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Short Communication

Seroprevalence and molecular characterization of *Leishmania* in dogs from an endemic area of zoonotic visceral leishmaniasis in Brazil

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ABSTRACT

Visceral leishmaniasis (VL) can cause large-scale and tenacious epidemics with high fatality rates. Current seroprevalence and circulating *Leishmania* species were evaluated in dogs domiciled in the municipality of Sabará, a small historic and touristic city in the Brazilian state of Minas Gerais. A total of 3926 dogs domiciled in seven different districts of Sabará were serologically tested for canine visceral leishmaniasis (CVL) by indirect enzyme-linked immunosorbent (ELISA) and immunofluorescence (IFA) assays, in a two-years census survey (2011–2012). The average positivity rate of canine infection was 3.4%. Three additional diagnostic tests – imprint/smear direct parasitological, molecular (LnPCR) and myeloculture – were performed in a random sample of fifty seropositive dogs composed of symptomatic (39) and asymptomatic (eleven) animals. LnPCR showed 100% of positivity for *Leishmania* DNA in, at least, one among four tissue samples tested (mesenteric lymph node, skin, spleen and bone marrow), independently of the clinical canine group. Higher and statistically equivalent positivity rates (98% and 96%) for *Leishmania* DNA were found in canine lymph node and spleen. Asymptomatic dogs showed expressive positivity rates in all three additional diagnostic techniques. *Leishmania infantum* was confirmed as the etiological agent of CVL in Sabará.

1. Introduction

Visceral leishmaniasis (VL) can cause large-scale and tenacious epidemics, with high case–fatality rates. In Brazil, rural epidemics were seen in ten-year cycles. Since 1999, a sharp expansion of VL has been observed over the country. VL reached urban areas with increasing numbers of human cases and high prevalence of canine cases [1–4].

The etiological agent of VL in Latin American is *Leishmania infantum*. Human infection occurs after biting of females of *Lutzomyia longipalpis* phlebotomine sand flies previously infected with the parasite [5]. Dogs (*Canis familiaris*) are considered the main domestic reservoirs in the zoonotic cycle of VL [6]; therefore, it is important to know the prevalence of canine VL infection (CVL) in a given place. To achieve that, the reliable identification of infected dogs, even in the absence of typical clinical signs of VL, is critical. A number of parasitological, serological and molecular tests have been developed and comparatively used to diagnose canine *Leishmania* infection [7]. In most cases, the final result will be determined by the clinical state of the dog, parasitemia level, quality of the biological sample and type of method chosen for diagnosis [8].

In the present work, we studied the current infection of dogs by *Leishmania* in a historic and touristic town – named Sabará – in the Brazilian state of Minas Gerais. Sabará is part of the so-called Gold Circuit of Minas Gerais which comprises small towns with historical heritage from the late 1700's. Over the last seven years, human and canine cases of VL have been reported to the Health Department of Sabará. In the last three years, the average number of human cases of VL was higher than 4.4. Hence, the city was classified as area of intense transmission of VL, according to the parameters established by the Brazilian Ministry of Health in the VL Control Program. Aiming at determining the current *Leishmania*

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infection in dogs domiciled in Sabará, we undertook a two-year canine census survey using the standard, antibody-based methods recommended by the Control Program of VL of the Brazilian Ministry of Health [9]. We also compared the performance of three additional diagnostic tests—imprint/smear direct parasitological, molecular (PCR) and myeloculture—in a sample of seropositive dogs and identified the etiological agent of CVL in Sabará at species level.

2. Materials and methods

2.1. Ethical procedures

The present study was approved by the Ethical Committee on Animal Experimentation of Fundação Oswaldo Cruz (CEUA/FIOCRUZ) under the license No. LW-11/10 (protocol No. P-28/10-3). All the procedures followed the technical rules established by the Federal Board of Veterinary Medicine (CFMV resolution No. 714/2002). The dog owners were previously informed of the project objectives and signed a Statement of Informed Consent regarding sample collection for biopsy.

2.2. Study area

Sabará (19°53′21″ S 43° 48′ 17″ W) is part of the Metropolitan region of the state capital Belo Horizonte (Fig. 1). The town occupies an area of 302 km^2 with 64 districts and about 134.300 inhabitants. Eight districts of Sabará—named Alvorada, Novo Alvorada, Alvorada Velho, Bom Retiro, Rio Negro, Ana Lúcia, Casa Branca, and Nova Vista— were selected for study based on recent reports of canine and human cases of VL.

2.3. Canine census survey

To determine the current positivity rates of canine VL, two census population surveys were performed in 2011 and 2012. The blood samples were taken to the Zoonoses Laboratory of the Health Department of Sabará. The presence of *Leishmania* antibodies was investigated by enzyme-linked immunosorbent assay (ELISA) followed by indirect immunofluorescence assay (IFA), according to the standard protocol adopted by the Brazilian Ministry of Health at that time [9]. Only dogs with positive results in both tests were considered seropositive to VL. When the ELISA was positive but the IFA antibody titer remained in the borderline (1:40) the result was undetermined. The positivity rates were calculated by dividing the number of seropositive dogs in a given district by the number of tested dogs in the district.

The screening-and-culling procedure of the seropositive dogs was conducted by trained technicians from the Center for Zoonosis Control (CCZ) of Sabará and followed the rules established by the Brazilian Ministry of Health for VL control.

2.4. Euthanasia and tissue collection from seropositive dogs

Fifty seropositive dogs for VL from the canine census survey were randomly selected for further clinical examination by a veterinarian. The dogs were classified as asymptomatic or symptomatic, according to the absence or presence of, at least, one clinical sign of VL – cutaneous alterations such as alopecia, dermatitis and ulcers; onycogryphosis; keratoconjunctivitis; loss of weight; emaciation and rigidity of posterior limbs [10,11]. After anesthesia with Thionembutal (30 mg/mL *via* i.v.), bone marrow aspirates were harvested by sterilely puncturing the tibial crest. The aspirates were inoculated in the appropriate culture medium for parasite isolation and also used for the preparation of slide smears. Subsequently, the dogs received an intravenous injection of saturated solution of KCl (0,5 mL/kg). Biopsied samples from spleen, mesenteric lymph node and skin tissues were submitted to further parasitological and molecular analysis.

2.5. Leishmania isolation

Bone marrow aspirates (two samples per dog) were inoculated into Novy-MacNeal-Nicolle-liver infusion tryptose (NNN/LIT) culture medium and incubated at 25 °C. The cultures were weekly examined for the presence of *Leishmania* promastigotes. The positive samples were gradually expanded to 100 million cells. After washing with PBS, the *Leishmania* biomass was frozen at −20 °C until use. Negative samples were discarded after five weeks of monitoring. *Leishmania* growing in any of the two samples per dog was considered as a positive result for that dog.

2.6. Direct parasitological test

The presence of *Leishmania* amastigotes was microscopically investigated in bone marrow smears, and tissue—spleen, lymph node and skin—imprints, in this order, after slide staining by Giemsa. In case of *Leishmania* visualization in any of the samples, the result was taken as positive in the direct parasitological test.

2.7. Molecular analysis

Total genomic DNA was extracted from tissue—spleen, lymph node and skin—fragments and bone marrow aspirates using the

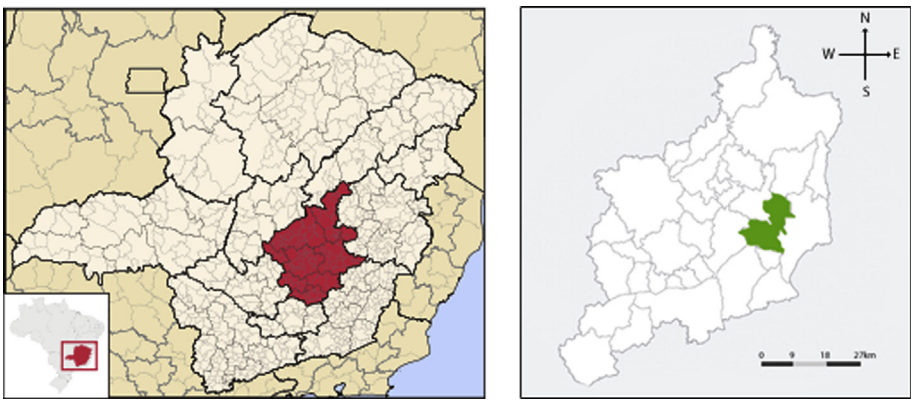

Fig. 1. Geographical localization of the municipality of Sabará, in the Metropolitan area of the state capital, in Minas Gerais, Brazil. The Metropolitan area of Belo Horizonte is marked in red (left map). Sabará is marked in green in the expanded map of the Metropolitan area (right map).

Illustra Tissue & Cells Genomic Prep Mini Spin and the Blood Genomic Mini Spin kits, respectively, according to the manufacturer's instructions (GE HealthCare). The presence of *Leishmania* DNA in the samples was investigated by LnPCR for the SSUrRNA gene, a technique that amplifies a conserved fragment of the *Leishmania* genus [12,13]. In the first step, ten to twenty ng of extracted DNA were amplified in the presence of R1 (5′ GGT TCC TTT CCT GAT TTA CG 3′) and R2 (5′ GGC CGG TAA AGG CCG AAT AG 3′) primers. The 603 bp DNA fragment amplified in the positive samples was diluted 1:40 in sterile water and used as template in the second amplification step with R3 (5′ TCC CAT CGC AAC CTC GGT T 3′) and R4 (5′ AAA GCG GGC GCG GTG CTG 3′) primers. In the positive samples for *Leishmania*, a 353 bp was expected to be amplified.

For the PCR amplifications we used the PureTaq Ready-To-Go PCR Beads (GE Healthcare) in a Veriti thermocycler (Applied Biosystems). Negative (no DNA) and positive (DNA extracted from *Leishmania chagasi* MHOM/BR74/PP75) were run as controls. The amplified products were visualized under UV light, after electrophoresis on 2% agarose and ethidium bromide staining.

LnPCR was considered positive when *Leishmania* DNA was detected in, at least, one of the test samples (overall positivity).

2.8. Specific identification of Leishmania

The amplified DNA fragments were extracted from agarose gels using a commercial kit (QIAquick Gel Extraction Kit, QIAGEN). After forward and reversed sequencing of both strands (BigDye® Terminator v3.1 Cycle kit in an ABI 3730, Life Technologies), the nucleotide segments were multiple aligned with *Leishmania braziliensis* (M80292.1), *Leishmania amazonensis* (M80293.1) and *Leishmania infantum* (M81430.1) sequences deposited in the GenBank, using BioEdit (www.mbio.ncsu.edu/BioEdit.html) and BLAST (www.ncbi.nlm.nih.gov/BLAST).

2.9. Statistical analysis

The overall performance of CVL diagnostic tests and LnPCR from different tissues were compared by Mann-Whitney test with 95% of confidence.

3. Results

3.1. Seroprevalence of canine visceral leishmaniasis

A total of 3926 dogs were serologically tested for CVL during the two-year study, with prevalence of 3.4% on the average (Table 1). Fifty dogs were randomly selected for further analysis and later examined for clinical classification for CVL. Eleven of them were asymptomatic (22%) and 39 (78%) were symptomatic ones. In Casa Branca, the district sample was restricted to eight dogs. Although

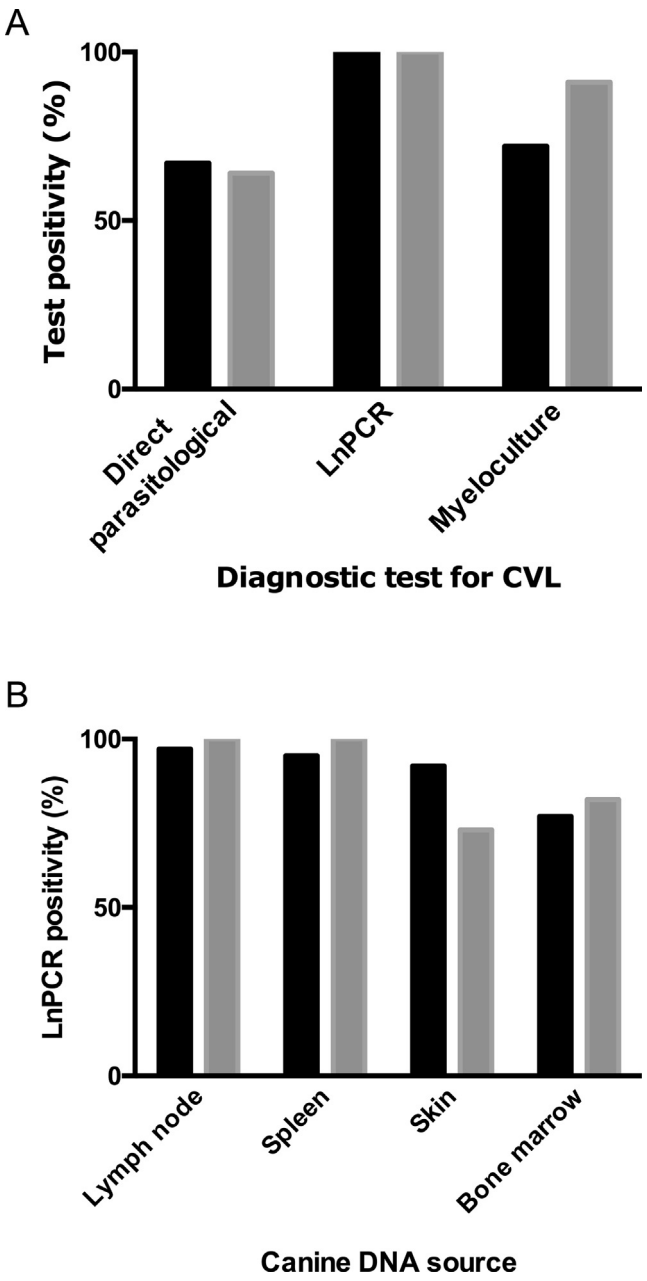

Fig. 2. Frequency distribution of additional diagnostic tests for CVL (A) and LnPCR positivity according to canine DNA source (B), in a random sample of 50 seropositive dogs for CVL. The dogs were later classified as symptomatic (dark gray) or asymptomatic (light gray), based on the presence or absence of CVL clinical signs. There was no statistical difference between the clinical canine groups, for any test or DNA source. The dogs were domiciled in Sabará (Minas Gerais state, Brazil). Period of study 2011–2012.

Table 1
Seroprevalence of canine visceral leishmaniasis (CVL) in the census survey performed in Sabará (state of Minas Gerais, Brazil) between 2011 and 2012. The blood samples were tested by indirect enzyme-linked immunosorbent (ELISA) and immunofluorescence (IFA) assays, according to the standard protocol adopted by the Brazilian Ministry of Health at that time [9]. Only dogs with positive results in both tests were considered seropositive to VL.

District	Dogs		Serology			Positivity rate
	Number	%	Positive	Negative	Undetermined	(%)
Alvorada	858	21.9	33	816	9	3.8
Alvorada Velho	331	8.4	5	322	4	1.5
Ana Lúcia	385	9.8	19	364	2	4.9
Bom Retiro	110	2.8	3	106	1	2.7
Nova Vista	1312	33.4	62	1231	19	4.7
Novo Alvorada	810	20.6	22	782	6	2.7
Rio Negro	120	3.1	4	115	1	3.3
Total	3926	100.0	148	3736	42	

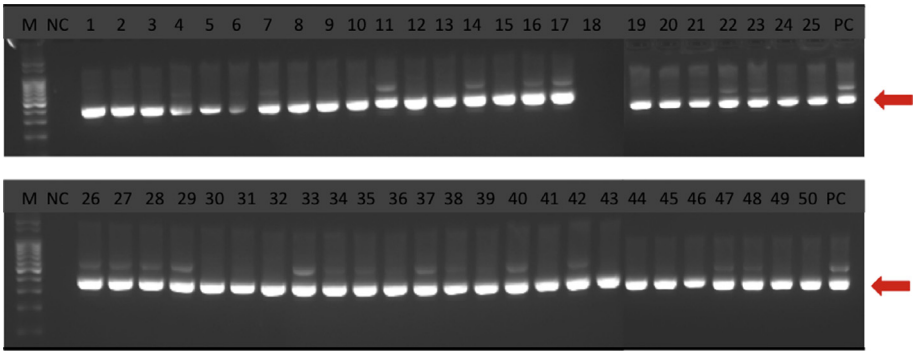

Fig. 3. Agarose gel electrophoresis analysis of mesenteric lymph node canine DNA after LnPCR. The gel was stained by ethidium bromide. Lanes: M-100 bp DNA ladder; 1 to 50-individual seropositive dogs for CVL; PC-Positive DNA control (*L. chagasi* MHOM/PP75); NC-negative control (no DNA). The red arrows point to the characteristic 353 bp band of *Leishmania* DNA in the samples. All the dogs were domiciled in Sabará (Minas Gerais state, Brazil). Period of study: 2011–2012.

they have been serologically tested, the number was considered non-representative and the district was excluded from the study.

3.2. Frequency distribution of additional diagnostic tests for CVL

The results of additional CVL diagnostic tests (imprint/smear direct parasitological, molecular (PCR) and myeloculture) in the randomly sampled dogs are shown in Fig. 2A.

LnPCR reached 100% of overall positivity. *Leishmania* DNA detection was unaffected by the clinical canine group. When the DNA source was taken into account, the highest overall positivity (98%) was observed for mesenteric lymph nodes (Fig. 2B). The corresponding agarose gel electrophoresis analysis is represented in Fig. 3. Only one sample (No. 18) gave a negative result for *Leishmania* DNA in lymph node. All the asymptomatic dogs (eleven dogs) were positive in lymph node LnPCR. A similar result (96%) was obtained when lymph node was replaced by spleen (96%) (Fig. 2B). There was no statistical difference between the overall positivity rates for lymph node or spleen DNA. The asymptomatic group was 100% positive for *Leishmania* DNA. In the symptomatic group, 37 among 39 dogs (95%) gave positive results. Lower positivity rates were observed for skin (88%) and bone marrow (78%) in the sampled canine group as a whole, with no statistical difference between the DNA sources. *Leishmania* DNA was detected in the skin of 92% (36/39) of the symptomatic dogs and in 73% (8/11) of the asymptomatic ones.

The overall positivity of imprint/smear direct parasitological assay was 66%. *Leishmania* parasites were detected in 26 among 39 (67%) of the symptomatic dogs and in seven among eleven (63%) asymptomatic ones. In myeloculture, *Leishmania* parasites developed in 38 among the 50 canine samples. The positivity rates were 72% for the symptomatic canine group. In the asymptomatic dogs, it reached 91% with the development of *Leishmania* parasites in ten among eleven samples.

Among the 50 seropositive dogs for CVL, 27 (54%) had the diagnosis confirmed by the three additional tests employed, independently of the clinical group they belonged to (data not shown). Seventeen and six dogs were positive for two or a single test, which correspond to 34% and 12% of the total number of dogs tested, respectively.

3.3. Identification of Leishmania parasites at species level

Nucleotide sequencing of the 353 bp fragments amplified by LnPCR confirmed the presence of *L. infantum* in all of them.

4. Discussion

Dogs play an important role as *L. infantum* domestic reservoirs and are able to present intense cutaneous parasitism [14,15]. The prevalence of infection can be much higher than the proportion of dogs that develop the symptomatic form of the disease [7,16]. Since the canine cases precede the human cases of VL, it is important to have a profile of the current situation of CVL in any given municipality. In Sabará, the prevalence of CVL was expressive (3.4% on the average).

Until very recently, ELISA and IFA were adopted as screening and confirmatory assays, respectively, in the VL Control Program of the Brazilian Ministry of Health. The procedure was considered to display enough sensitivity and specificity, low cost and easy implementation [9]. As a more appropriate alternative, they have been routinely replaced by the Dual-Path Platform (DPP®) chromatographic immunoassay for screening followed by a confirmatory ELISA.

In our study, 66% and 76% among 50 seropositive dogs for CVL displayed positive results in direct (imprint/smear) parasitological and myeloculture, respectively. Because they are based on *Leishmania* visualization, these tests are considered the gold standard for CVL diagnosis. In our hands, there was no direct parasitological detection in 14% (17/50) of the seropositive dogs. Known drawbacks of parasitological tests are that they are time-consuming, laborious and require trained personnel. In addition, *Leishmania* visualization can be rather difficult when the parasite load is low [17]. Therefore, the possibility that those samples could be false negatives can not be discarded. On the other hand, seropositive dogs with no parasite detection may represent false positives in antibody-based tests (ELISA and IFA).

It is known that molecular tests are fast, highly sensible, easily performed and independent of parasite growing [18–21]. Herein, PCR amplification displayed 100% of positivity for, at least, one among the four tissues tested. The best biological sample to be used in the molecular diagnosis of canine VL is controversial [22,23]. In the present study, LnPCR reached almost 100% of positivity when lymph node and spleen were used as DNA source, independently of the clinical state of the animal. This result is in accordance with other author reports [24,25]. Still, in practical terms, the best sample would be canine skin because it is more easily obtained. In our hands, the positivity of LnPCR for skin DNA reached 88%.

Although asymptomatic dogs comprised only eleven dogs in the 50 sampled animals, they showed expressive positivity rates on direct parasitological tests (64%) an even superior (91%) in myeloculture. *Leishmania* DNA was detected in, at least, one tissue in

100% of them. The presence of parasites particularly in the skin of 73% of the asymptomatic dogs confirms the potential infectivity to the insect vector [26] and denotes the epidemiological importance of this clinical group in the transmission cycle of VL.

5. Conclusions

The detection of *Leishmania* parasites or *Leishmania* DNA in the sampled seropositive dogs indicates that antibody-based techniques are still useful for CVL diagnosis in large-scale programs for VL control. The role played by asymptomatic and not only symptomatic dogs in the transmission cycle of VL must be taken into account.

Competing interests

The authors declare that there is no conflict of interests concerning the publication of the present article.

Acknowledgments

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