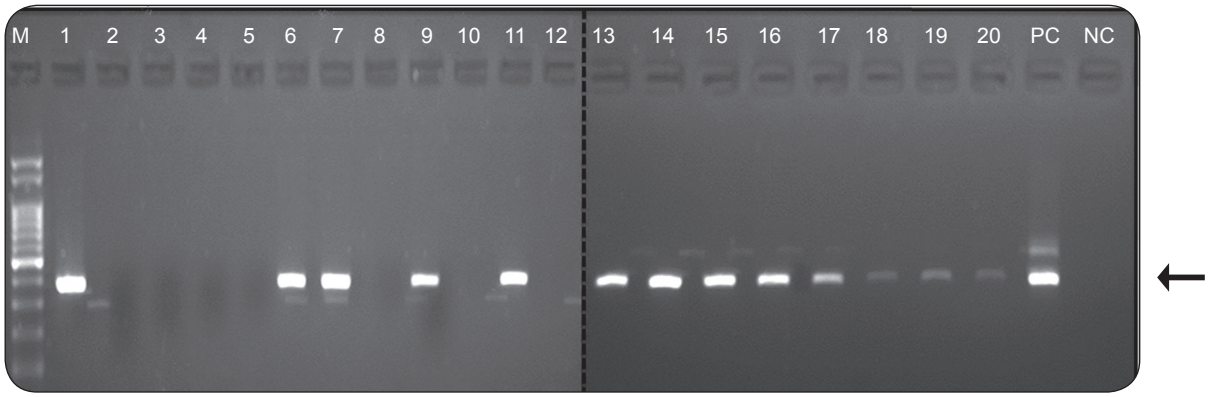

FIGURE 3 - Agarose gel electrophoresis of the LnPCR products after amplifying the total phlebotomine sandfly DNA for the SSUrRNA gene of *Leishmania* sp. The characteristic 353bp fragment (indicated by an arrow) was present in the following samples: *L. umbratilis* (18 and 19); *L. antunesi* (1, 6, 7, 9, 11, 13, 14, 15, 16, 17 and 20). PC: positive control (*Le. braziliensis* M2903); NC: negative control (no DNA); and M: 100bp DNA ladder.

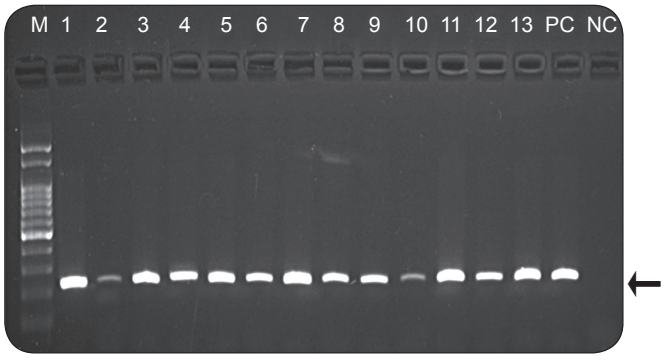

FIGURE 4 - Agarose gel electrophoresis of the PCR products obtained after amplifying the total phlebotomine sandfly DNA with primers for the cacophony IV6 gene of *Lutzomyia*. The characteristic 220bp fragment is indicated by an arrow. Samples: *L. umbratilis* (11 and 12); *L. antunesi* (1 to 10, 13); PC: Positive control (*L. longipalpis* DNA); NC: negative control (no DNA); and M: 100 bp DNA ladder.

DISCUSSION

The phlebotomine sandfly fauna of the State of Mato Grosso is quite diversified, consisting of more than 100 species whose spatial distribution varies according to the different biomes present[21,24]. In the rural *Cerrado* (tropical savannah), which was under study, roughly 50% of the captured specimens were *L. antunesi*. *L. longipalpis* and *L. cruzi,* which are both proven vectors of the visceral leishmaniasis (VL) in Brazil, were not captured at that location. This result differed from the capture reports in urban areas with *Cerrado* fragments[23,25]. Surprisingly, even in the absence of *L. longipalpis* and *L. cruzi* and no reported cases of VL, *Le. infantum* was present in a pooled sample of *L. antunesi* from Nova Mutum. A previous finding of promastigotes in *L. antunesi* with a VL focus in the Marajó Island (State of Pará) led the authors to suggest that the sandfly species could be involved as a secondary VL vector on that island. The authors also suggested the possibility of *Le. infantum* infection in *L. antunesi,* although the inoculation of isolated promastigotes from the sandflies produced no skin lesions in hamsters[26].

Regarding ACL vectors, *L. whitmani* (vector of *Le. braziliensis, Le. guyanensis* and *Le. shawi*), *L. flaviscutellata* (vector of *Le. amazonensis*), *L. ubiquitalis* (vector of *Le. lainsoni*), *L. umbratilis* (vector of *Le. guyanensis*) and *L. antunesi* (epidemiologically shown to transmit *Le. lindenbergi*) were captured in Nova Mutum. *L. ubiquitalis* has been previously found in forested areas in Brazil, Peru and Bolivia and is the only known vector of *Le. lainsoni* so far[27].

Studies on natural *Leishmania* infection in the State of Mato Grosso are rare, with the exception for two that were published by Missawa[12,13] regarding proven VL vectors[12]. In Brazil, estimated infection rates of ACL vectors by *Leishmania* ranged from 0.16% to 7.1%: 0.4% in Bahia[11] and Maranhão State[28], from 0.8% to 7.1% in Minas Gerais[29], 2% in Rio de Janeiro[30], 0.3% in Porto Alegre[31], and 0.16% in Serra dos Carajás, Pará[32]. In Nova Mutum (MT) we found minimum infection rates of 0.8% for *Le. (V.) braziliensis* or *Le. (V.) guyanensis* and 0.08% for *Le. (L.) infantum* in *L. antunesi,* For *L. ubiquitalis,* the minimum rate of infection was 6.67% for *Le. braziliensis* or *Le. guyanensis*. There are no previous reports of *Leishmania* infection in any of these phlebotomine sandflies species. Vásquez-Trujillo[33] found *L. antunesi* naturally infected with *Leishmania* spp. and suggested its involvement in the transmission cycle of ACL in Colombia.

Genetically speaking, *Le. braziliensis* and *Le. guyanensis* are closely related species. We attempted to distinguish them in the *Leishmania* infected sandflies using hsp70[34] methodology. However, only one pooled sample of *L. antunesi* showed the expected amplified product (1300bp) (data not shown). After gel extraction, that DNA fragment provided insufficient sequencing results.

In a study conducted at the Reference Center for Leishmaniasis in Cuiabá (MT) using isozyme typing and molecular analysis, most (94.1%) ACL cases were because of infection by *Le. braziliensis,* whereas *Le. amazonensis* was the causative parasite in the others[35]. In another study that investigated patients from MT that were diagnosed with *Le. amazonensis*, *Le. braziliensis* and *Le. shawi* were determined to be the infecting species[36]. Although we were unable to discriminate between *Le. braziliensis* and *Le. guyanensis* in our

infected sandfly samples, it seems probable that the parasite was *Le. braziliensis,* as opposed of *Le. guyanensis*.

Based on the occurrence of autochthonous human cases of ACL and on the presence of *L. antunesi* and *L. ubiquitalis* that are infected by *Leishmania* (most likely *Le. braziliensis*), it is possible to hypothesize that those phlebotomine sandfly species might be involved in the zoonotic cycle of ACL in the rural *Cerrado* of Nova Mutum (MT).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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