Acta Tropica 148 (2015) 128–136

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Epidemiological aspects of vector, parasite, and domestic reservoir in areas of recent transmission and no reported human cases of visceral leishmaniasis in Brazil

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ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form 4 March 2015
Accepted 6 April 2015
Available online 14 April 2015

Keywords:
Canine visceral leishmaniasis
Lutzomyia longipalpis
Lutzomyia lloydi
cortelezzii complex
Leishmania infantum
Leishmania braziliensis

ABSTRACT

About 97% of the human cases of the American visceral leishmaniasis (VL) occur in Brazil. In the last few years, the disease expanded to medium- and large-sized cities, in which surveillance and control actions have been intensified, in an effort to control VL spreading. Our two-year study was conducted in Belo Horizonte, the sixth most populous city in Brazil, which is endemic for VL. We focused in two particular districts of recent transmission of the disease, with no reported human cases and submitted to minor surveillance and control actions. Our aim was to draw an epidemiological profile of the local situation concerning *Lutzomyia* vector, *Leishmania* parasites, and the main domestic reservoirs (dogs). *Lutzomyia longipalpis* comprised 96.5% of the total phlebotomine sand flies captured and displayed an expressive minimal infection rate by *Leishmania infantum* (16.7%). Positive correlations were found between the population densities of *L. longipalpis*, rainfall and temperature. *L. infantum* was also detected in the *cortelezzii* complex and, for the first time, in *Lutzomyia lloydi*. *Leishmania braziliensis*, an etiological agent of the American cutaneous leishmaniasis, was also identified in *L. longipalpis*. Among the 1408 dogs serologically tested by standard enzyme-linked and fluorescence immune assays (ELISA/IFA) 3.6% were positive for VL. *L. infantum* DNA and *Leishmania* parasites were identified in 100% and 72.5% of the seropositive dogs, respectively. The co-positivity of other diagnostic tests for VL—*Leishmania*-nested PCR, imprint and myeloculture—was compared to the standard serology. Both symptomatic or asymptomatic dogs displayed an equal average number of positive diagnostic tests for VL. The districts studied display favorable conditions for the rapid spreading of human infection, in terms of *L. longipalpis* population density, and presence of *L. infantum* in both vector and main reservoir.

1. Introduction

Leishmaniases are present in four out of the five continents and are endemic in 98 countries, with more than 350 million individuals currently at risk. The visceral form of leishmaniasis (VL) causes large-scale and tenacious epidemics, with high fatality rates (WHO, 2014). About 97% of the human cases of VL in the Americas occur in Brazil, where more than 70,000 official notifications and more than 3800 deaths were recorded over the last three decades (Werneck, 2010; PAHO, 2013). The epidemiological triad of VL in the country involves *Leishmania infantum* (syn. *Leishmania chagasi*) parasites as the etiological agents, *Lutzomyia longipalpis* phlebotomine sand flies as the main vectors, and dogs (*Canis familiaris*)

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http://dx.doi.org/10.1016/j.actatropica.2015.04.002
0001-706X/

as the principal domestic reservoirs (Deane and Deane, 1955). It has been observed that canine VL usually precedes human cases of VL (Brazilian Ministry of Health, 2009). Other synantropic reservoirs of VL are foxes (*Dusicyon vetulus* and *Cerdocyon thous*), opossums (*Didelphis albiventris* and *Didelphis marsupialis*) and rodents (*Rattus rattus*, *Nectomys squamipes*, *Trichomys apereoides*, *Proechimys canicollis*, *Coendu prehensilis*) (Quinnell and Courtenay, 2009), although the epidemiological role played by the latter in the urban transmission remains uncertain.

Originally, VL was limited to rural environments; however, in the course of time, the disease underwent a clear epidemiological transition, with increasing incidences in urban areas. In Brazil, it reached medium- and large-sized cities (Brazilian Ministry of Health, 2006). This expansion was due to a set of socioeconomic, physical, and biological factors induced by human activities, which culminated in the adaptation of vectors and reservoirs to urban areas (Lainson, 1989; Rangel and Maurício, 2008).

Most of the extant studies conducted in Brazil do not address the entire epidemiological chain of VL (see Cabrera et al., 2003; Guerra et al., 2004 for exceptions). On the other hand, those that do provide a detailed analysis of the full transmission cycle are not recent, and thus may not reflect the current disease characteristics and prevalence (Deane, 1956; Marzochi et al., 1985). Moreover, most of these studies were performed in endemic regions, where the transmission cycle is fully active and the epidemiological transmission risk (ETR) of VL is high. The actions of the Surveillance and Control Program of VL of the Brazilian Ministry of Health vary according to the local ETR and are generally more intense in the areas with high ETR, where the VL has been established for a number of years. The ETR classification is based on the average number of reported human cases in the last three years (n), and varies from sporadic ($n < 2.4$), to medium ($2.4 \leq n < 4.4$) or intense ($n \geq 4.4$) (Brazilian Ministry of Health, 2009).

Our study was conducted in Belo Horizonte, the sixth most populous city in Brazil, which is an area of intense ETR for VL, as a whole. However, the average number of human cases varies from district to district and it has been noted that VL is spreading from the Northern to the Southern districts of the city [unpublished data]. The actions of the Surveillance and Control Program of VL concentrate in the districts with intense or medium ETR. Despite the application of systematic control actions—such as the continuous removal of seropositive dogs, early diagnosis/treatment of human cases, and chemical vector control—the VL is still in expansion in the urban areas of Brazil. In the last two quinquennia (2001–2005 and 2006–2010), the prevalence of canine VL remained stable around 18%, despite intensive screening and culling actions. During the same period, the overall number of human cases increased by 41% [unpublished data]. There is no consensus about the efficacy of these control actions adopted, particularly the canine culling (Dye, 1996; Courtenay et al., 2002; Ribeiro et al., 2013).

The two districts presently studied are located in the South of Belo Horizonte. Despite the presence of canine cases of VL, no human cases have been reported therein. Our aim was to evaluate the current epidemiological status of vector, parasite, and canine reservoir in those districts with recent transmission of VL. Due to their sporadic ETR, they have been submitted to minor actions of the Surveillance and Control Program of VL.

2. Materials and methods

2.1. Ethical procedures

The present study was approved by the Ethics Committee on Animal Experimentation of Fundação Oswaldo Cruz (CEUA/FIOCRUZ) under the license no. LW-21/11 (protocol no. P-85/10-2). All the procedures followed the technical norms established by the Federal Board of Veterinary Medicine (CFMV resolution no. 714/2002). Euthanasia was performed at the Zoonosis Control Center in Belo Horizonte, according to the screening-culling procedure of the Program for Visceral Leishmaniasis Control of the Brazilian Ministry of Health. The dog owners were informed of the project objectives and voluntarily signed the Statement of Informed Consent regarding the sample collection for biopsy.

2.2. Study area

Our study was developed in two districts of Belo Horizonte (19°55′15″ S, 43°56′16″ W), the capital of the Brazilian state of Minas Gerais (Fig. 1). Belo Horizonte occupies an area of 331.4 km^2 with population of 2,375,151 inhabitants and an average human development index (HDI) of 0.810 (UNDP, 2013). The two districts, namely Salgado Filho and Miramar, were selected due to the absence of reported human cases of VL. Hence, minor control actions of the Surveillance and Control Program of VL have been applied therein.

2.3. Entomological survey

Entomological captures were performed from September 2010 to August 2012, during three consecutive nights from 5:00 pm to 9:00 am, always in the first week of each month, and included nine houses per district (Fig. 1). The houses were selected based on previous canine cases of VL in the neighborhood, in 2010, as well as on environmental conditions that favor the rearing of phlebotomine sand flies such as shadowed areas, presence of domestic animals, and fruit trees. Two HP light traps (Pugedo et al., 2005) were mounted in the peri- and intradomiciles of the houses.

The phlebotomine sand flies of both genders were stored in 70% ethanol until species identification. The non-engorged females from the third day of capture per month, in particular, were preserved in 6% DMSO instead. The head and the last three abdominal segments of every female were removed and slide-mounted with Berlese liquid for species identification. The remaining body parts of the females from the third days of capture were used for natural infection assays.

The captured phlebotomine sand flies of both sexes were identified using specific descriptions, taxonomic keys, and comparison with specimens from the Reference Collection of Phlebotomine Sand Flies of the Centro de Pesquisas René Rachou/FIOCRUZ. We adopted the species classification proposed by Young and Duncan (1994). Due to their morphological similarity, *Lutzomyia sallesi* and *L. cortelezzii* species were considered as *cortelezzii* complex. Specimens with missing or incomplete characters that impaired their identification were considered *Lutzomyia* sp.

2.4. Climate data

Monthly climate data (maximum temperature, relative humidity, and total rainfall) were collected at a meteorological station of the Fifth District of the Brazilian Institute of Meteorology, located in Belo Horizonte, Minas Gerais state. Average values of each climate variable and their respective standard deviations were calculated on annual basis. Concerning rainfall, months with precipitation indices higher and lower than the annual average were considered rainy or dry, respectively. For comparison purposes, we normalized the data by taking the highest precipitation value, in each year, as 100%. Similarly, the highest number of *L. longipalpis* specimens captured per year was also taken as 100% for the respective year.

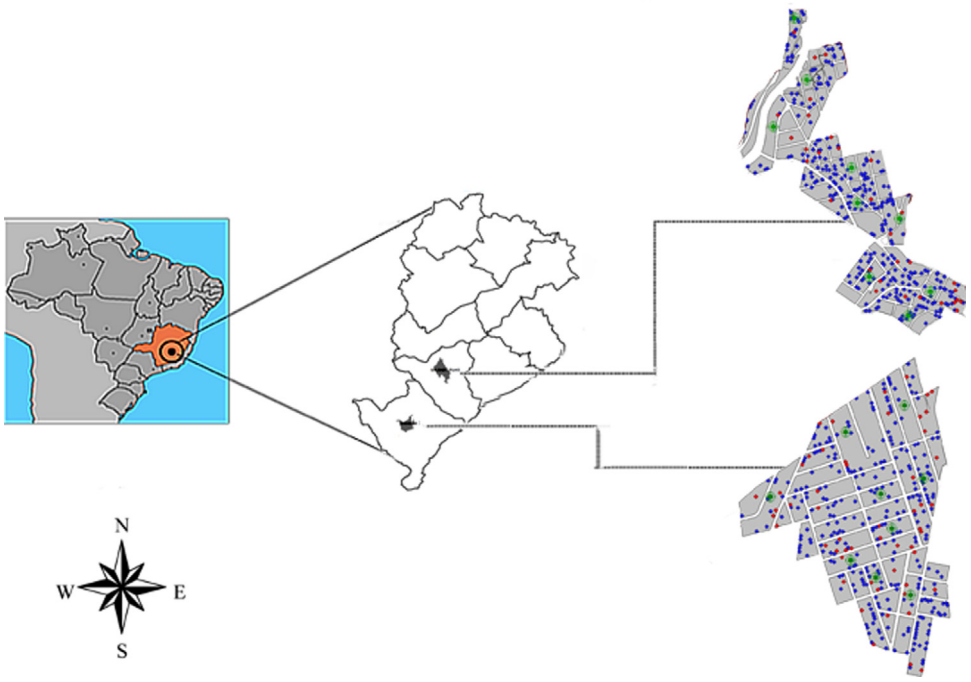

Fig. 1. Geographical localization of the two studied districts (top right – Salgado Filho; top bottom – Miramar) in the city of Belo Horizonte, Minas Gerais state, Brazil. Phlebotomine sand fly capture sites (nine per district) are indicated by green asterisks. Period of capture: September 2010 to August 2012. Seropositive and seronegative dogs from the canine census survey denoted by red and blue dots, respectively. One dot may represent more than one dog. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

2.5. *DNA extraction from phlebotomine sand flies and dogs*

Total DNA was extracted from the phlebotomine sand fly females captured every third day per month, using commercial kits (Puragene® Core KitA from QIAGEN). When appropriate, up to ten specimens from the same species, capture site and date were combined as a single test sample for DNA extraction. The reliability of the extraction was verified by the amplification of the IV S6 region from a constitutive gene (cacophony) for *Lutzomyia* (Lins et al., 2002) and the DNA was used as template in a nested PCR reaction for *Leishmania* (*Ln*PCR). Negative (no DNA) and positive (DNA from *Lutzomyia*) controls were run in parallel. Total DNA was also extracted from canine spleen, mesenteric lymph node, and skin by the Cell and Tissue Genomic Prep™ kit (GE Healthcare). The GFX™ Genomic Blood DNA Purification kit (GE Healthcare) was used to extract DNA from bone marrow aspirates. The extracted canine DNA served as template in *Ln*PCR. All the amplification fragments were analyzed by electrophoresis on 2% agarose gels followed by ethidium bromide staining and UV visualization.

2.6. *Nested PCR for* Leishmania *(Ln*PCR*)*

Total DNA from phlebotomine sand flies and dogs was submitted to *Ln*PCR for the SSUrRNA gene that amplifies a conserved fragment for *Leishmania* (van Eys et al., 1992; Cruz et al., 2002, 2006). Negative (no DNA) and positive (DNA extracted from *L. infantum* MHOM/BR74/PP75) controls were run in parallel.

2.7. *Minimal infection rate of* L. longipalpis *by* L. infantum

The minimal infection rate (MRI) by *L. infantum* was estimated using the formula MRI = number of positive test samples of *L. longipalpis* × 100/total number of *L. longipalpis* specimens captured (Paiva et al., 2006).

2.8. Leishmania *identification*

The fragments amplified by *Ln*PCR were purified from agarose gels using the QIAquick Gel Extraction kit (QIAGEN) and submitted to DNA sequencing, in both directions, using the BigDye® Terminator v3.1 Cycle kit and the ABI 3730 analyzer (Life Technologies). The nucleotide segments were aligned with *Leishmania braziliensis* (M80292.1), *L. amazonensis* (M80293.1), and *L. infantum* (M81430.1) DNA sequences deposited in the GenBank® database. BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html), BLAST (www.ncbi.nlm.nih.gov/BLAST), and MacVector NTI® tools were employed in sequence editing and alignment.

2.9. *Collection of canine samples*

A census survey of the local population of dogs was performed in 2011. After collection in filter paper, the blood eluates were tested by immunofluorescence indirect assay (IFA) (Camargo and Rebonato, 1969) and enzyme-linked immunosorbent assay (ELISA) (Voller et al., 1979), using the appropriate kits produced by Biomanguinhos (Fiocruz, RJ, Brazil). Dogs for which absorbance values were equal to or higher than three times the standard deviation of the cutoff value in ELISA and that were positive at ≥1:40 dilution in IFA were considered seropositive for VL, according to the diagnostic parameters adopted by the Brazilian Ministry of Health. The seropositive dogs were examined by veterinary physicians and classified as asymptomatic or symptomatic, depending on the absence or presence of signs and symptoms suggestive of *Leishmania* infection. The signs and symptoms observed were lymphoid adenopathy, slight decrease of weight and/or opaque hair, cutaneous alterations (depilation, furfuraceous eczema, ulcers), onychogryphosis, keratoconjunctivitis and rigidity of posterior limbs (Mancianti et al., 1988). Oligosymptomatic cases were considered as a part of the symptomatic group, rather than separately.

After bone marrow aspiration, the seropositive dogs were euthanized at the Zoonosis Control Center in Belo Horizonte. Spleen,

Table 1
Phlebotomine sand flies captured in two districts of Belo Horizonte, in the Brazilian state of Minas Gerais, with recent transmission profile and no reported cases of human leishmaniasis. Study period: September 2010–August 2012.

Species	Male	Female	Total	%
cortelezzii complex	36	69	105	2.02
Lutzomyia intermedia	4	3	7	0.13
L. ischyracantha	0	2	2	0.04
L. lloydi	0	1	1	0.02
L. longipalpis	4516	498	5014	96.54
L. whitmani	2	5	7	0.13
Lutzomyia spp.	23	35	58	1.12
Total	4581	613	5194	100.0
%	88.20	11.80	100.0	

mesenteric lymph node, and skin fragments were collected and used for preparation of imprints by slide apposition. Slide smears were prepared with bone marrow aspirates. All the slides were examined for the presence of *Leishmania* parasites, after Giemsa staining. The DNA extracted from canine tissues was used as a template in *Ln*PCR and the bone marrow aspirates were also seeded in NNN/LIT culture medium for *Leishmania* isolation attempts.

2.10. Statistical analysis

The influence of climate variables on the population density of *L. longipalpis* was evaluated and the results expressed as simple Spearman correlation coefficient (r_s), for each pair of variables.

The standard ELISA/IFA serological methodology for canine visceral leishmaniasis was taken as reference and the performance of the additional diagnostic tests was evaluated relative to that. Since all the dogs were positive by the standard method, the performance of other diagnostic tests was measured by their precision or accuracy, which express the total of correct classifications. In addition, given that the reference was not the certainty of absence of the disease but a serological result, we used the term co-positivity (that is equivalent to relative sensitivity). The overall co-positivity of the additional diagnostic assays was compared by the chi-square test whereas the co-positivity in the two clinical groups of dogs—symptomatic and asymptomatic—and tissue positivity by *Ln*PCR were compared by the Fisher's exact test. The Mann–Whitney test was employed to compare the number of positive tests among the three tests, in the two clinical groups of dogs.

All the statistical analysis was performed using the Prism 6 software (GraphPad Inc., USA) with 5% of significance level.

3. Results

3.1. Phlebotomine sand flies survey

During the study period, a total of 5194 phlebotomine sand flies belonging to six species were captured (Table 1). *L. longipalpis* was the most prevalent among them. *Lutzomyia whitmani* and *Lutzomyia intermedia*—proven vectors of the American cutaneous leishmaniasis (ACL)—were also present, albeit at lower rates. *L. longipalpis* males were more abundant than females, with an overall male/female ratio of 9:1. *L. longipalpis* specimens were mostly captured (88.1%) in the peridomiciles (data not shown).

3.2. Climate and fluctuation of L. longipalpis population

During the two years of study, the temperature varied from 24 °C to 31 °C with an average of 27.0 ± 1.6 °C. Humidity ranged between 48.0% and 78.0%, averaging at 63.0 ± 7.8%. Major variations occurred in the total precipitation along each year, as expressed by the high standard deviations and the respective variation coefficients: 151.3 ± 143.8 mm (CV = 95.0%) for the first year and 167.9 ± 216.8 mm (CV = 129.1%) for the second year. Five rainy (October, November, December, January, and March) and seven dry months were readily identified in each year and the population densities of *L. longipalpis* markedly increased after rainfall peaks (Fig. 2).

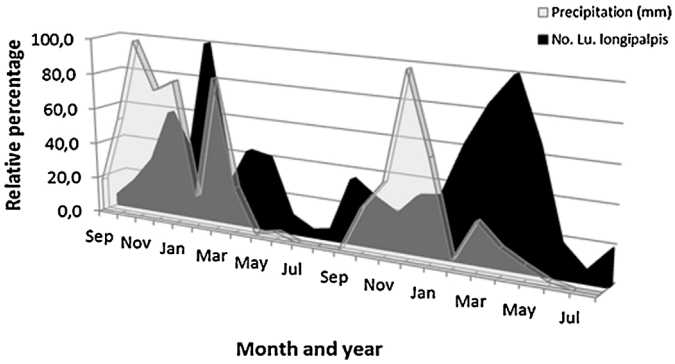

Fig. 2. Pluviometry and population fluctuation of *L. longipalpis* phlebotomine sand flies in the two districts with no reported cases of VL in Belo Horizonte, Minas Gerais, Brazil. Period of study: September 2010 to August 2012.

Positive correlations were found between the monthly population densities of *L. longipalpis* and the three climate variables with correlation coefficients (r_s) of 0.4177 for rainfall, 0.5430 for temperature and 0.3480 for humidity. However, statistical significance was found only for rainfall (*P*-value = 0.0422) and temperature (*P*-value = 0.0061).

3.3. Detection of Leishmania DNA in phlebotomine sand flies

The presence of *Leishmania* DNA was investigated in 93 test samples prepared with 152 *Lutzomyia* females. The preparation of pooled samples was applicable for *L. longipalpis* and *L. whitmani*; otherwise, single specimens were employed as test samples. All the test samples displayed the expected 220 bp fragment for the *Lutzomyia* cacophony gene (data not shown). *Leishmania* DNA was detected in 29 of the 93 test samples, as indicated by the presence of a 353 bp fragment following *Ln*PCR (Fig. 3A).

Table 2 summarizes the *Leishmania* infection ratios and species identification in the *Lutzomyia* test samples. Positivity for *Leishmania* sp. means that species identification attempts were unsuccessful. The MRI of *L. longipalpis* by *L. infantum* was estimated as 16.7%.

3.4. Canine serology and co-positivity of other diagnostic tests for VL

Among the 1408 dogs tested for VL, 51 (3.6%) were seropositive by ELISA/IFA. The seropositive dogs were distributed throughout the two districts, with no clusterization (Fig. 1). The dogs were also

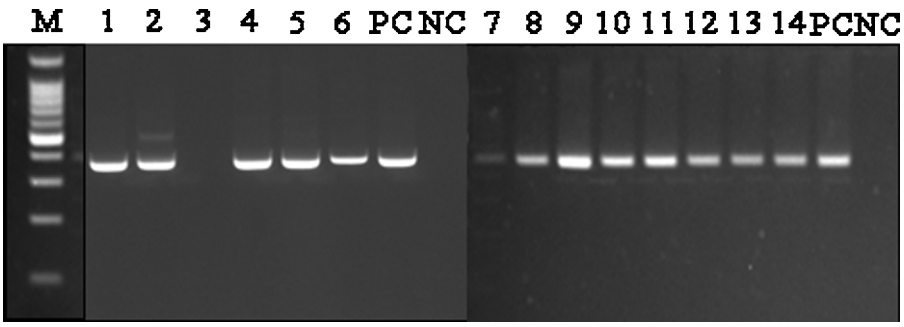

Fig. 3. Representative agarose gel of *Leishmania* nested PCR (*Ln*PCR) products of phlebotomine sand fly (A) and seropositive dog (B) DNA amplified with primers for the *Leishmania* genus, after ethidium bromide staining. Samples: M. 100 bp DNA ladder (the strongest band in the gel corresponds to 500 bp); phlebotomine sand fly DNA (1–6); canine DNA (7–14). PC. Positive control [*L. braziliensis* (MHOM/BR/75/M2903)]. NC: negative control (no DNA).

Table 2
Leishmania parasites identified in *Lutzomyia* DNA after *Leishmania*-nested PCR (*Ln*PCR) and nucleotide sequencing. Total DNA was extracted from non-engorged females from the third day of capture per month. The study was developed in two districts with recent transmission profile and no reported cases of human visceral leishmaniasis, in Belo Horizonte, state of Minas Gerais (Brazil). Study period: September 2010 and August 2012.

Species	Number of females		Number of test samples	*Leishmania* species in positive samples		
	Available	Per test sample		*L. infantum*	*L. braziliensis*	*Leishmania* sp.
cortelezzii complex	10	1	10	1	0	1
L. intermedia	1	1	1	0	0	0
L. lloydi	1	1	1	1	0	0
L. longipalpis	138	1–10	80	23	2	1
L. whitmani	2	2	1	0	0	0
Total	152	–	93	25	2	2

tested by one molecular-based (*Ln*PCR) assay and two parasite-based (imprint and myeloculture) assays. A representative result of *Ln*PCR is shown in Fig. 3B. The number of positive dogs and the positivity rates varied according to the canine tissue analyzed: 51 dogs (100%) for the spleen, 41 dogs (80%) for the bone marrow, 35 dogs (69%) for the skin, and 29 dogs (57%) for the mesenteric lymph node (Fig. 4). While the difference in positivity was significant when lymph node was compared to bone marrow ($P = 0.001$) and to skin ($P = 0.031$), this was not the case for bone marrow comparison to skin. The number of positive dogs by imprint or

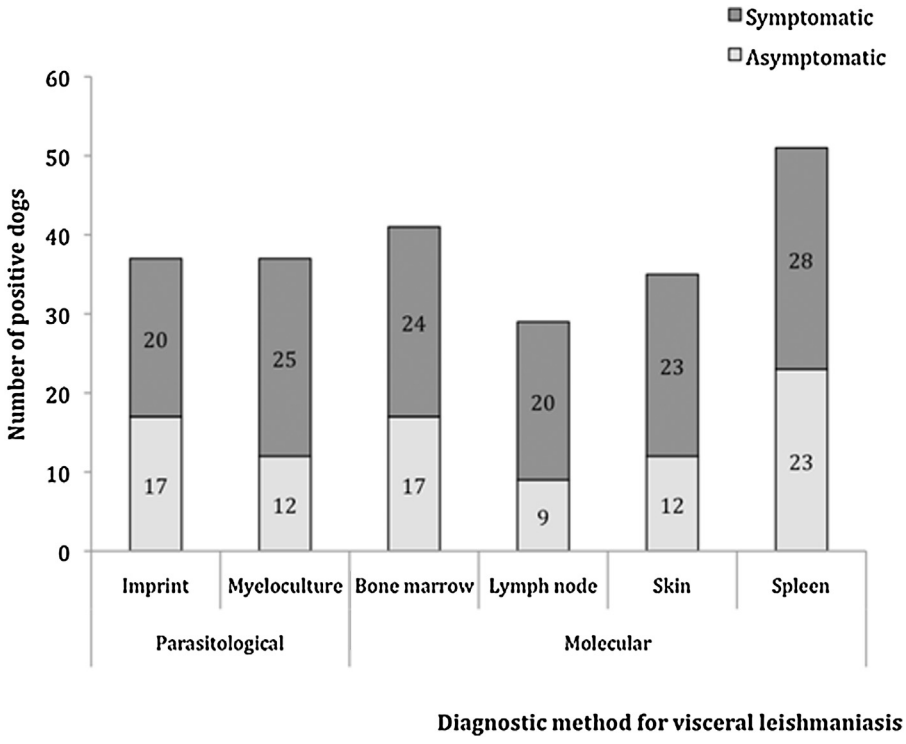

Fig. 4. Frequency distribution of asymptomatic and symptomatic seropositive dogs for canine visceral leishmaniasis ($n = 51$), according to the diagnostic methods employed herein. The numbers inside the bars indicate the number of positive dogs per diagnostic method and clinical group. Molecular diagnosis was performed by *Leishmania* nested PCR (*Ln*PCR) with DNA from the tissue samples specified as templates.

Table 3
Contingency table of the co-positivities (relative sensitivity) for diagnostic tests of canine visceral leishmaniasis. The reference was the ELISA-IFAT result, as adopted by the Surveillance and Control Program of visceral leishmaniasis of the Brazilian Ministry of Health, at the time of our study. The fifty-one sero-positive dogs ($n = 51$) were sampled from two districts with recent transmission profile and no human cases of leishmaniasis in Belo Horizonte, in the Brazilian state of Minas Gerais.

Test		IFAT-ELISA			
		Negative		Positive	
		No.	%	No.	%
*Ln*PCR	Negative	–	–	0	0
	Positive	–	–	51	100
Imprint	Negative	–	–	14	27.5
	Positive	–	–	37	72.5
Myeloculture	Negative	–	–	14	27.5
	Positive	–	–	37	72.5

myeloculture was the same (37 dogs). The co-positivity of these parasite-based methods—72.5% in both cases—was significantly lower (*P*-value = 0.0002), when compared to canine spleen *Ln*PCR (Table 3).

Among the 51 seropositive dogs in our sample, 28 were clinically symptomatic whereas 23 were asymptomatic. *Ln*PCR with canine spleen tissue, in particular, gave 100% of co-positivity with the ELISA/IFA, for both clinical conditions. The imprint results were not statistically different for the two clinical groups (*P*-value = 1.000) (Table 4). Differently, the myeloculture displayed 89.3% of co-positivity for the symptomatic dogs compared to 52.2% in the asymptomatic ones. These percentages were significantly different with *P*-value = 0.0045.

The presence of *Leishmania* assessed by either of the two parasite-based methods was confirmed in 72.5% of the seropositive dogs (Fig. 5). However, the identified dogs were not necessarily the same in both tests. A combined analysis of the results of the three additional tests showed that 28 dogs (54.9%) were positive in all three tests, 18 dogs (35%) were positive by *Ln*PCR and one of the parasite-based tests, and 9.8% were identified as positive by *Ln*PCR but with no confirmation of the presence of *Leishmania* parasites (Fig. 5).

The average number of positive tests in the symptomatic canine group was 2.607 with a standard deviation of 0.629. This value decreased to 2.261 ± 0.689 in the asymptomatic group (data not shown). The difference in the total of positive tests, according to the clinical canine group, was not statistically significant (*P*-value = 0.066).

3.5. *Infecting* Leishmania *in dogs*

L. infantum was identified as the infecting parasite in the spleen tissue of all the seropositive dogs.

4. Discussion

Regarding the phlebotomine sand fly fauna, *L. longipalpis*—the main vector of American VL—was the predominant species (96.5%) in our study, thus confirming the high adaptation of this phlebotomine species to urban environments (Barata et al., 2004; Silva et al., 2007; Michalsky et al., 2009, 2011). *L. longipalpis* was more abundant (88.1%) in the peridomicile of the houses, as observed in other studies (Michalsky et al., 2009; Missawa and Dias, 2007; Barata et al., 2013, among others). The predominance of *L. longipalpis* males over females (M/F = 9.0) was expressive (Michalsky et al., 2009; Ximenes et al., 1999; Souza et al., 2004).

Increased *L. longipalpis* population density was correlated to increased rainfall and temperature, consistently to previous reports (Michalsky et al., 2009; Souza et al., 2004; Dias et al., 2007; Oliveira et al., 2008). This effect may help in planning more effective chemical spraying actions to control *L. longipalpis*, which is one of the main strategies employed to limit the VL spreading in Brazil (Silva et al., 2007).

The minimal rate of infection (18.8%) of *L. longipalpis* by *Leishmania* was surprisingly high compared to average values reported in the literature (Missawa et al., 2010). However, it is close to that reported before (19%) for an endemic district for VL, in Belo Horizonte (Saraiva et al., 2010). Mostly (92.0%) of the infecting *Leishmania* was *L. infantum*, the etiological vector of VL. The high rate of natural infection by *L. infantum* associated to the expressive population density of *L. longipalpis* indicates very favorable vector conditions for the rapid spreading of VL. *L. infantum* was also found in the *cortelezzii* phlebotomine complex which was—together with *L. braziliensis* DNA—reported previously (Saraiva et al., 2009; Carvalho et al., 2008). In addition, we detected *L. infantum* DNA in *L. lloydi*, which is a novel finding.

Recently, several reports have been published on the presence of genetic material of etiological agents of cutaneous leishmaniasis in visceral leishmaniasis vectors and vice versa. These findings might be explained by variations in permissivity of the sand fly species to *Leishmania* infection. *L. longipalpis*, which was found infected by *L. braziliensis* (present study, Paiva et al., 2010) has been considered a permissive species (reviewed by Kamhawi, 2006). Obviously, vector competence for leishmaniasis is a complex matter and cannot be ascertained solely on the presence of *Leishmania* DNA in a given phlebotomine species.

The high rate of natural *Leishmania* infection in *L. longipalpis* was not followed by equally expressive prevalence of canine visceral leishmaniasis (CVL). The prevalence of CVL varies widely in urban areas (Dantas-Torres et al., 2006; Almeida et al., 2009; Naveda et al., 2006, among others) and there is no minimum prevalence that could be used as a marker of human risk. In previous studies performed in endemic cities of Minas Gerais state, we observed CVL prevalences between 1% and 2% in certain districts, with average prevalence around 4.5% for the whole city (Michalsky et al., 2007; Dias et al., 2011). Districts with no human cases of the VL, showed canine prevalence of 28.3% (Michalsky et al., 2009), whereas prevalences about 1.5% were found, independently of the absence or presence human cases of VL (Barata et al., 2004). A variable that was previously shown to correlate with CVL prevalence is vector population density (França-Silva et al., 2005) and in Montes Claros (Monteiro et al., 2005). Those studies, however, were based on monthly population density and CVL prevalence. In conclusion, the transmission scenarios of VL in urban areas are complex, highly heterogeneous and involve a network of variables (Werneck, 2008).

In our study, we did not investigate any other possible synanthropic reservoir for VL. However, *Leishmania* infection up to 67.7% and around 25% were reported for rodents and marsupials, respectively, in Belo Horizonte (Technical notes on Veterinary and Zootechnics, 2012; Marcelino et al., 2011; Schallig et al., 2007). Although further studies will be needed to assess the role played by

Table 4
Contingency table of the co-positivity of diagnostic tests of canine leishmaniasis, according to clinical group. The reference was the ELISA-IFA result, as adopted by the Brazilian Ministry of Health at the time of our study (2010–2012). *Ln*PCR with spleen tissue gave 100% co-positivity, in both cases, and was not included. The dogs ($n = 51$) were sampled from two districts with recent transmission profile and no human cases of visceral leishmaniasis in Belo Horizonte, in the Brazilian state of Minas Gerais.

Test		Asymptomatic		Symptomatic		*P*-value
Imprint	Negative	6	26.1%	8	28.6%	1.000
	Positive	17	73.9%	20	71.4%	
Myeloculture	Negative	11	47.8%	3	10.7%	0.0045
	Positive	12	52.2%	25	89.3%	

other synanthropic reservoirs, they might be important to maintain the zoonotic cycle of VL in Belo Horizonte.

At the time of the present study, the screening and confirmatory tests adopted by the Brazilian Ministry of Health in the canine serological surveys for VL were ELISA and IFA, respectively (Brazilian Ministry of Health, 2006). Although none of the molecular approaches dependent on invasive tissue biopsy would be applicable to canine surveys, our findings confirm that 100% of the seropositive samples by ELISA/IFA carried *L. infantum* DNA. Of the 51 sero- and molecularly positive dogs, only 10% showed no parasitological confirmation by imprint or myeloculture analysis, which are considered the gold standard methods for VL diagnosis. However, 100% carried *Leishmania* DNA in their spleen. It is worth noting that spleen was the best tissue sample for PCR amplification, reaching 100% positivity, while the lowest positivity (57%) among the four tissues tested was obtained for lymph nodes. Spleen was also shown to be a better choice than lymph node when tissue aspirates were used as samples (Barrouin-Melo et al., 2004). Currently, the Brazilian Ministry of Health replaced the ELISA/IFA set by the rK28-based immunochromatography (Dual Path Platform CVL, produced by the Biomanguinhos Institute at Fiocruz, RJ, Brazil) and ELISA, in an effort to increase the specificity and precision in the diagnosis of VL. In a recent comparison of diagnostic tests for VL, by our group, the immunochromatography reached up to 77.3% of co-positivity compared to ELISA-IFA. Although the canine sample was limited to 44 dogs, those data reinforced that more sensitive and specific tests need to be developed before the efficient diagnosis of canine VL can be performed (Regina-Silva et al., 2014).

Asymptomatic dogs represented 45% of our seropositive sample. *L. infantum* DNA was confirmed in all of them. Using parasite-based

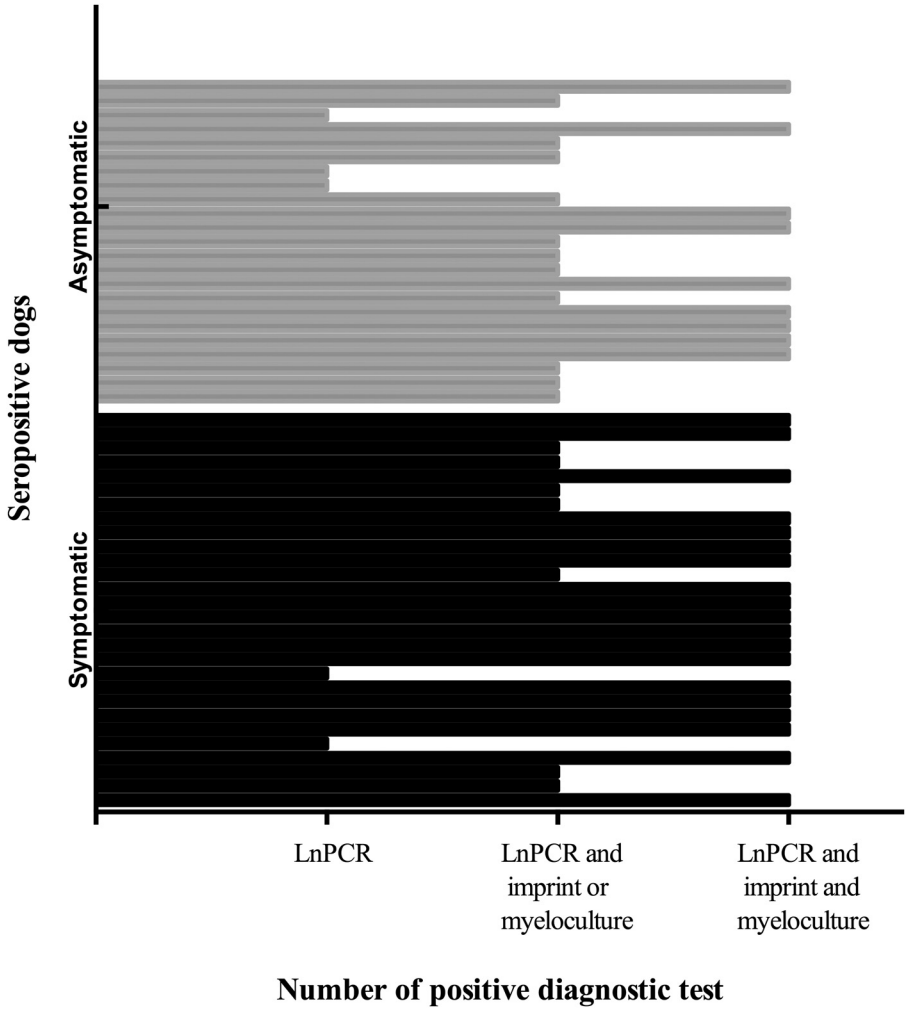

Fig. 5. Frequency of positive molecular (*Ln*PCR with spleen tissue) and parasitological (imprint and/or myeloculture) results in seropositive dogs (ELISA/IFA) for visceral leishmaniasis, according to the clinical condition. Our study was developed in two districts with no reported human cases of the disease in Belo Horizonte, Minas Gerais, Brazil. Period of study: September 2010 to August 2012.

methods, 52.2% were confirmed positive by myeloculture and 73.9% by imprint. Although previous molecular studies in endemic areas of VL have confirmed that the prevalence of infection is much higher than the number of dogs that actually develop the symptomatic form of the disease (Solano-Gallego et al., 2001; Alvar et al., 2004), those expressive positivity rates indicate the epidemiological importance of asymptomatic dogs in the transmission cycle of VL. Using xenodiagnoses, an infectivity rate of 33% was reported before for asymptomatic dogs (Michalsky et al., 2007). Hence, the presence of *Leishmania* in various tissues of this clinical group, particularly in the exposed skin, suggests that the animals may be acting as VL reservoirs (Michalsky et al., 2007; Madeira et al., 2004).

Although our data do not support any prediction of the local VL transmission strength, the districts under study display favorable conditions for the rapid spreading of *Leishmania* infection, in terms of *L. longipalpis* population density, and presence of *L. infantum* in both vector and main reservoir. The reason why no human cases have been reported, so far, is unknown. The districts studied share the same human occupation and environmental characteristics of any other urban area in Belo Horizonte. The occurrence of human asymptomatic infection, in the studied area, is a possibility. Moreno et al. (2006) reported no human cases of VL in area with 5–10% of canine VL prevalence, except for eight bad-nourished children that did not developed the disease. Asymptomatic cases with no evolution to acute phase were also observed in the Northeast of Brazil (Jeronimo et al., 2000; Caldas et al., 2002; Gama et al., 2004).

In conclusion, both population density and natural *Leishmania* infection of vectors as well as CVL prevalence are favorable to outbreaks of human VL, in the districts under study. Even with the actual absence of human cases, control actions are urgently needed to avoid it. As recently pointed out by other authors, more than 30 years after the beginning of the process of visceral leishmaniasis' urbanization, transmission scenarios of VL are still poorly understood and further studies will be needed until we comprehend all the determining variables involved in the transmission of VL (Belo et al., 2013).

Conflict of interests

The authors declare that they have no competing interests.

Authors' contributions

FOLS—phlebotomine experiments, including identification, molecular biology assays, data preparation an article writing; EMM—canine infection experiments, including necropsies, imprints, molecular biology assays, data analysis, article writing; CLFD—sequence alignments, data analysis, article writing and critical review; VOPF—project planning and supervision; JEMP—databank and experimental data analysis; SRS—canine necropsies and tissue biopsies; DMA—parasitological diagnosis; MAS—data collection and organization of entomological and canine databank; ACVMRL—myelocultures; AJAC—entomological captures, specimens preparation and figure preparation; GLLMC—data interpretation and statistical analysis; ESD—project planning and supervision, general data analysis, article review. All the authors approved the submitted version of the article.

Acknowledgements

We wish to express our gratitude to Dr. França-Silva for collaborating in canine necropsies. We acknowledge the financial support of the Brazilian funding agency Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG, grant PPSUS 09/2009).

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