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Hepatozoon canis and *Leishmania* spp. coinfection in dogs diagnosed with visceral leishmaniasis

Coinfecção com *Hepatozoon canis* e *Leishmania* spp. em cães diagnosticados com leishmaniose visceral

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Abstract

This study describes the occurrence of dogs naturally co-infected with *Hepatozoon canis* and two *Leishmania* species: *L. infantum* or *L. braziliensis*. Four dogs serologically diagnosed with Visceral Leishmaniasis were euthanized. Liver and spleen samples were collected for histopathological analysis and DNA isolation. *H. canis* meronts were observed in tissues from all four dogs. *H. canis* infection was confirmed by PCR followed by sequencing of a fragment of 18S rRNA gene. *Leishmania* detection and typing was confirmed by ITS1' PCR-RFLP and parasite burden was calculated using ssrRNA quantitative qPCR. A DPP - Dual Path platform test was performed. One out (Dog #2) of four animals was asymptomatic. Dogs #1 and #4 were infected by *L. infantum* and were DPP test positive. Dogs #2 and #3 were infected by *L. braziliensis* and were DPP test negative. Furthermore, visceral dissemination was observed in Dogs #2 and #3, since *L. braziliensis* was detected in liver and spleen samples. The visceral dissemination of *L. braziliensis* associated with systemic signs suggested that this co-infection could influence the parasite burden and disease progression.

Keywords: Hepatozoonosis, canine visceral leishmaniasis, *Leishmania infantum*, *Hepatozoon canis*, co-infection, *Leishmania braziliensis.*

Resumo

O presente estudo descreve a ocorrência de coinfecção com *Hepatozoon canis* e duas espécies de *Leishmania* (*L. infantum* ou *L. braziliensis*) em cães. Quatro cães sorologicamente diagnosticados com leishmaniose visceral foram eutanasiados. Amostras do baço e fígado foram submetidas à histopatologia e extração de DNA. Merontes de *H. canis* foram observados nos quatro cães. A infecção por *H. canis* foi confirmada por PCR e sequenciamento de um fragmento do gene 18S rRNA. A infecção por *Leishmania* e tipagem foram realizadas por PCR-RFLP do região intergênica ITS1. A carga parasitária foi calculada pela qPCR quantitativa baseada no gene ssrRNA. O teste DPP - Dual Path platform foi realizado. Apenas o Cão #2 era assintomático. Os cães #1 e #4 estavam infectados com *L. infantum* e foram positivos no DPP. Os cães #2 e #3 estavam infectados com *L. braziliensis* e foram negativos no DPP. Além disso, visceralização foi observada nos cães #2 e #3, nos quais *L. braziliensis* foi detectada em amostras de baço e fígado. A visceralização da *L. braziliensis* associada a sinais clínicos sistêmicos sugerem que esta coinfecção pode ter influenciado na carga parasitária e progressão da doença.

Palavras-chave: Hepatozoonose, leishmaniose visceral canina, *Leishmania infantum*, *Hepatozoon canis*, coinfecção, *Leishmania braziliensis.*

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Introduction

Canine visceral leishmaniasis (CVL) is a zoonotic disease endemic in various countries. In Brazil, visceral leishmaniasis is caused by the protozoa *Leishmania infantum*. The dog is considered an important urban reservoir and the detection of canine cases may precede the occurrence of human visceral leishmaniasis (BELO et al., 2013). The current public health policy in Brazil demands the culling of infected dogs -based on serologic tests - for VL control (BRASIL, 2006). Cross-reactivity using *Leishmania infantum* antigen with sera from *Leishmania braziliensis*-infected dogs is commonly observed (BRASIL, 2006), but dog culling, in this case, is not recommended. Dogs infected with *L. braziliensis* commonly present localized skin lesions without systemic dissemination (MADEIRA et al., 2005). The urban reservoir of *L. braziliensis* is still not known in Brazil.

Canine hepatozoonosis is a systemic disease caused by Apicomplexa protozoa of the genus *Hepatozoon,* that is transmitted by ingestion of infected ticks belonging to the genus *Rhipicephalus* (RUBINI et al., 2008; KAUR et al., 2012; MIRANDA et al., 2014). Domestic dogs can be infected by two species: *H. canis* and *H. americanum* (O'DWYER, 2011). The infected animals usually present low parasitemia (MUNDIM et al., 2008). Clinically, *Hepatozoon* infected-animals can present anorexia, lack of appetite, fever, pale mucosa, lethargy, diarrhea and vomiting (CHHABRA et al., 2013). Hematological alterations, such as anemia, leucocytosis and neutrophilia or lymphopenia, have also been observed (KAUR et al., 2012). Many of these clinical signs described for canine hepatozoonosis are also observed in CVL.

In Brazil, *Hepatozoon* has been observed since 1979, when Massard studied the infection (O'DWYER, 2011). Molecular characterization indicated that Brazilian *H. canis* was closely related to isolates from Japan, presented 99% nucleotide identity with isolates from Israel and was found to be distantly related to *H. americanum* (RUBINI et al., 2005). In the following years, canine hepatozoonosis has also been described in several Brazilian regions (RUBINI et al., 2008; MIRANDA et al., 2014; GONÇALVES et al., 2014; RAMOS et al., 2015; DEMONER et al., 2016). Many of these areas are also endemic for CVL, and co-infection has already been described in dogs from Rio Grande do Norte based only on molecular analyses (GONÇALVES et al., 2014). Herein, we described the occurrence of co-infection of dogs with *H. canis* and *L. infantum* or *L. braziliensis,* using molecular and histopathological analyses. In addition, the co-infection of *H. canis* and *L. braziliensis* could have lead to visceral dissemination, which may lead to a false CVL diagnosis.

Material and Methods

Animals and samples

Four seropositive dogs (IFAT - Bio-Manguinhos and ELISA - Bio-Manguinhos) for *Leishmania,* two from Barra do Garças (Latitude: 15° 53' 24" S; Longitude: 52° 15' 24" W) and two from Cuiabá (Latitude: 15° 35' 46" S; Longitude: 56° 05' 48" W), Mato Grosso state, were included in this study. Clinical evaluations were performed before euthanasia and were performed by veterinarians at the Zoonosis Control Center (Centro de Controle de Zoonoses - CCZ). The procedure included the administration of 1.0 ml/Kg of 1.0% Thiopental intravenously (Thiopentax®, Cristália) for anesthesia. Once the absence of corneal reflex due to deep anesthesia was confirmed, 10 mL of 19.1% potassium chloride (Isofarma) was intravenously administered. During necropsy, fragments from spleen and liver were collected for histopathological analysis and molecular techniques. Samples were also forwarded to the *Leishmania* Collection of the Oswaldo Cruz Institute (CLIOC, www.clioc.fiocruz.br) for parasite isolation and typing. In addition, serum samples were collected and tested with the rapid Dual Path Platform test (DPP CVL, BioManguinhos, FIOCRUZ), which detects anti-rK26/39 *Leishmania* antibodies (GRIMALDI et al., 2012).

Ethics statement

The animals included in this study were naturally infected and destined to euthanasia as recommended by the politics of Brazilian Ministry of Health. All procedures performed in the present study were in accordance with the ethical standards of the institution or practice. The present research uses tissues fragments after euthanasia, so the study did not need to be submitted for The Ethics Committee on Animal Use (CEUA) according to Brazilian's Law 11794/08.

Leishmania spp. isolation

Spleen samples were cultured in tubes with biphasic medium containing Schneider liquid medium for *Drosophyla* (Sigma) with 10-20% fetal bovine serum and NNN medium as solid phase. The tubes were maintained at 25 °C in Biological Oxygen Demand (B.O.D.) incubator (Contemp). The cultures were evaluated every 5 days for promastigotes growth and maintained at least for 4 weeks.

DNA extraction

Total DNA was isolated from approximately 10 mg of both spleen and liver samples using QIAmp® DNA Mini Kit (Qiagen, CA, USA), which included a prior phase of digestion with 20 µl of proteinase K (20 mg/mL) for 1 h at 56 °C. DNA was dissolved in 50 µl of Tris EDTA buffer (AE buffer) and quantified via NanoDrop® (Thermo Fisher Scientific, Waltham, MA, USA).

Molecular detection of Leishmania spp.

The molecular detection of *Leishmania* spp. by PCR targeting the ITS1 intergenic region was employed (GRAÇA et al., 2012). Restriction Fragment Length Polymorphism (RFLP) was performed for *Leishmania* species identification. The ITS1 PCR product was submitted to digestion using Sau 3AI and loaded on 12.5% polyacrylamide gel (Genephor). Two *Leishmania* reference strains were used as controls: IOC/L 579 (Li - *Leishmania* (*L.*)

infantum- MHOM/BR/1974/PP75) and IOC/L 566 (Lb - *Leishmania (V.) braziliensis*- MHOM/BR/1975/M2903).

Determination of Leishmania spp. burden by quantitative polymerase chain reaction (qPCR)

Leishmania parasite burden in spleen samples was estimated by quantitative real-time PCR (Step One equipment, Applied Biosystems, Molecular Probes, Inc.) using the detection system Power Sybr Green Master Mix (Applied Biosystems, Molecular Probes, Inc.). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) primers were chosen to normalize the concentrations of canine DNA in each sample; the primers described by Prina et al (PRINA et al., 2007) were used to amplify a product corresponding to the small subunit ribosomal RNA (ssrRNA), a multi-copy gene of *Leishmania* spp. parasites. DNA (100 ng in 2 µl) was added to a final PCR reaction volume of 20 µl containing Power Sybr Green 1X, 300 nM of each primer for HPRT and 500 nM for ssrRNA PCR assays. Reactions were submitted to a initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation, annealing and extension (95 °C for 15 seconds, 60°C for 1 minute and 68 °C for 30 seconds) with the option for a melt curve ranging from 60 °C to 95 °C and an incremental temperature of 0.3 °C for second and a Tm 78 ± 2 °C. Reactions were performed in duplicate and both targets were run within the same plate for the same sample.

The number of cells from peripheral blood mononuclear cells (PBMC) of non-infected dogs and cultures of *L. infantum* promastigotes (MCAN/BR/2007/CG-1) were determined by a cell counter (Z1™ COULTER COUNTER®, Beckman Coulter, Fullerton, CA, USA). The total DNA was isolated from 1.0×10^6 PBMC cells and 1.0×10^7promastigotes. Standard curves for the HPRT (R^2=0.966, slope= –2.98, efficiency=1.13, y-intercepto=34.143) and ssrRNA genes (R^2=0.943, slope= –2.67, efficiency=1.18, y-intercepto=28.117) were prepared using serial 10-fold dilutions from 10^{-2} to 10^7 of total purified DNA. All reactions were performed in duplicate in microtubes (Applied Biosystems). The parameters were according with MIQE guidelines (BUSTIN et al., 2009).

Molecular detection of H. canis

H. canis was detected by PCR based on the amplification of a fragment of 666 pb of 18S rDNA gene, using HepF (5' – ATA-CAT-GAG-CAA-AAT-CTC-AAC- 3') and HepR (5' – CTT-ATT-ATT-CCA-TGC-TGC-AG-3') primers (INOKUMA et al., 2002) and the GoTaq® DNA Polymerase kit (Promega, WI, USA). PCR was performed with a denaturation step at 94 °C for 3 minutes, followed by 37 cycles of denaturation, annealing and extension (94°C for 45 seconds, 57 °C for 45 seconds and 72 °C for 1 minute), and a final extension step (72 °C for 7 minutes).

PCR products were purified using ExoSAP® IT (GE Healthcare) accordingly to the manufacturer's recommendations and sequenced using the BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) in an automated Applied Biosystems ABI 3500 DNA genetic analyzer (Applied Biosystems). All sequences obtained were analyzed in the software package Phred/Phrap/Consed Version: 0.020425.c (University of Washington, Seattle, WA, USA) and presented Phred values above 20 Sequences were edited using BioEdit software, version 7.2.5 (HALL, 1999) and compared for similarity using BLAST to the sequences available in GenBank (NCBI, 2016).

Phylogenetic analysis were based on the sequences obtained in the study and sequences retrieved in from GenBank. Multiple alignment analysis was performed using the Clustal X version 2.0 (LARKIN et al., 2007), and aphylogenetic tree was constructed based on the Neighbor-Joining algorithm andthe Kimura 2-parameter model for generating the distance matrix. As implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 6 (TAMURA et al., 2013) Bootstrap of 1000 replicates were performed to estimate the confidence of the branching patterns (FELSENSTEIN, 1985). Histopathology

Spleen and liver fragments were fixed in 10% buffered formalin for one to four weeks, embedded in paraffin and sliced in 5-µm thick sections to be mounted on microscope slides. The sections were stained with hematoxylin and eosin (HE) and examined by light microscopy (Nikon Eclipse E400 – Tokyo, Japan). Structural changes of spleen lymphoid tissue, the cell population in the red pulp and the parasite burden were analyzed.

Results

The sampled dogs presented different clinical signs and *Leishmania* spp. parasite burdens, but all four were co-infected with *Leishmania* spp. and *Hepatozoon* spp. Each case is presented separately and differential diagnosis is summarized in Table 1.

Table 1. Results obtained by the diagnosis methods used to characterize *Hepatozoon* and *Leishmania* species.

Dog number	*Hepatozoon* spp.		*Leishmania* spp.			
	Parasitological detection (HE)	PCR (18S rRNA sequence)	Parasitological detection (culture)	DPP	ITS1 PCR (PCR-RFLP)	Amastigotes/10^6cells
1	+	np	+	+	(*L. (L.) infantum*)	1.10×10^8
2	+	*H. canis*	-	-	(*L. (V.) braziliensis*)	1.64×10^4
3	+	*H. canis*	-	-	(*L. (V.) braziliensis*)	1.43×10^3
4	+	np	-	+	(*L. (L.) infantum*)	4.13×10^6

(+) positive; (-) negative; (np) not performed. HE: hematoxylin and eosin; DPP: Dual Path Platform Test. PCR-RFLP: Polymerase Chain Reaction Followed by Restriction Fragment Length Polymorphism.

Description of cases

Dog 1: A male dog from Cuiabá, state of Mato Grosso, presenting cachexy and ascites. Parasite isolation was positive and the species identified as *L. infantum* in the spleen and liver (Figure 1). The splenic histopathological analysis showed an organized white pulp and many mature meronts of *H. canis* (Figure 2a-e). Histological alterations in the liver were not observed. Molecular analysis of *Hepatozoon canis* was not performed (Table 1).

Dog 2: Asymptomatic male dog from Barra do Garças, state of Mato Grosso. Although parasite isolation was negative, PCR-RFLP confirmed *L. braziliensis* infection in spleen and liver samples (Figure 1). The histopathological analysis of the spleen showed an organized white pulp and mature meronts of *H. canis*. The liver presented an intense non-granulomatous inflammation, with vacuolar degeneration, intense fibrosis and colestasis. Amastigotes were observed in the liver (Figure 2f). = *Hepatozoon* spp. DNA was only detected in spleen and the sequences showed 100% identicalness with *H. canis* by BLAST analysis (Tables 2, 3, Figure 3).

Dog 3: A male dog from Barra do Garças, state of Mato Grosso, presenting cachexy, lymphadenomegaly, onychogryphosis and conjunctivitis. Parasite isolation was negative; species detection was performed confirming *L. braziliensis* infection in a spleen sample (Figure 1). In the liver sample, the digestion of ITS1 PCR product generated inconclusive results. Histopathological analysis of the spleen showed intense disorganization of white pulp, perisplenitis, absence of granuloma and mature meronts of *Hepatozoon canis*. The liver presented intense and diffuse non-granulomatous inflammation, with vacuolar degeneration and amastigotes. *Hepatozoon* spp. DNA was detected in the spleen and liver and the sequences showed 100% identicalness with *H. canis* by BLAST analysis (Tables 2, 3, Figure 3).

Dog 4: A female dog from Cuiabá, state of Mato Grosso, presenting alopecia, onychogryphosis, dermatitis and conjunctivitis. Parasite isolation was negative, though the infection by *L. infantum* was confirmed in the spleen and liver (Figure 1) by PCR-RFLP. Histopathological analysis of the spleen showed moderate disorganization of the white pulp, amastigotes and mature meronts of *H. canis*. The liver presented only a moderate and diffuse non-granulomatous inflammation. Molecular detection of *H. canis* was not performed.

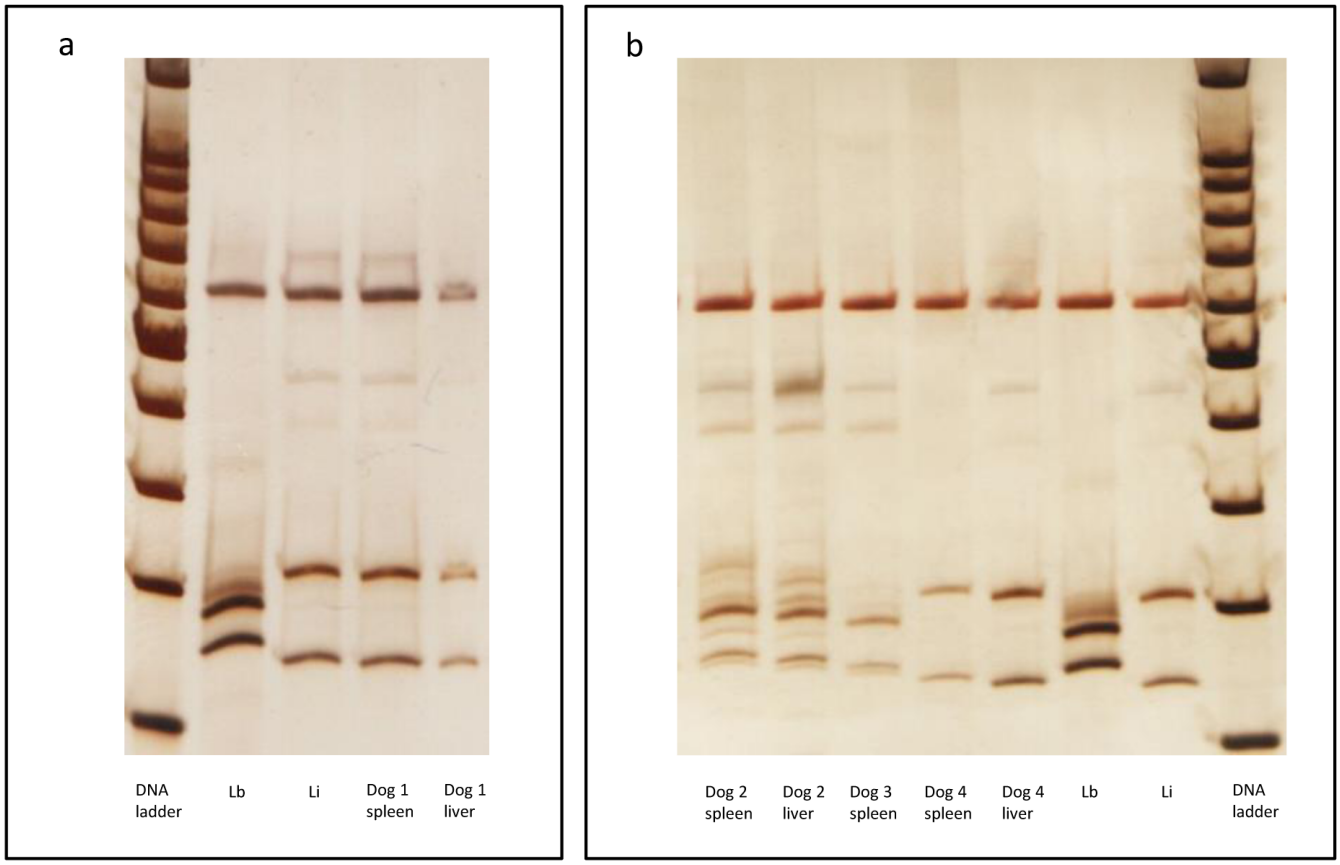

Figure 1. Molecular detection of *Leishmania* sp using ITS1 PCR-RFLP after Sau 3AI digestion. Silver stained polyacrylamide gel (12.5%) (Genephor). (a) Dog #1; and (b) Dogs #2, #3 and #4 analysis. The following *Leishmania* reference strains were used: Li - IOC/L 579 (*Leishmania (L.) infantum*- MHOM/BR/1974/PP75) and Lb - IOC/L 566 (*Leishmania (V.) braziliensis*- MHOM/BR/1975/M2903). DNA Ladder = 100bp from Promega. ITS: internal transcribed spacer; PCR RFLP: Polymerase Chain Reaction Followed by Restriction Fragment Length Polymorphism

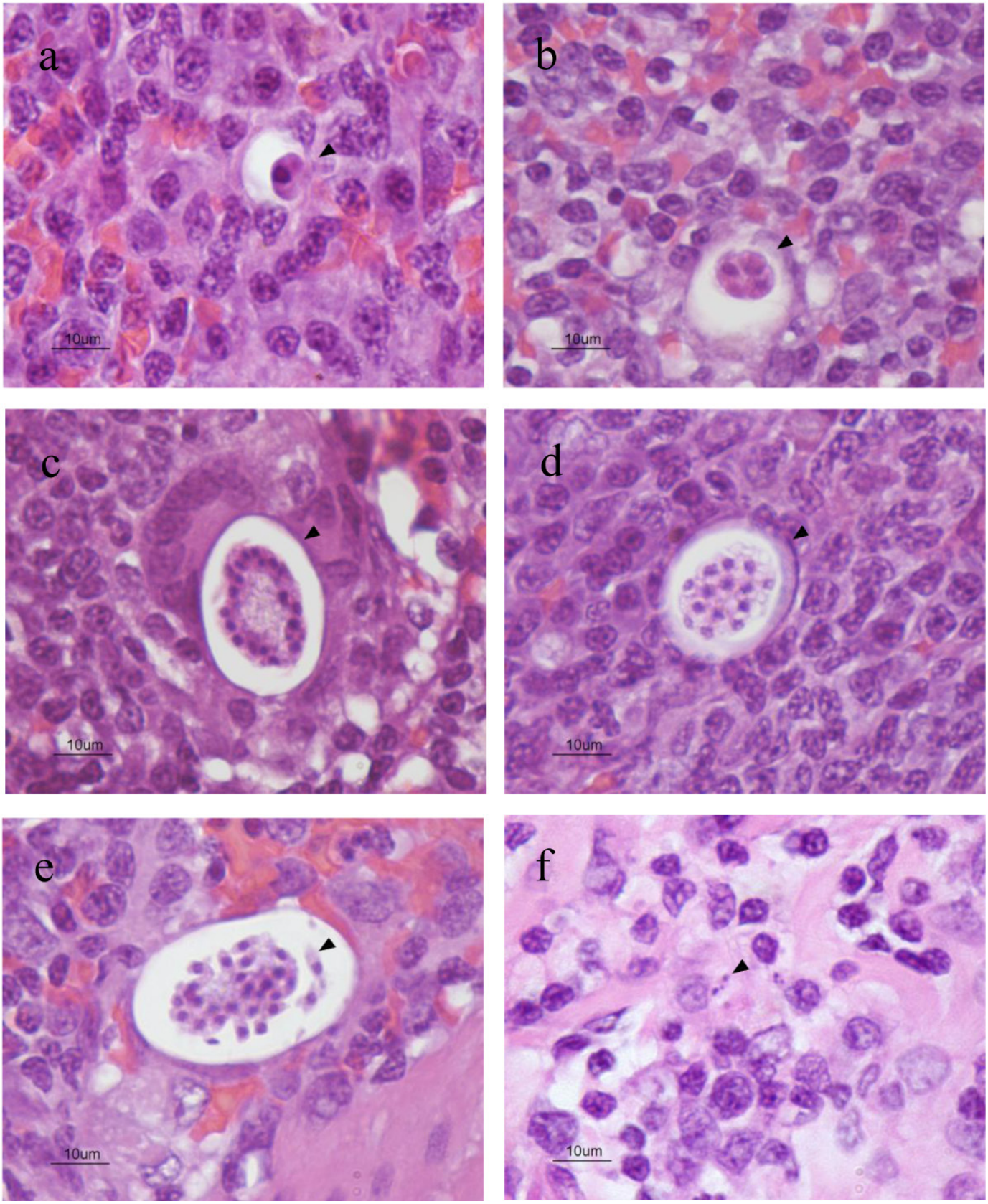

Figure 2. Evidence of the parasitic forms of *Hepatozoon* spp. and *Leishmania* spp. in the spleen and liver of infected dogs. (a-e) spleen and (f) liver. (a) A monozoic tissue cyst and (b) a mature meront containing four macromerozoites of *Hepatozoon canis*; (c) a mature meront containing micromerozoites arranged at the periphery presenting a "wheel spoke" pattern of *Hepatozoon canis* inside a multinucleated giant cell and (d) a meront containing 20-30 micromerozoites; (e) micromerozoites of *Hepatozoon canis* being released; (f) amastigotes of *Leishmania braziliensis*.

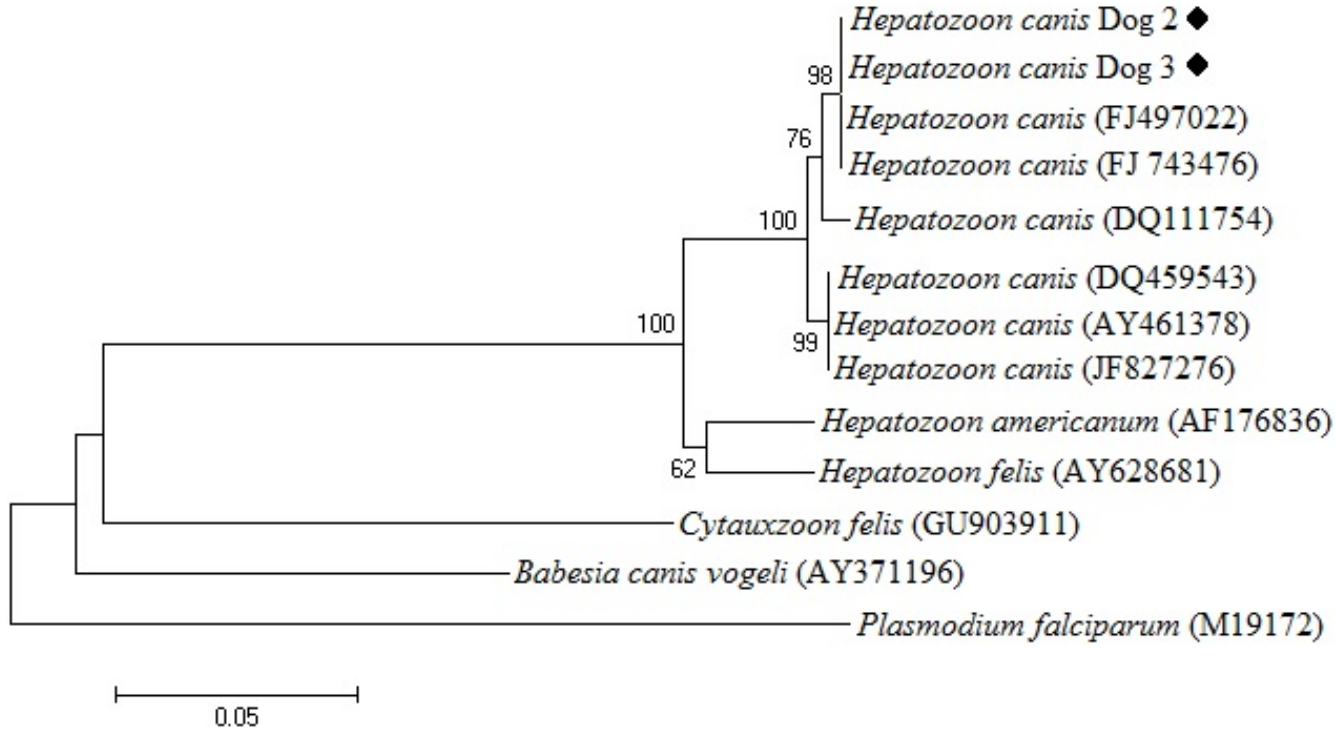

Figure 3. Neighbor-Joining Tree based on the 18S rDNA gene with 666 bp for *Hepatozoon* spp. related to sequences obtained with the HepF and HepR oligonucleotides. The Kimura 2-parameter model was used. Numbers at node indicate bootstrap values over 1000 replicates. Scale indicates the evolutionary distance of 0.05 nucleotides per position in the sequence. *Cytauxzoon felis*, *Babesia canis vogeli* and *Plasmodium falciparum* were used as outgroup.

Table 2. *Hepatozoon* species included in the alignment and in the Neighbor-Joining tree.

Specie	Code in *GenBank*	Host	Country
Hepatozoon canis	AY461378	Dog	Spain
Hepatozoon canis	DQ111754	Dog	Sudan
Hepatozoon canis	DQ439543	Dog	Venezuela
Hepatozoon canis	FJ497022	Dog	Croatia
Hepatozoon canis	FJ743476	Dog	Brazil
Hepatozoon canis	JF827276	Dog	Italy
Hepatozoon americanum	AF176836	Dog	Spain
Hepatozoon felis	AY628681	Cat	Spain
Hepatozoon canis Dog 2	_	Dog	Brazil
Hepatozoon canis Dog 3	_	Dog	Brazil

Table 3. Data from the analysis of genetic identity using Blast software.

Sample	GenBank accession numbers of genetically similar strains	Percentage of identity
Dog 2 (spleen)	*Hepatozoon canis* FJ497022.1	100%
	Hepatozoon canis FJ743476.1	100%
	Hepatozoon canis AY15006.1	100%
Dog 3 (spleen)	*Hepatozoon canis* FJ497022.1	100%
	Hepatozoon canis FJ743476.1	100%
	Hepatozoon canis AY461375.2	100%
Dog 3 (liver)	*Hepatozoon canis* KC509526.1	100%
	Hepatozoon canis FJ743476.1	100%
	Hepatozoon canis AY150067.2	100%

Discussion

In the present study, *H. canis* infection and co-infection with *Leishmania* spp. were confirmed through histopathological and molecular analyses in the spleen for the first time in dogs from Barra do Garças, Mato Grosso state - Brazil. *In silico* analysis showed that the ssrRNA and 18S rRNA primers used in this work were specific for *Leishmania* spp. and *Hepatozoon* spp., respectively (data not shown), and did not generate cross-reaction. We observed 100% identicalness between our sequences with *H. canis* from Croatia and other regions of Brazil, suggesting either a geographic expansion of this infection or a recent increase in the efficiency in detecting these parasites. Recently, canine hepatozoonosis has been described in several Brazilian regions (RUBINI et al., 2008; MIRANDA et al., 2014; GONÇALVES et al., 2014; RAMOS et al., 2015; DEMONER et al., 2016). Many of these areas are also endemic for CVL and co-infection was indeed described in dogs from Rio Grande do Norte, but only through molecular analysis (GONÇALVES et al., 2014).

The pathogenesis of *H. canis* infection is influenced by immunodeficiency conditions, an immature immune system in young pups, or a concurrent infectious agent (BANETH, 2011). Conditions that impair the immune responses increase the susceptibility to new infection with *H. canis* or allow existing infections to reactivate. Co-infections with *Toxoplasma, Leishmania, Babesia* or *Ehrlichia* predispose dogs to clinical illness (BANETH, 2011). Detection of *L. braziliensis* parasites DNA in the liver and spleen shows the probability of the visceralization of this species in dogs presenting comorbidities. Although DNA detection may not show the presence of viable parasites, additional results (observation of amastigotes and ELISA and IFAT positivity) confirmed the infection. Currently, *L. braziliensis* is considered a dermotrophic species in the human leishmaniasis disease complex, with isolation and detection of this species in the viscera only in cases of immunologically compromised patients (SILVA et al., 2002; GONTIJO et al., 2002). In wild and synantropic animals (considered potential reservoirs for *L. braziliensis*), such a distinction regarding tissue tropism does not exist (ROQUE et al., 2010; ROQUE & JANSEN, 2014), and the parasite may be detected in many organs. In dogs, this species has only been detected in cutaneous lesions (MADEIRA et al., 2005; DANTAS-TORRES, 2007). The visceralization detected herein by *L. braziliensis* may be a consequence of the impaired immune system of the dogs. Such an immunological deficit may be due to either *Hepatozoon* infection alone or the increased virulence due to co-infection. These hypotheses are difficult to verify, but the first is more likely since the two dogs infected with *L. braziliensis* showed lower parasite burden than the dog infected by *L. infantum*, and only one was symptomatic. If we consider that there is a relationship between clinical signs and parasite burden (TEIXEIRA-NETO et al., 2010), the clinical signs described in dog #3 must have been related to the infection by *Hepatozoon*. Visceralization of *L. braziliensis* strains was previously reported in dogs (OLIVEIRA et al., 2013). Although the authors attributed dissemination and tissue tropism of populations to a distinct genetic profile, they did not mention if there were comorbidities in the evaluated dogs.

Therefore, other studies are needed to highlight the potential influence of the immune response that both infections could have on each other. The understanding of this co-infection is crucial since the impaired immune response to leishmaniasis may lead to an increased severity of the disease and, consequently, to a high *Leishmania* parasite burden and dissemination. This may be - at some point - related to the potential of dogs as reservoirs. The visceralization of *L. braziliensis*, whether related with the co-infection with *Hepatozoon* or not, raises a question about its impact on *Leishmania* parasite burden and consequently on the role of dogs as a source of infection to vectors.

In this study, two animals were infected by *L. braziliensis* and, for this reason, were unnecessarily submitted to euthanasia. They were DPP negative, which suggests this test may be a good tool for the differential diagnosis and euthanasia. Although cross-reaction could be observed by ELISA based on DPP antigens with sera from dogs infected with *Leishmania braziliensis*, this cross-reaction is less frequent than that observed when using crude antigens (PORROZZI et al., 2007). Nevertheless, it is not expected that this cross-reaction occurs in immunochromatographic tests. Because only two animals were evaluated in the present study, this hypothesis must be better verified. In spite of that, the data points out the importance in performing an etiological diagnosis at the species level. Since 2012, DPP® (rK39 antigen, Biomanguinhos) has been used as a screening test for CVL followed by confirmation with an ELISA test to identify animals for euthanasia when they are positive by both methods. However, when the samples used herein were collected, DPP® had not been used as a method to diagnose CVL yet. For these two dogs, clinical signs attributed to *Leishmania* infection could actually be related to *Hepatozoon* infection, since *L. braziliensis* species does not lead to such clinical profile. *Hepatozoon* infection could in turn cause immunosuppression leading to the visceralization of *Leishmania braziliensis*. In this context, the importance of a complementary diagnosis in the veterinary clinical evaluation, either molecular or serological, is clear. Additionally, the impact of co-infection with *Leishmania* spp. and *Hepatozoon* spp. or other pathogens (*Ehrlichia canis*, *Babesia* spp., *Anaplasma* spp., etc.) on clinical manifestations, the immune response and the parasite burden needs to be clarified.

Conclusion

In the present work, *Hepatozoon canis* infection and co-infection with *Leishmania braziliensis* were identified for the first time in Barra do Garças, Mato Grosso state, Brazil. From this report, the main points were addressed: i) visceral dissemination of *L. braziliensis* associated with systemic signs suggested that this co-infection could have influenced the parasite burden, the parasite spread, morbidity, clinical signs and, possibly, the role of co-infected dogs as reservoirs; ii) it is important for veterinarians to promote a differential diagnosis for co-infected dogs, with an emphasis on defining the involved *Leishmania* species.

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