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Communication

[*Comunicação*]

Cytological and molecular detection of *Leishmania* spp. in different biological tissues of dogs in areas endemic for visceral leishmaniasis

[*Detecção citológica e molecular de Leishmania spp. em diferentes amostras biológicas de cães em áreas endêmicas para leishmaniose visceral*]

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Leishmaniasis, a zoonotic disease caused by protozoa of the genus *Leishmania*, is a serious global public health problem. It is estimated that 1.3 million new cases and 20,000 to 30,000 deaths occur annually (Leishmaniasis, 2018). In the Americas, Brazil is the country with the highest occurrence rate of visceral leishmaniasis (VL), which is considered the most serious and fatal form of the disease caused by *Leishmania* (*Leishmania*) *infantum* (syn: *L.* (*L.*) *chagasi*), being transmitted by *Lutzomyia longipalpis* and *L. cruzi* species sandflies (Brasil, 2014).

As the main source of the vector infection, the dogs play an important role in the domestic transmission cycle of VL, which makes it essential to develop researches for canine visceral leishmaniasis (CanL). In this context, it is important to investigate the prevalence of the infection by methods of detection of *Leishmania*-specific antibodies (serology), DNA detection (PCR) or parasitological (cytology, histopathology, immunohistochemistry, culture) (Quaresma *et al*., 2009). These methods contribute to the epidemiological surveillance providing results to the Brazilian Ministry of Health, that propose specific measures of surveillance, prevention and control of the disease.

However, the distribution of CanL throughout Brazil's territory, its sympatric occurrence with other microorganisms, and the limitations of the sero-epidemiological studies can result in the occurrence of cross reaction with other phylogenetic similar species which underscore the need for the correct classification of parasites of the genus *Leishmania* (Coutinho *et al*., 2011). Thus, this study aimed to evaluate the occurrence of *Leishmania* spp. in dogs domiciled in an endemic region by parasitological and molecular techniques in different biological tissues.

A transversal study was conducted in the region of Baixada Cuiabana, Mato Grosso, Brazil. The investigation involved dogs of both sexes and different breeds, age ≥ 6 months, from the municipalities Cuiabá (15°35'56"S and 56°06'01"W), Várzea Grande (15°38'49"S and 56°07'58"W), and Santo Antônio de Leverger (15°51'56"S and 56°04'36"W). This study was conducted in accordance with the ethical principles approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Mato Grosso under protocol number 23108.014950/11-5.

With the written approval of the owners, the dogs were sedated intramuscularly with ketamine (10mg/kg) and acepromazine (0.2mg/kg) to collect samples of blood, lymph nodes and bone

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marrow aspirates by jugular venipuncture, popliteal lymph node puncture and sternal puncture, respectively. Immediately after sampling, the collected material was distributed to glass slides and cytology smears were stained with Romanowsky and analyzed under an optical microscope with 1000x magnification for observation of *Leishmania* amastigotes.

DNA extraction from clinical samples was done using phenol-chloroform method (Gomes *et al*., 2007). For *Leishmania* spp., the nPCR was performed with the external primers TRY927F (5'-GAAACAAGAAACACGGGAG-3') and TRY927R (5'-CTACTGGGCAGCTTGGA-3') and internal primers SSU561F (5'-TGGGATAACAAAGGAGCA-3') and SSU561R (5'-CTGAGACTGTAACCTCAAAGC-3'), amplifying a DNA fragment of 700pb, corresponding to the 18S rRNA gene (Smith *et al*., 2008). To detect possible false-positive and false-negative reaction, samples of *L.* (*L.*) *infantum* (MHOMQ/GB/1994/PP75) DNA were used as positive control, and ultrapure water as negative control in all the reactions. DNA and amplification product quality and integrity were analyzed by electrophoresis in 1.5% agarose, stained with GelRed (Biotium, Hayward, CA, USA) and observed in ChemiDoc XRS$^+$ (Bio-rad Laboratories, Inc., Hercules, CA, USA) using the software Image Lab (Bio-rad Laboratories, Inc., Hercules, CA, USA).

Positive samples were purified with an Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, USA) and sequenced on the automatic model ABI-PRISM 3500 Genetic Analyzer. (Applied Biosystems/PerkinElmer, Foster City, CA). The sequences were deposited in GenBank database using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) to confirm the specie of *Leishmania*. Statistical analyses were performed with the aid of Epi Info version 7 (Center for Disease Control and Prevention [CDC], Atlanta, GA, USA). The agreement between the techniques was investigated using the kappa (*k*) test. The confidence interval employed in all statistical analyses was 95%.

Of the 205 dogs evaluated, *Leishmania* DNA was detected in 34 dogs (16.58%), while 12 dogs (5.85%) were positive on the parasitological diagnosis. The overall prevalence detected by combining the cytological and molecular tests was 16.58% (34/205), being the highest infection rate found in the municipality of Cuiabá, followed by the municipalities of Várzea Grande and Santo Antônio de Leverger (Table 1).

Table 1. Cytological examination and nested PCR results of the investigation of *Leishmania* spp., in different biological samples obtained from 205 dogs residing in Baixada Cuiabana

City	Parasitological diagnosis			nPCR			Total +/*n*[d]
	BM[a]	LN[b]	BL[c]	BM[a]	LN[b]	BL[c]	
Cuiabá	0	0	0	4	7	2	11/50 (22%)
Várzea Grande	9	9	2	15	14	4	18/82 (21,95%)
*Santo A. de Leverger	1	1	1	1	5	1	5/73 (6,84%)
Total +/*n*	10/205 (4,87%)	10/205 (4,87%)	3/205 (1,46%)	20/205 (9,75%)	26/205 (12,68%)	7/205 (3,41%)	34/205 (16,58%)

* Santo Antônio de Leverger; [a] Bone marrow; [b] Lymph node; [c] Blood; [d] Positive in at least one clinical sample.

Sensitivity and specificity values were calculated separately for bone marrow, lymph node and blood nPCR technique, considering the direct parasitological examination as the gold standard, due to the possibility of visualizing the amastigotes forms. The results of sensitivity, specificity and Kappa coefficient by nPCR were for bone marrow (94.87%,100%, 0.643), lymph node (91.8%, 100%, 0.522) and blood (98%,100%, 0.592), respectively.

The 53 amplified samples in the nPCR (GenBank accession number of KU948433 to KU948485) were similar to the 18S rRNA gene of *L. infantum* (XR_001203206. 1). The sequences presented little variation in the percentage of similarity with *L. infantum:* two samples had 87% and 88% similarity, two presented 89%, three presented 91%, 42 samples presented 99% and four samples showed 100% similarity.

L. infantum is present and infecting the canine population of Baixada Cuiabana, representing a serious public health problem, since dogs constitute one of the main sources of the vector infection and play an important role in the ecology and epidemiology of this disease (Brasil, 2014). Previous studies conducted in Cuiabá and Várzea Grande reported prevalence of 22.1% (Almeida *et al*., 2012) and 23% (Fujimori *et al*., 2016), similar results to those observed in this study. In relation to the municipality of Santo Antônio de Leverger, the prevalence of 10.85% found in serological survey (Unpublished dates; Souza, 2018; UFMT) was higher than that found in our study. According to Missawa and Borba (2009), several factors can be associated with different prevalence in the same municipality, including the area where the dogs reside, the technique used and the type of biological material analyzed.

Regarding the diagnostic methods, the parasitological test is routinely used for the diagnosis of CanL, however it is infeasible in epidemiological studies, due to low sensitivity. We observed that nPCR shows high sensitivity and specificity and although blood had higher sensitivity than other tissues, the proportion of positive results was lower, demonstrating the preference of the etiological agent for the host's mononuclear phagocytic system, resulting in lower parasite loads in the bloodstream (Laurenti, 2009). Several biological tissues can be used for the investigation of *Leishmania* spp., but there is still no consensus on what constitutes the best tissue sample, in this premise, we highlight bone marrow as the best biological tissue, that showed Kappa index greater than 0.643, which represents a substantial agreement.

A neglected disease in Brazil, the occurrence of CanL and identification of the etiologic agent in the target areas of sero-epidemiological surveys are important, since the cross reactivity among other phylogenetic similar species, e.g., *Leishmania* spp. and *Trypanosoma cruzi* in canine infection has already been reported (Troncarelli *et al*., 2009). Furthermore, in 2013 the first case of canine *T. cruzi* infection was reported in Cuiabá, ratifying the importance of providing data for future epidemiological surveys (Almeida *et al*., 2013).

The 18S rRNA genomic region showed satisfactory results, molecularly characterizing the CanL. The simultaneous use of parasitological examination and nPCR allowed to identify the *Leishmania* species circulating in the region and to guarantee the correct diagnosis of CanL, reducing the occurrence of false negative and false positive results, frequently observed in sero-epidemiological studies. Although no other species of *Leishmania* was detected in this study, the investigation of different agents in endemic areas for VL is necessary, generating knowledge about the circulation of species in the region, since euthanasia is one of the control measures for CanL in Brazil (Brasil, 2014).

Causative agent of CanL is present in the region of Baixada Cuiabana. Complementary diagnostic, such as nPCR, showed great potential to detect and identify *L. infantum*. Bone marrow was considered the most adequate sample for the diagnosis of the disease.

Keyword: visceral canine leishmaniosis, Leishmania infantum, PCR, parasitology, Kala-azar

RESUMO

Devido à ampla distribuição da leishmaniose visceral (LV) no Brasil e à importância dos cães no ciclo de transmissão dessa zoonose, o presente estudo teve como objetivo avaliar a ocorrência de Leishmania spp. e caracterizar a espécie circulante em diferentes tecidos biológicos de cães da Baixada Cuiabana, Mato Grosso, Brasil. Amostras de sangue, linfonodo e medula óssea foram coletadas de 205 cães para realização de análise parasitológica por citologia e análise molecular por meio da nested PCR (nPCR) e do sequenciamento . Dos 205 cães estudados, 34 (16,58%) animais foram positivos pela nPCR, dos quais 12 possuíam formas amastigotas de Leishmania spp. na citologia. Amostras positivas na nPCR foram sequenciadas e caracterizadas como Leishmania (Leishmania) infantum. A sensibilidade da nPCR nas amostras de medula óssea, linfonodo e sangue foi de 94,87%, 91,8% e 98%, respectivamente, enquanto a especificidade foi de 100% para todas as amostras. O presente estudo relata a ocorrência de LV canina em 16,58% dos cães analisados, caracterizando a L. infantum como agente causador. Entre as amostras

avaliadas, a medula óssea foi a única a apresentar concordância substancial entre as técnicas de nPCR e citologia (k = 0,643), sendo considerada a amostra mais adequada para o diagnóstico da doença. Os resultados ampliam o conhecimento de espécies de Leishmania infectando cães no Brasil, destacando a importância da identificação etiológica em áreas com escassos dados moleculares.

Palavras-chave: leishmaniose visceral canina, Leishmania infantum, *PCR, parasitologia, Kala-azar*

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