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First report of natural infection of phlebotomines for *Leishmania (Leishmania) chagasi* captured in Ponta Porã, on the border between Brazil and Paraguay

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ABSTRACT

Objective: Visceral leishmaniasis has been reported in all Brazilian regions and, in Mato Grosso do Sul State, the occurrence of cases has increased significantly. The objective of this study was to identify the natural infection of phlebotomines by *Leishmania* in Ponta Porã, a Brazilian county bordering Paraguay. **Methods:** By using light CDC and Shannon traps, 185 sandfly females were captured, dissected and arranged in 107 pools subjected to DNA extraction and amplification by Polymerase chain reaction. **Results:** From the samples subjected to amplification, the fragment of 120 bp characteristic of *Leishmania* sp was observed in one of the groups, which is composed by a female species of *Evandromyia cortelezzii* (Brèthes, 1923). **Conclusions:** The minimum infection rate calculated for Ponta Porã was 0.54%, and the occurrence of flagellates and the confirmation of natural infection by *Leishmania chagasi* pose a serious concern for the transmission of visceral leishmaniasis in the region.

1. Introduction

The leishmaniasis are widely spread, with high prevalence in tropical regions like Brazil[1]. These diseases involve protozoa of the genus *Leishmania* transmitted by the bites of sand flies, hematophagous insects of nocturnal or crepuscular habits. These insects are considered natural vectors of the leishmaniasis[2].

Despite presenting a differential distribution along the digestive tract in the vectors, the *Leishmania* species take a flagellate form indistinguishable among the species[3]. The rates of naturally-infected phlebotomines in endemic areas and the correct identification of *Leishmania* species in a given sand fly species are crucial in the epidemiology of

leishmaniasis[4].

As a consequence of the environment changes imposed by man, these insects have adapted to rural, urban and peri-urban environments and the species have become resistant to adverse conditions, being able to explore new environments and getting closer to the peridomestic[5].

The research of flagellates in the vector's digestive tract and the identification of the parasite species are normally carried out by microscopic observation after dissection or by an attempt to isolate the agent from insects by culture. Female phlebotomines, however, are also hosts of some species of *Trypanosoma* and *Endotrypanum*, which go through a promastigote stage indistinguishable from *Leishmania* spp.[6].

The polymerase chain reaction (PCR) is a molecular technique used to identify the parasite in various biological materials, helping with the diagnosis of human and canine leishmaniasis as well as detecting its presence in the phlebotomines. This technique has been successfully applied to the analysis of several samples of insects coming

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from the country, in view of the difficulty in detecting, identifying and characterizing infectious organisms by conventional methods[7–9].

In the diagnosis of leishmaniasis, one of the main targets of the molecular techniques developed is the kinetoplastic DNA (kDNA)[10]. The kDNA is composed by two molecules, the minicircles of 1.42 Kb and the maxicircles of about 36 Kb. PCR can be used to increase the sensitivity in the detection of *Leishmania* by hybridization methods through the amplification of the minicircle sequencing[11].

The infection rate of visceral leishmaniasis has grown rapidly in many regions of South America, including the border areas between Brazil and Paraguay[12]. In 2007 the first case of human visceral leishmaniasis transmission in Ponta Porã, Mato Grosso do Sul, was confirmed and ten more cases were confirmed until March 2011, totaling 11 cases.[13].

The constant and abundant presence of *Leishmania* vectors in Ponta Porã is a reason for alert, as the county is likely to pass from the condition of vulnerable to a moderate or intense area of visceral leishmaniasis transmission. In addition to the growing confirmation of cases, the city is a land border with Pedro Juan Caballero County, in Paraguay[14].

Considering the scarcity of studies about bordering counties with these characteristics, the objective of this work was to detect the natural infection of phlebotomine sand flies by *Leishmania* using dissection and PCR for the molecular identification of the parasite.

2. Material and methods

2.1. Area of study

Ponta Porã is located in southern Mato Grosso do Sul, micro region of Dourados, between the geographic coordinates 22° 53' and 10'' S and 55° 42' and 32'' W. Average altitude is 655 m and population in 2009 was estimated at 75 941 inhabitants[15].

The total area is 5 328.62 km²; tropical climate with dry winter and high concentration of rainfall from November to March. The monthly average temperature is 23 °C and the vegetation is typical of Cerrado (savanna) and Atlantic Forest. Ponta Porã is a border city with a high growth in tourism, next to Pedro Juan Caballero, Republic of Paraguay. It is a land border, with no geographical barriers, which facilitates the transit of people and animals between the two countries[15]. According to Marcondes, possible vectors can also cross barriers. In places of arid climates such as Africa and Asia, the phlebotomines can fly for several kilometers whereas the American species generally move for only dozens of meters[16].

2.2. Collections

Phlebotomine sand flies were captured with CDC automatic light traps fortnightly installed at seven sites in Ponta Porã, both intra and in the peridomiciles, totaling 14 ecotopes. Collection was carried out in the period between April 2009 and March 2010, from 6 pm to 6 am, disregarding the summer time change. The sites were chosen so that they could comprise the whole urban area, considering the healthcare regions already defined for the municipality actions. The traps were so installed: Site 1: Inside and outside a property measuring 2.500 m², a place used for breeding horses located at Jardim Ivone; Site 2: In the intra- and peridomicile of a property (123m²) located at Jardim das Paineiras; Site 3: A house located in São João neighborhood; collections occurred both inside the house and in the peridomicile (in the hen-house); Site 4: Pousada do Bosque Hotel, located in the central area, where there is a remaining forest with preserved headspring and three residences—the traps were installed inside and outside the hen-house of one of the residences; Site 5: A house located at Bairro Granja, near a military area, 50 meters far from a stream; Site 6: An area of 230 m² where the traps were installed inside the house and in the peridomicile of a hen-house; Site 7: An area of 500 m² at Bairro São Francisco, with extensive swine and poultry breeding.

At Pousada do Bosque, captures using white Shannon traps, with a stand containing fluorescent lamps connected to a 12-volt battery were also carried out. Phlebotomine sand flies were captured manually with the aid of collector tubes labeled with the collecting time and inserted in a damp plaster pot until exam.

The phlebotomine sand flies captured were separated from other insects; males were placed on Petri dishes and taken to the Parasitology Laboratory of the Federal University of Mato Grosso do Sul, where they were clarified according to Forattini's methodology and then arranged on plates.

Dissection was used for studying the flagellates in the females' digestive tract. This method is most commonly used in natural infection research and requires skill to be performed. The females were immobilized in refrigerator and placed on a dry blade for removal of wings and paws. Dissection was performed under a stereoscopic microscope with the aid of a scalpel. A drop of sterile saline (0.9%) was placed on the plates, where dissection was carried out. By making two sections in the final portion of the abdomen, the digestive tract was removed with zigzag movements, covered with glass slide and examined under an optical microscope (400× magnification) for flagellates research. The phlebotomine identification is done using head, thorax and abdomen structures in accordance with Galati's keys[17].

2.3. Minimum infection rate

As phlebotomines are mostly packed in groups of samples, the minimum infection rate was calculated by using the following formula: Minimum rate (MR)= Number of positive

groups \times 100 / total number of insects^[18].

2.4. Extraction of *Leishmania* DNA

The extractions were carried out individually or in sample groups of one to six phlebotomines per tube, classified according to date of capture, collection site and species caught. The phenol–chloroform method was used.

2.5. Polymerase chain reaction

The DNA extracted from the samples was used to perform the detection of parasites by PCR, firstly standardized.

PCR Conditions: The DNA extracted was amplified using primers 13A and 13B. The reaction mixture (25 μ L) was prepared containing buffer 1X of Invitrogen, 0.2 mM of dNTPs, 1.5 mM of MgCl₂, two units of Taq polymerase (Invitrogen), 5 μ L of DNA, 19.75 μ L of water, 0.16 pmol of each primer 13A (5′-GTG GGG GAG GGG CGT TCT-3′) and 13B (5′-ATT TTA CAC CAA CCC CCA GTT-3′), described by Rodgers *et al.*, to amplify the conserved fragment of minicircle kDNA of *Leishmania*. DNA amplification was performed in thermocycler BIOERÒ model XP cyler using initial sequencing of 95 °C for 5 min^[10].

Subsequently, 30 cycles were performed, each divided into three steps: Denaturing (94 °C for one minute), annealing (54.5 °C for one minute) and polymerization (72 °C for 30 seconds). The material was then kept at 72 °C for 10 min (final extension) and the amplified product was stored at 4 °C until analysis.

In all reactions a positive control (C+) and a negative control (C-) (mix + water) were included. The reactions were standardized with DNA control provided by the Laboratory de Leishmaniasis of René Rachou/Fiocruz Research Station (Belo Horizonte, Brasil): *Leishmania chagasi* (*L. chagasi*) (MHOM/BR/74/PP/75), which were also the positive controls of the reactions. The expected final product was 120 bp.

For the amplification of the ITS-1 Region, primers L5.8S (5-TGATACCACTTATCGCACTT-3) / LITSR (5-CTGGATCATTTCCGATG-3) were used. The reactions were also standardized with DNA control provided by the same laboratory and research station (Belo Horizonte, Brasil): *L. chagasi* (MHOM/BR/74/PP/75), also serving as positive control of the reactions, with 320 bp as the expected final product.

2.6. Electrophoresis in agarose gel

The analysis of the amplification of DNA fragment of *Leishmania* was carried out using 12 μ L of PCR product plus 3 mL of sample buffer (0.25% of bromophenol blue, 0.25% xylene cyanol and 30% glycerol) by horizontal electrophoresis in 2% agarose gel, in TBE buffer (Tris base, boric acid and EDTA) 1X, pH 8.0 to 80 v and 400 mA for 13A and 13B.

After electrophoresis, the agarose gel was stained with

ethidium bromide (0.5 μ g/mL) and the amplification products were visualized and photographed under ultraviolet. Molecular marker of DNA 25 bp and the positive control were used to detect the presence of a fragment of 120 bp for the genus *Leishmania*.

2.7. Purification with PCR product

PCR products for ITS-1 region were purified using the purification kit QIAquick PCR (QIAGEN, Chatsworth, CA) according to the manufacturer's specifications.

2.8. Sequencing

The positive sample was sequenced at the National Laboratory of Biosciences (LNBio), National Research Station of Energy and Materials (CNPEM)/ (Brazilian Association of Light Technology Síncrotron) ABTLuS in Campinas, São Paulo.

2.9. Alignment

The analysis and alignment of the edited nucleotide sequence were performed with the MEGA(Molecular Evolutionary Genetics Analysis) program version 5.0 and Blast of the NCBI (National Center for Biotechnology Information) site .

3. Results

In the urban area of Ponta Porã, 185 phlebotomine females were captured with Shannon and CDC light traps. These specimens belonged to three sub tribes, seven genera and eight species according to Galati's nomenclature^[19]. The representative species of *Lutzomyia* subtribe were: *Lutzomyia longipalpis* (*L. longipalpis*) (Lutz & Neiva, 1912), *Evandromyia cortelezii* (*E. cortelezii*) (Brèthes, 1923), *Sciopemyia sordellii* (Shannon & Del Ponte, 1927), *Pintomyia pessoai* (Coutinho & Barreto, 1940) and *Pintomyia monticola* (Costa Lima, 1932). That of *Bumptomiyia* subtribe was *Brumptomiyia brumpti* (Larouse, 1920); and those of *Psychodopygina* subtribe were *Nyssomyia whitmani* (Antunes and Coutinho, 1939) and *Psathyromyia shannoni* (Dyar, 1929). *L. longipalpis* was the most frequent species (71.35%) (Table 1).

The 185 females were sorted out in 107 sample groups. The fragment of 120 bp, characteristic of *Leishmania* spp., was observed in one of the 107 groups of these samples. The minimum infection rate calculated for Ponta Porã was 0.54%. The positive sample had a 100% identity whit *L. chagasi* by the alignment in the nucleotideblast program (Blastn) (ACCESSION GU045591)(Figure 1). A female of *E. cortelezii* captured on Shannon traps, installed in the central area of the city was found to be infected by flagellates in the midgut

Table 1

Number of phlebotomines dissected for species captured in the urban area of Ponta Pora, Mato Grosso do Sul, Brazil between April 2009 to March 2010.

Species	No. of females	Percentage (%)	PCR
<i>Brumptomyia brumpti</i>	1	0.54	–
<i>Evandromyia cortelezzii</i>	7	3.78	<i>L. chagasi</i>
<i>Lutzomyia longipalpis</i>	132	71.35	–
<i>Nyssomyia whitmani</i>	14	7.57	–
<i>Psathyromyia shannoni</i>	15	8.11	–
<i>Pintomyia monticola</i>	2	1.08	–
<i>Pintomyia pessoai</i>	12	6.49	–
<i>Sciopemyia sordellii</i>	2	1.08	–

and hindgut of the digestive tract (Figure 2), accounting for 14.3% of all samples of this species.

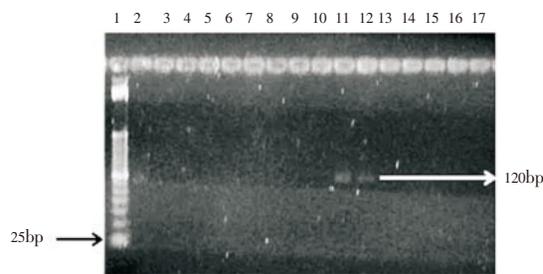


Figure 1. Result of the amplification of DNA extraction test in phlebotomines using primers 13A (5′-GTG GGG GAG GGG CGT TCT-3′) and 13B (5′-ATT TTA CAC CAA CCC CCA GTT-3′), described by Rodgers *et al* for the genus *Leishmania*.

1– Molecular marker of 25 bp; 2 to 10– Negative samples; 11– Positive sample (*E. cortelezzii*); 12– Positive control; 13– Negative control; 14 to 15– Negative samples.



Figure 2. Flagellates seen during dissection of midgut and hindgut of digestive tract of the *E. cortelezzii* female captured in the urban area of Ponta Porã, Mato Grosso do Sul.

4. Discussion

The loss of DNA during the washings with phenol/chloroform/isoamyl alcohol is unquestionable and the minimal DNA amount lost during the extraction process in insects may yield false negative results[18]. In the present study only one specimen, among the 107 groups of samples, was positive for *Leishmania* infection.

The analysis of the infection rate of the different species of *Leishmania* in the vector insects occurring in endemic areas helps to understand the epidemiology of the disease and also to adopt measures for its prevention and control[4].

DNA amplification through PCR is a practical and advantageous alternative because of its high specificity and sensitivity[19]. The use of primers that amplify the specific sequences of the parasite constitutes a tool to help the studies of vectorial incrimination[18]. In a study about diagnosis of American cutaneous leishmaniasis, PCR technique was used in 54 skin and mucosa biopsies using primers 13A and 13B of the conserved region of kDNA minicircle, PCR was positive in 81.5% samples[19].

Non-coding regions of DNA variables are useful in identifying strains of *L. chagasi*[20]. Among these regions, ITS-1 has generally been very informative because it differs in size and sequence among the species[21]. Studies carried out with the purpose of identifying a target for the right diagnosis of leishmaniasis show that the ITS-1 region is effective in detecting all known *Leishmania* spp.. Thus the amplification of the region in this study is justified[21].

In a study conducted in Parana State[22], among 2 487 females only one showed natural infection by flagellates, confirming the findings of the Ponta Porã study. Low natural infection rates of phlebotomines have been described, showing that the probability of flagellate detection by dissection is very small and that the method is time-consuming[23,24]. Hence the reason to employ faster and more sensitive techniques, such as PCR, as the technique success disregards the amount, stage and location of *Leishmania* in the vector's digestive tract[25].

Like in other Brazilian states, in Mato Grosso do Sul visceral leishmaniasis is widely distributed, and human cases increase every year. Even so studies about etiology and the particularities in the transmission process are scarce.

Ponta Porã presents a wide variation in native vegetation in view of the great demand for large areas for agriculture. It is noteworthy that, according to Ponta Porã official socioeconomic information, 16 558 cubic meters of wood, 31 974 cubic meters of wood in logs and 1 060 tons of charcoal have been produced in the past few years[26]. This clearly shows the removal of natural vegetation, causing the vectors

to migrate to regions where they can have food and favorable environmental conditions, including urban and household spaces.

The predominance of *L. longipalpis* in relation to other species of phlebotomines observed during the surveys is also found in other studies developed in Mato Grosso do Sul State^[18,28]. These results are compliant with the findings of Almeida *et al*^[14] in Ponta Porã.

The occurrence of some phlebotomine species in urban areas is due to the insects' ability to adapt to places where there are deep changes in their natural habitats, provoking restriction of biological space^[29–31]. The collection of the naturally-infected female of *E. cortelezzii* in urban areas confirms the findings. In addition, the species has been captured through the Shannon trap, used to capture the anthropophilic species in a given region^[32,33].

In this study, the infection of *E. cortelezzii* female by *L. chagasi* was detected for the first time in Ponta Porã; the minimum infection rate was 0.54%. The variation in the value of minimum infection rate occurs according to the amount of positive phlebotomine captured. In a study developed in Minas Gerais State the minimum rate of infection was 3.9% for *L. chagasi* where as in São Luis, Maranhão State, it was 0.25%^[19,29].

E. cortelezzii had already been collected in Mato Grosso do Sul^[28], inside the houses and in the peridomiciles in Ponta Porã and in Antônio João county, another land border with Paraguay^[9,14]. In both counties the transmission of visceral leishmaniasis was seen. Natural infection by *L. chagasi* was observed in a study carried out in Santa Luzia, Minas Gerais^[4].

Although the requirements of vector incrimination of *E. cortelezzii* have not been met, the increase of its frequency in the peridomicile, its growing anthropophilic character, its gradual adaptation to anthropic environments and the environmental conditions favorable to the persistence of the enzootic cycle of *Leishmania* are factors indicating its vector potential. Even though, more studies about this species should be conducted^[4,34].

According to the technical standards of the Manual for Surveillance and Control of Visceral Leishmaniasis, Ponta Porã used to be classified as a vulnerable area for visceral Leishmaniasis transmission until the first human cases, increasing in a yearly basis, were reported in 2007^[35].

The minimum infection rate calculated for Ponta Porã was 0.54%, and the detection of the natural infection vector is a reason why healthcare professionals should be alert to visceral Leishmaniasis transmission in the region. The results obtained show that the infection circulates in the phlebotomines found in the peridomicile. Moreover, it is necessary to continue this study in Ponta Porã to determine the possible presence of infection in other phlebotomine species. With the molecular tools available today, studies on the species-specific character of the *Leishmania* spp. can be conducted in loco. In addition, studies should also

be carried out in other areas of the state, so that the relation between infection rate and number of the cases may be established.

Preventive measures are needed so that the vector behavior and the health of the population may be monitored. Examples of these measures are monitoring systematized collection, verifying natural infection, and monitoring human and canine cases. The results from the present study point towards a need for epidemiological surveillance to follow up the human occupation of the environment that is taking place in the Ponta Porã region, especially because of its importance as a tourist center.

Conflict of interest statement

We declare that we have no conflict of interest.

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