

The molecular detection of different *Leishmania* species within sand flies from a cutaneous and visceral leishmaniasis sympatric area in Southeastern Brazil

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Over the last 20 years, there has been an increase in the number of leishmaniasis cases in Brazil. Belo Horizonte (BH) is one of the most highly populated Brazilian cities that is affected by visceral leishmaniasis (VL). The health services in BH are coordinated by a central nucleus that is subdivided into nine sanitary districts. Historically, the highest level of human VL cases was found in the northeast sanitary district (NSD). The objective of our study was to detect *Leishmania* infection in the phlebotomine sand flies collected in the NSD by dissection and molecular approaches. Following the occurrence of human VL cases in 2005, entomological captures were performed from July 2006-June 2007. Out of the 245 sand flies dissected, only three *Lutzomyia longipalpis* spp contained flagellates. The female sand flies were grouped into 120 pools according to date, collection site and species, with approximately 10 individual sand flies in each pool. Subsequently, the DNA was extracted and *Leishmania* spp and other parasites were detected and identified by polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism. *Leishmania infantum* was present in at least 19% of the *L. longipalpis* collected, in 3.8% of the *Nyssomyia whitmani* collected, in 33.3% of the *Evandromiya termitophila* collected and in 14.3% of the *Nyssomyia intermedia* collected. When the females of the *cortelezzii* complex were compared with each other, 3.2% of the females were infected with *Leishmania braziliensis*, whereas 3.2% of the females were infected with trypanosomatids.

Key words: *Leishmania* spp - sand flies - natural infection - Belo Horizonte

Several leishmaniasis studies have used molecular techniques to detect, identify and characterize *Leishmania* in humans and reservoirs (Degraeve et al. 1994, Cortes et al. 2004, Volpini et al. 2004, Carvalho et al. 2008). Molecular techniques are highly sensitive in the detection of *Leishmania* spp infections in phlebotomines. This sensitivity increases the understanding of the vector competence and leishmaniasis epidemiology (Aransay et al. 2000, Perruolo et al. 2006).

At the complex level, the identification of *Leishmania* spp in phlebotomines has employed various molecular techniques such as polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (PCR-RFLP) and probe hybridization (Volpini et al. 2004, de Pita-Pereira et al. 2005, Carvalho et al. 2008). Several DNA targets such as the rRNA gene, minexon-derived RNA gene, repeated genome sequences and the kDNA minicircle (Aransay et al. 2000, Paiva et al. 2006) are used in the PCR detection and identification of *Leishmania* spp.

Sensitivity and specificity are the main advantages of molecular methods regardless of the number, stage and location of the parasites in the phlebotomine digestive tract (Perez et al. 1994, de Pita-Pereira et al. 2005). Even in situations where excess insect DNA exists, the presence of a single parasite is enough for DNA detection by PCR (de Bruijn & Barker 1992). Several studies using molecular methods to evaluate *Leishmania* infection rates in phlebotomines have been performed in Brazil (de Pita-Pereira et al. 2005, 2008, Paiva et al. 2006, Kato et al. 2007, Carvalho et al. 2008, Saraiva et al. 2009, Rocha et al. 2010). In addition, studies in other trypanosomatids have been reported (Wallace & Hertig 1968, Arias et al. 1985, Naiff et al. 1989).

Leishmaniasis has occurred in record numbers in several Brazilian states (Souza et al. 2009). The detection and identification of *Leishmania* spp in naturally infected sand flies is important for disease prevention and to understand leishmaniasis epidemiology (Michalsky et al. 2002).

In several Brazilian cities, visceral leishmaniasis (VL) has become a serious public health problem. City urbanization and the adaptation of *Lutzomyia longipalpis* to the peridomicile have been linked to environmental changes as well as the migration, interaction and spread of sylvatic reservoirs and infected dogs to normally low transmission areas. Recently, VL outbreaks and epidemics have been reported in large Brazilian cities such as Belo Horizonte (BH), which is the capital of the state of Minas Gerais (MG) (MS 2006).

Financial support: LeishEpiNetSA (015407), CAPES, CNPq

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Received 13 July 2010

Accepted 27 October 2010

Phlebotomine fauna studies in BH have demonstrated the presence of vectors for American cutaneous leishmaniasis (ACL) and VL (Souza et al. 2004, Resende et al. 2006). In a study performed in BH from 2001-2003, Souza et al. (2004) reported the presence of the important leishmaniasis vectors *Lu. longipalpis*, *Nyssomyia whitmani* and *Nyssomyia intermedia*. However, they did not detect the presence of *Leishmania* in these sand fly species. Here, we studied the *Leishmania* spp infection rates in the phlebotomine population of BH's northeast sanitary district (NSD). We used DNA-based techniques to identify the *Leishmania* spp in an area where both ACL and VL are present.

SUBJECTS, MATERIALS AND METHODS

Study areas and phlebotomine collection - The research was performed in the Southeast region of Brazil (19°55'S 43°57'W) in BH. With a population of over 2.4 million people, BH is the fifth largest city in the country. This high-altitude city has a tropical wet and dry climate that corresponds to the Aw and As Köppen climate classification categories. The average annual temperature is approximately 21°C. The health services are coordinated by a central nucleus that is subdivided into nine sanitary districts encompassing the north, northeast, east, south central, west, Venda Nova, Pampulha and Barreiro.

Our study has been performed in the NSD, which has a population of 274,060, distributed into 69 neighbourhoods. Its territorial extension is 39.60 km² and has 19.46 km² of green area (PBH 2008) (Fig. 1). This is an endemic area of human and canine VL and cutaneous leishmaniasis. With 257 cases between 1994-2009, the NSD accumulated the highest number of human VL cases in BH. In 2006, 2007 and 2008, 23, 21 and 41 cases of VL occurred, respectively, with five cases resulting in death. The positive tests for canine leishmaniasis were high, reaching 10%. In each sanitary district of BH, several ACL cases were registered, but none were reported: in 2007, 25 cases were registered, in 2008, 27 cases, and in 2009, 44 cases (SINAN 2010).

The entomological study was performed from July 2006-June 2007. Phlebotomine sand flies were collected in the peridomestic area of residences where human VL cases had been reported in 2005 and in two green areas that were close to the urban areas. The insects were captured by 24 HP light traps (Pugedo et al. 2005) that were placed overnight from 6:00 pm-8:00 am. Using a Shannon light trap, one collection was performed each season, for a total of four collections.

Processing and packaging of collected phlebotomines - All phlebotomines were identified according to the Galati classification system (Galati 2003). The male specimens were prepared and mounted. The female specimens were subjected to dissection and DNA extraction. Subsequently, the extracted DNA was used in the PCR and PCR-RFLP reactions to identify the *Leishmania* spp.

Sand fly dissection was performed following a modification of a method by Carvalho (1973). The female specimens were immersed in Mycostatin (Squibb) solution at 100,000 U/mL for 15 min, immersed in a benzalkonium chloride solution (1:1000) (Zephiran, Winthrop) for 2 min and washed twice with sterile distilled water. All instruments and solutions were sterilized and the immersion and washing steps were performed in laminar flow. All insects were dissected in saline with 50 U/mL of amikacin sulphate on a glass slide and the digestive tract was examined under an optical microscope with 400X magnification.

The female digestive tract halves that had flagellates forms in the gut were transferred to an Eppendorf tube with saline containing 50 U/mL of amikacin sulphate. The tube was centrifuged at 500 g in an Eppendorf 5415 microcentrifuge for 5 min and then the supernatant was discarded. The pellet was resuspended in 300 µL of liver infusion tryptose (LIT) medium and transferred to tubes containing Novy-MacNeal-Nicolle plus LIT.

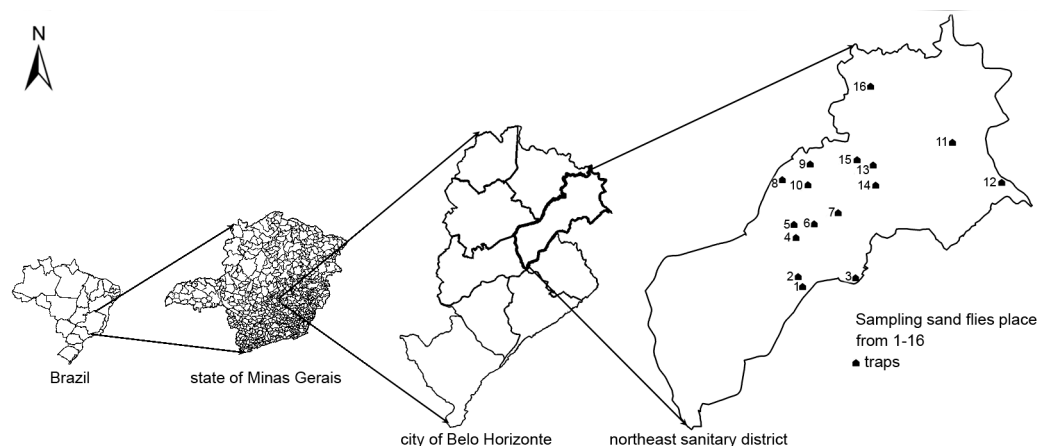


Fig. 1: administrative division of the municipal district of Belo Horizonte (BH), its location in the state of Minas Gerais and in Brazil, the location of the northeast sanitary district in BH and of the phlebotomine sampling sites in this regional from July 2006-June 2007. 11, 12: green areas; 1-10, 13-16: urban areas.

The other half of the material was stored in Eppendorf tubes and frozen at -70°C for the execution of the molecular assays. Females that did have flagellated forms in their gut were frozen. Depending on the number of specimens collected, females of the same species that were from the same trap and the same collection date were processed individually or in pools that contained up to 10 specimens. Female sand flies that had blood and flagellated forms in their digestive tracts were processed individually. The DNA was extracted using the Genra Puregene Blood Kit (Qiagen) according to the manufacturer's instructions.

Primers that amplified the IVS6 region of the cacophony gene of new world sand flies (5'-GTGGC-CGAACATAATGTTAG-3' and 5'-CCACGAACAAGT-TCAACATC-3') (Lins et al. 2002), were used to test the samples by PCR to exclude the possibility of PCR inhibition and to serve as a control for the extraction procedure. Two microlitres of hydrated DNA solution from each sample was added to PCR reactions that were performed with the GoTaq Green Master Mix PCR kit (Promega). The protocol was based on a study by de Pita-Pereira et al. (2005). Ten microlitres of amplified product was examined by electrophoresis on 2% agarose gel stained with ethidium bromide, visualized under ultra-violet transillumination and photographed. A positive control (DNA obtained from a *Lu. longipalpis* that was reared in the laboratory) and a negative control (a reaction without DNA) were used in all reactions.

Using a protocol adapted from de Pita-Pereira et al. (2005), primers that amplified the 120-bp sequence corresponding to the conserved region of *Leishmania* kDNA (5'CCG CCCCTATTTTACACCAACCC3', 5'GGCCAC-TATATTACCAACCC3' and 5'GGGGAGGG GGT-TCTGCGAA3') (Michalsky et al. 2002) were utilized to evaluate the presence of *Leishmania* DNA in all samples. The 10 μL PCR reaction was composed of 1 μL of dNTPs, 1 μL of enzyme buffer (Applied Biosystem, Roche), 0.6 μL of magnesium chloride (Applied Biosystem, Roche), 0.2 μL of Taq Gold (Applied Biosystem, Roche), 1 μL (5 pmol) of each initiator, 5.2 μL of water (double distilled and deionised) and 1 μL of sample DNA. A 5% polyacrylamide gel was used to electrophorese 4 μL of the amplified product and then the gel was stained with silver for visualization.

The amplified PCR product was digested with Hae III and analyzed in a 10% agarose gel to identify individual *Leishmania* spp in sand flies or a pool of sand flies. Digestion with this endonuclease distinguishes the *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania infantum* species (Volpini et al. 2004, de Andrade et al. 2006). Following the method described by de Andrade et al. (2001), a hybridization reaction of the PCR products (DOT-BLOT) was used to confirm the results of the PCR-RFLP. Because the exact rate of natural *Leishmania* infections in the positive pools was not known, it was assumed that only one female in each positive pool was infected. This procedure is adopted by many authors as the minimum infection rate (Paiva et al. 2006).

RESULTS

Out of the 245 female specimens that were dissected, only three of the specimens had flagellated forms freely moving in the middle portion of the intestine. These females were *Lu. longipalpis* spp and came from collection point 15, which was a house in an urban area (Table I). Because of contamination, it was not possible to isolate the flagellated forms in culture medium.

Both *Evandromyia sallesi* and *Evandromyia cortelez-zii* females were identified as belonging to the *cortelez-zii* complex. Because these sand flies are indistinguishable by morphological characteristics (Carvalho et al. 2009), the procedure was adopted.

During DNA extraction, the females were grouped into 120 samples. All of the samples amplified the 200 bp fragment corresponding to the cacophony gene, which was expected by PCR. The presence of the 200 bp fragment confirmed the high quality of the DNA obtained from all samples.

Using the primers described by Michalsky et al. (2002), 15 samples tested positive for *Leishmania* spp (Fig. 2). Among the positive samples, three females (analyzed individually) had blood in the digestive tract; one sample was *Lu. longipalpis*, one sample was *Ny. whitmani* and one sample was *Ny. intermedia* (Table I).

Twelve PCR product samples had restriction fragments characteristic of *L. infantum* (120 bp, 80 bp, 60 bp, 40 bp) following digestion with *Hae* III and one sample had a restriction fragment profile (80 bp and 40 bp) characteristic of *L. braziliensis*. Using this technique, there were no conclusive restriction fragment profiles for samples 47 and 70 (Fig. 3).

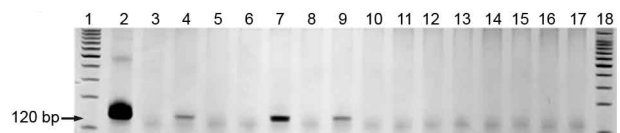


Fig. 2: silver stained polyacrylamide gel (5%) showing the polymerase chain reaction products with initiators for the conserved region kDNA minicircle of *Leishmania* sp. in DNA samples extracted from phlebotomine females. Lanes 1, 18: standard molecular weight 100 bp; 2: positive control (DNA from promastigotes of *Leishmania infantum* (MHOM/BR/1967/BH46)); 3: negative control without DNA; 4-17: samples relating to collection of July 2007.

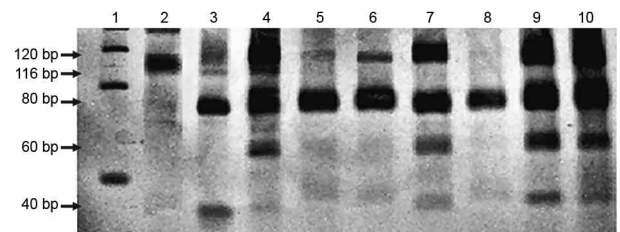


Fig. 3: polyacrylamide gel (10%), silver stained, showing the polymerase chain reaction-restriction fragment length polymorphism products. Lane 1: standard molecular weight 50 bp; 2: strain PH8 of *Leishmania amazonensis*; 3: strain M2903 of *Leishmania braziliensis*; 4: strain PP75 of *Leishmania infantum*; 5-10: samples positive to *L. infantum*.

TABLE I
 Samples of phlebotomines that tested positive for infection by *Leishmania* sp., site and capture date, sample type and techniques used to study the natural infection

Sample number	Trap type	Collection place ^a	Species	Specimens (n)	PCR-RFLP	Radioactive probes	Flagellates in the gut	Blood in the gut
1	HP	Green area 11	<i>Nyssomyia whitmani</i>	3	<i>Leishmania infantum</i>	<i>L. infantum</i>	-	-
4	HP	Urban area 15	<i>Ny. whitmani</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	-	+
6	HP	Green area 11	<i>Ny. whitmani</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	-	-
17	HP	Green area 11	<i>Ny. whitmani</i>	2	<i>L. infantum</i>	<i>L. infantum</i>	-	-
19	HP	Urban area 15	<i>Lutzomyia longipalpis</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	+	-
22	HP	Green area 11	<i>Ny. whitmani</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	-	-
47	HP	Green area 12	<i>cortellezzii</i> complex	1	Trypanosomatids	Does not belong to <i>donovani</i> complex	-	-
51	HP	Green area 12	<i>cortellezzii</i> complex	2	<i>Leishmania braziliensis</i>	Does not belong to <i>donovani</i> complex	-	-
52	HP	Urban area 15	<i>Lu. longipalpis</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	+	+
62	Shannon	Urban area 15	<i>Lu. longipalpis</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	+	-
70	HP	Urban area 15	<i>Nyssomyia intermedia</i>	1	<i>Leishmania</i> sp.	<i>L. infantum</i>	-	+
79	HP	Green area 12	<i>Evandromiya termitophila</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	-	-
82	HP	Urban area 15	<i>Lu. longipalpis</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	-	-
84	HP	Green area 12	<i>Ny. whitmani</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	-	-
88	Shannon	Green area 12	<i>Ny. whitmani</i>	1	<i>L. infantum</i>	<i>L. infantum</i>	-	-

^a: see Fig. 1; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.

Most of the sand fly infections were caused by *L. infantum* and the sand fly infection rates for this species were as follows: 19% infected with *Lu. longipalpis* (4 positive samples and 1 sample with blood in the gut), 3.8% infected with *Ny. whitmani* (7 positive samples and 1 individual with blood in the gut), 33.3% infected with *Evandromyia termitophila* and 14.3% infected with *Ny. intermedia* (1 positive sample with blood in the gut). For the *cortelezzii* complex, the sand fly infection rates were 3.2% infected with *L. braziliensis* and 3.2% infected with trypanosomatids (Table II).

Because the majority of the samples were characterized by PCR-RFLP as *L. infantum*, the samples were verified by a hybridization technique that used ³²P-labelled probes for the entire minicircle of the *Leishmania donovani* complex. Sample 70 tested positive by hybridization for *L. infantum* (*L. donovani* complex), whereas sample 47 tested negative by hybridization for *L. infantum* (*L. donovani* complex) (Table I).

DISCUSSION

Compared with some studies that used molecular approaches, the infection rates encountered in the phlebotomines from the NSD were high (Rodríguez et al. 1999, Luz et al. 2000). In the NSD, the highest *L. infantum* infection rate (19%) was found in *Lu. longipalpis*, which is the main vector of this species in Brazil. When compared to similar studies in other parts of Brazil, this infection rate is much higher. For example, Paiva et al. (2006) observed a *L. infantum* infection rate of 3.9% in *Lu. longipalpis* in the state of Mato Grosso do Sul by PCR, while Savani et al. (2009) observed a 0.25% infection rate of *Lu. longipalpis* by *L. infantum* and an infection rate of 1.25% by *L. amazonensis*.

We suggest three factors that could be responsible for the high infection rates observed in this study: (i) the majority of the samples were collected from the peridomiciles where human VL cases occurred in 2005, (ii) the high *Leishmania* spp infection rate (Oliveira et al. 2006) of the phlebotomines could be due to the favourable environmental conditions for vector development and the high prevalence of canine infection (in areas where zoonotic VL occurs, domestic dogs are considered to be

the most important infection source for phlebotomines) and (iii) molecular methods are more sensitive than the specific techniques traditionally used.

In the present study, the rates of *L. infantum* infection for *Ny. whitmani* and *Ny. intermedia* were 3.8% and 14.3%, respectively. This is the first report of *L. infantum* infection in these phlebotomine species. *Ny. whitmani* was the species most frequently captured in the area and PCR has previously shown that *Ny. whitmani* found in the metropolitan area of BH is infected with *L. braziliensis* (Carvalho et al. 2008). In the municipal district of Rio de Janeiro, de Pita-Pereira et al. (2005) has calculated that *Ny. intermedia* has a *L. braziliensis* infection rate of 15.6%. In Brazil, both of these species are the main vectors of *L. braziliensis* (Lainson & Rangel 2003). It is interesting to note that *Nyssomyia neivai*, a species that is phylogenetically close to *Ny. intermedia* (Marcondes 1996, Andrade Filho et al. 2003), has been shown to be infected with *L. infantum* north of MG (Saraiva et al. 2009). These species have similar habitats and are found in sympatry in some localities (Andrade Filho et al. 2007, Saraiva et al. 2008).

For the *Ny. whitmani* samples with and without blood in the gut, both PCR and hybridization reactions were positive for *L. infantum*. The hybridization reaction was positive in an individual *Ny. intermedia* sample that had blood in the gut. Unlike *Ny. intermedia*, these results suggest that *L. infantum* could colonize the *Ny. whitmani* gut.

Comparing the three *Ev. termitophila* specimens collected in this study, one had an *L. infantum* infection rate of 33.3%. Given the small number of insects collected, this result should be analyzed carefully. To the best of our knowledge, the infection of *Ev. termitophila* by any *Leishmania* spp has not been studied.

The *cortelezzii* complex specimens were naturally infected by *L. braziliensis* and trypanosomatids. This is the first report on *L. braziliensis* infection of females of the *cortelezzii* complex and it demonstrates the presence and circulation of this parasite regionally. In MG, both Carvalho et al. (2008) and Saraiva et al. (2009) encountered *L. infantum*-infected *Ev. cortelezzii* and *Ev. sallesi*.

Infected specimens from the *cortelezzii* complex were collected in the green area of collection point 12

TABLE II
Positivity rates for *Leishmania* by phlebotomine species and sample type

Species	Specimens collected (n)	Infected specimens/groups (n)	<i>Leishmania</i> species	Positivity rates for <i>Leishmania</i> (%)
<i>Nyssomyia whitmani</i>	181	7	<i>Leishmania infantum</i>	3.80
<i>Lutzomyia longipalpis</i>	21	4	<i>L. infantum</i>	19
Complexo <i>cortelezzii</i>	31	1	<i>Leishmania braziliensis</i>	3.20
Complexo <i>cortelezzii</i>	31	1	Trypanosomatids	3.20
<i>Nyssomyia intermedia</i>	7	1	<i>L. infantum</i>	14.30
<i>Evandromyia termitophila</i>	3	1	<i>L. infantum</i>	33.30

(Table I). In this same area and study period, 16 small mammal specimens, which included rodents and carnivores, were collected and were positively infected with *L. braziliensis* (LA Melo 2008, personal communication). Further studies on the behaviour of the *cortelezzii* complex species are necessary so that their possible participation in the local *L. braziliensis* transmission cycle can be determined.

Although we have used a highly sensitive PCR to detect the conserved region of the kDNA minicircle in infected sand flies (de Pita-Pereira et al. 2005, Carvalho et al. 2008), we used other molecular techniques to confirm the etiological identification, PCR-RFLP and hybridization. We also knew that trypanosomatids other than *Leishmania* would be present in the wild sand flies (Wallace & Hertig 1968, Arias et al. 1985, Naiff et al. 1989) and recent studies (Rocha et al. 2010) have shown that PCR-RFLP is unable to differentiate some *Leishmania* species from other trypanosomatids. In this study, the trypanosomatids that were present in the *cortelezzii* complex could be *Leishmania* spp other than *L. infantum* or *L. braziliensis* or other trypanosomatids that could be found in these sand flies.

When the results obtained by the molecular techniques and the observation of dissected intestines by microscope were compared, only three specimens were positive for *Leishmania* in by microscopic analyses, whereas 15 tested positive by molecular techniques, including the previous three mentioned above. This represents a 500% increase in the number of positive results obtained when the techniques are compared. This result illustrates why molecular techniques are considerably more sensitive and specific in *Leishmania* detection in sand flies.

The samples characterized as *L. infantum* by PCR-RFLP have been confirmed by the hybridization with radioactive probes, which are successfully used in the identification of *Leishmania* in vertebrate and phlebotomine tissues (de Pita-Pereira et al. 2005, Oliveira et al. 2005, de Andrade et al. 2006).

These results reveal an important circulation of *L. infantum* in the ecotopes studied in the NSD. Phlebotomine species that have never been shown to be infected naturally by any *Leishmania* species tested positive for *L. infantum*.

Because of the recent development of VL in Brazil, the characteristics of VL urbanization in the NSD must be studied in more detail. In addition, health authorities should consider a possible outbreak of urban or peri-urban *L. braziliensis*.

It is important to note that the occurrence of a natural infection is not enough to define a species as a vector in the leishmaniasis cycle. To define a phlebotomine species as the vector of a particular *Leishmania* sp., it must fit the parameters described by Killick-Kendrick (1990) and must be accepted and adopted by the Brazil Ministry of Health (MS 2007). Thus, these data only attest to the circulation of *L. infantum* and do not mean that *Ny. whitmani*, *Ny. intermedia* and *Ev. termitophila* are its vectors. Similarly, the *cortellezzi* complex can not be considered a vector of *L. braziliensis*.

ACKNOWLEDGEMENTS

To Northeast community, to the collaboration in the field sampling process, and to David Lee Nelson, by the English language review.

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