Molecular and serological detection of tick-borne pathogens in dogs from an area endemic for *Leishmania infantum* in Mato Grosso do Sul, Brazil

Detecção sorológica e molecular de patógenos transmitidos por carrapatos em cães de uma área endêmica para *Leishmania infantum* do estado do Mato Grosso do Sul

Keyla Carstens Marques de Sousa[1]; Marcos Rogério André[1]; Heitor Miraglia Herrera[2]; Gisele Braziliano de Andrade[2];
Marcia Mariza Gomes Jusi[1]; Luciana Ladislau dos Santos[2]; Wanessa Teixeira Gomes Barreto[2];
Rosangela Zacarias Machado[1]; Gilson Pereira de Oliveira[1]*

[1]Faculdade de Ciências Agrárias e Veterinárias – FCAV, Universidade Estadual Paulista – Unesp, Jaboticabal, SP, Brasil

[2]Universidade Católica Dom Bosco – UCDB, Campo Grande, MS, Brasil

Abstract

Tick-borne pathogens affect a wide range of vertebrate hosts. To identify tick-borne pathogens among dogs from Campo Grande, MS, Brazil testing seropositive for *Leishmania infantum* (syn. *L. chagasi*), a serological and molecular study was conducted to detect *Ehrlichia canis, Anaplasma platys* and *Babesia vogeli* in 60 serum and spleen samples. A confirmatory diagnosis of *L. infantum* based on serological and molecular assays was also performed, as was sequence alignment and phylogenetic analysis to assess the identity of the parasite species infecting these animals. IgG antibodies to *Ehrlichia* spp., *B. vogeli* and *L. infantum* were found, respectively, in 39 (65%), 49 (81.6%) and 60 (100%) of the sampled dogs. Twenty-seven (45%), fifty-four (90%), fifty-three (88.3%), two (3.3%) and one (1.6%) dog were positive, respectively, for *E. canis, Leishmania* spp., *Leishmania donovani complex, Babesia* sp. and *Anaplasma* sp. in PCR assays. After sequencing, the amplicons showed 99% of identity with *E. canis, B. vogeli, A. platys* and *Leishmania chagasi* isolates. The findings of this study indicate that *L. infantum*-seropositive dogs from Campo Grande are exposed to multiple tick-borne pathogens, which should therefore be included in the differential diagnosis of dogs with clinical suspicion of leishmaniasis.

Keywords: *Ehrlichia canis, Babesia vogeli, Anaplasma platys, Leishmania infantum,* dogs, co-infection.

Resumo

Patógenos transmitidos por carrapatos atingem uma variedade de hospedeiros vertebrados. Para identificar os agentes patogênicos transmitidos por carrapatos entre cães soropositivos para *Leishmania infantum* no município Campo Grande-MS, foi realizado um estudo sorológico e molecular para a detecção de *Ehrlichia canis, Anaplasma platys* e *Babesia vogeli* em 60 amostras de soro e baço, respectivamente. Adicionalmente, foi realizado o diagnóstico confirmatório de *L. infantum* por meio de técnicas sorológicas e moleculares. Também foi realizado o alinhamento e análise filogenética das sequências para indicar a identidade das espécies de parasitas que infectam esses animais. Anticorpos IgG anti-*Ehrlichia* spp., anti-*B. vogeli* e anti-*L. infantum* foram detectados em 39 (65%), 49 (81,6%) e 60 (100%) dos cães amostrados, respectivamente. Vinte e sete (45%), cinquenta e quatro (90%), cinquenta e três (88,3%), dois (3,3%) e um (1,6%) cães mostraram-se positivos na PCR para *E. canis, Leishmania* spp., *Leishmania donovani complex, Babesia* sp. e *Anaplasma* sp., respectivamente. Após o seqüenciamento, os amplicons mostraram 99% de similaridade com isolados de *E. canis, B. vogeli* e *A. platys* e *Leishmania chagasi*. Os resultados deste estudo indicaram que os cães soropositivos para *L. infantum* de Campo Grande, MS, são expostos a vários agentes transmitidos por carrapatos, e, portanto, devem ser incluídos no diagnóstico diferencial em cães com suspeita clínica de leishmaniose.

Palavras-chave: *Ehrlichia canis, Babesia vogeli, Anaplasma platys, Leishmania infantum,* cães, co-infecção.

*Corresponding author: Gilson Pereira de Oliveira
Faculdade de Ciências Agrárias e Veterinárias Júlio de Mesquita Filho,
Universidade Estadual Paulista – UNESP, Campus de Jaboticabal, Via de
Acesso Prof. Paulo Donato Castellane, s/n, Zona Rural, CEP 14884-900,
Jaboticabal, SP, Brasil
e-mail: gilsongpod@fcav.unesp.br

Introduction

Tick-borne pathogens are emerging agents responsible for diseases in both humans and animals worldwide (SHAW et al., 2001). Ehrlichiosis, anaplasmosis and babesiosis are tick-borne diseases of worldwide occurrence in dogs, whose main vector is the brown dog tick, *Rhipicephalus sanguineus* (DAGNONE et al., 2003; NAKAGHI et al., 2008; OLIVEIRA et al., 2008). Dogs can be infected by several agents of the Anaplasmataceae family (DUMLER et al., 2001), such as *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia chaffeensis*, *Anaplasma platys*, *Anaplasma phagocytophilum*, *Neorickettsia risticii* (INOKUMA et al., 2001) and *N. helminthoeca* (HEADLEY et al., 2006).

Ehrlichia canis is a common pathogen among domestic dogs in Brazil, and is considered primarily responsible for canine monocytic ehrlichiosis (NAKAGHI et al., 2008; DAGNONE et al., 2009). Clinical manifestations of the disease include fever, weight loss, anorexia, bleeding disorders and lymphadenopathy (CASTRO et al., 2004; NAKAGHI et al., 2008). *Anaplasma platys*, a bacterium that parasitizes platelets, causing canine cyclic thrombocytopenia (COHN, 2003), has also been reported in dogs in Brazil (DAGNONE et al., 2009). Furthermore, *A. phagocytophilum* also has been detected molecularly in dogs in the state of Rio de Janeiro, Brazil (SANTOS et al., 2011). Although dogs infected with *A. platys* and *A. phagocytophilum* usually present mild symptoms or are asymptomatic (HARRUS et al., 1997; KOHN et al., 2008; RAR; GOLOVLJOVA, 2011), some cases may be serious or fatal, particularly when co-infections are involved (DANTAS-TORRES, 2008). The confirmation of the occurrence of *E. ewingii* (OLIVEIRA et al., 2009) and *A. phagocytophilum* (SANTOS et al., 2011) in dogs from Minas Gerais and Rio de Janeiro, respectively, showed that other Anaplasmataceae agents circulate among dogs in Brazil, although the vector tick species involved in these transmission routes remains unknown.

Babesiosis, which is caused by intraerythrocytic protozoa of the genus *Babesia* sp., is an emerging disease transmitted to humans and animals by ticks (IRWIN, 2009). In Brazil, apart from a single report of *B. gibsoni* in a dog in the state of Paraná (TRAPP et al., 2006), dogs are more often parasitized by *B. vogeli* (PASSOS et al., 2005; FURUTA et al., 2009). Clinical manifestations of canine babesiosis are characterized by fever, lethargy, anorexia, jaundice, pale mucous membranes, splenomegaly, and weight loss. The prognosis for diseases caused by piroplasm species is usually good, except when the disease is caused by *B. rossi* and *B. gibsoni* (IRWIN, 2009; SOLANO-GALLEGO; BANETH, 2011).

Visceral leishmaniasis (VL), an infectious disease that affects both humans and animals, is caused by protozoan parasites of the genus *Leishmania*, such as *Leishmania (Leishmania) donovani* and *Leishmania (Leishmania) infantum*. The latter is endemic in the New World in regions extending from the southern United States to northern Argentina, including Brazil (KUHLS et al., 2011). The diagnosis of VL has proved to be problematic, mainly because of the wide variety of non-specific clinical symptoms, including a broad spectrum of presentations (BLAVIER et al., 2001). The immunosuppression observed in VL may promote the occurrence of co-infections with other agents such as *Ehrlichia,*

Babesia, Dirofilaria, Toxoplasma and *Neospora* in endemic regions (FEITOSA et al., 2000; PAULAN et al., 2013). On the other hand, a primary infection with tick-borne agents is likely to be a contributing factor to the establishment of canine VL, as was shown for ehrlichiosis in a longitudinal study of naturally exposed naïve dogs (MEKUZAS et al., 2009). The present study aimed to verify the occurrence of *E. canis*, *A. platys* and *B. vogeli* among *L. infantum*-seropositive dogs from Campo Grande in the Brazilian state of Mato Grosso do Sul (MS), using serological and molecular techniques.

Materials and Methods

Between June 2011 and March 2012, serum and spleen samples were collected from 60 *L. infantum*-seropositive dogs from the Center for Zoonosis Control (CZC) of Campo Grande, Mato Grosso do Sul and subjected to an enzyme-linked immunosorbent assay (ELISA). Blood samples for the serological tests were drawn from each dog by cephalic venipuncture and stored without anticoagulant (EDTA). During necropsy, fragments of spleen were collected aseptically and stored in absolute ethanol for DNA extraction. Additionally, serological and molecular assays were performed for a confirmatory diagnosis of *L. infantum*.

A *Leishmania* sp. strain isolated in Araçatuba, São Paulo, Brazil, characterized as *donovani* complex, probably *L. infantum*, using molecular techniques described by Cortes et al., (2004), was used in this study. The parasites were maintained on RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Canyon City, USA) at 25 °C and were used to prepare the crude antigens for IFAT and ELISA.

The ELISA method employed here was described in detail by Oliveira et al. (2008). Briefly, 100 mL of crude *Leishmania* sp. antigen diluted in 0.05M sodium carbonate-bicarbonate buffer (pH 9.6) was added to each well on ELISA plates (Nunclon TM surface; Nunc, Denmark) and 5mg mL protein concentration was used. After overnight incubation at 4 °C, the plates were washed three times with 0.05% PBS Tween-20. The plates were blocked with 200 mL containing 6% skim milk for 2 hours at 37 °C to reduce nonspecific binding. The blocking agent was removed, and individual dog serum diluted (1:400) in PBS Tween-20 with 5% skim milk added to each well, followed by incubation for 90 minutes at 37 °C and washing as described above. One hundred mL of alkaline phosphatase conjugated anti-dog IgG (Sigma-Aldrich, St. Louis, USA) diluted at 1: 4.000 in PBS Tween-20 with 5% normal rabbit serum was added to each well, followed by incubation for 90 minutes at 37 °C. The plates were washed and the substrate (p-nitrophenyl phosphate) diluted in diethanolamine buffer, pH 9.8, was added. Absorbance at 405 nm was read after 45 minutes of incubation at room temperature, using an ELISA reader (Dynex Technologies, USA). The discriminating absorbance value (cut-off) was determined as being 2.5 times the mean absorbance value of the negative control.

Ehrlichia spp. antigen was obtained from *E. canis* (Jaboticabal strain)-infected DH82 cells maintained in culture in the Immunoparasitology Laboratory of UNESP at Jaboticabal, São

Paulo (AGUIAR et al., 2007). *Babesia* spp. antigen was prepared by intravenous inoculation of *B. vogeli* (Jaboticabal strain) into a splenectomized three month-old dog negative for hemoparasites by PCR and serology (FURUTA et al., 2009). Giemsa-stained blood smears were examined microscopically twice daily day for the presence of parasites. After peak parasitemia was observed on the fifth day after inoculation, infected blood was collected with Alsever's solution (113.7 mM glucose, 27.2 mM sodium citrate, 71.8 mM sodium chloride).

Slides containing air-dried fixed *B. vogeli* trophozoite-infected blood, *E. canis*-infected DH82 cells and *Leishmania* sp. promastigotes were tested by IFAT (Indirect Fluorescent Antibody Test), as described previously (FURUTA et al., 2009; NAKAGHI et al., 2008; OLIVEIRA et al., 2008). Antigen slides were removed from storage (–20 °C) and allowed to thaw at room temperature for 30 min. Ten microliters of twofold dilutions of sera (cut-off of 1:64 for *Ehrlichia* spp., 1:40 for *B. vogeli* and 1:40 for *L. infantum*) were placed in wells on antigen slides. Canine sera known to be positive for *E. canis*, *B. vogeli* and *L. infantum* were obtained from symptomatic dogs with ehrlichiosis, babesiosis and leishmaniasis treated in the Governador Laudo Natel Veterinary Hospital of UNESP at Jaboticabal, SP, Brazil, and used as positive control. Negative control serum samples were obtained from dogs (unexposed to these agents, according to negative PCR and IFAT results) that were kept at the kennel of the Department of Veterinary Pathology of UNESP at Jaboticabal. Slides were incubated at 37 °C in a moist chamber for 45 min, washed 3 times in PBS (pH 7.2) for 5 min, and air dried at room temperature. FITC-labeled anti-dog IgG conjugate (Sigma-Aldrich®, St. Louis, MO, USA)

was diluted according to the manufacturer's instructions (dilution of 1:32) and added to each well. These slides were incubated again at 37 °C, washed 3 times in PBS, then in distilled water, and air dried at room temperature. Next, coverslips were placed on the slides, which were examined under a fluorescence microscope.

DNA was extracted from spleen fragments using the QIAamp DNA Blood and Tissue Mini Kit (QIAGEN, Valencia, California, USA) according to the manufacturer's instructions. Each sample of extracted DNA was used as a template in 25 μL of reaction mixture containing 10x PCR buffer, 1.0 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate (dNTPs) mixture, and 1.5 U Taq DNA Polymerase (Invitrogen, Carlsbad, California, USA) with 0.5 μM of genus specific primers for *Ehrlichia* spp. (*16S* rRNA gene) (MURPHY et al., 1998), *Anaplasma* spp. (*16S* rRNA gene) (MASSUNG et al., 1998), *Babesia* spp. (*18S* rRNA gene) (CARRET et al., 1999), *Leishmania* spp. (kinetoplast DNA) (MICHALSKY et al., 2002) and *L. donovani* complex (CORTES et al., 2004) (Table 1). Positive controls of *E. canis* and *B. vogeli* DNA samples were obtained from dogs infected experimentally with Jaboticabal strains of *E. canis* (CASTRO et al., 2004) and *B. vogeli* (FURUTA et al., 2009), respectively. *A. platys* DNA positive control was obtained from a dog from Campo Grande, MS naturally infected with *A. platys* (DAGNONE et al., 2009). *L. infantum* DNA positive control was obtained from parasites maintained in culture. Ultrapure sterile water was used as negative control. To prevent PCR contamination, the DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separate rooms. The reaction products were purified using a Silica Bead DNA Gel Extraction

Table 1. Description of primers, PCR product size and references used in PCR assays for *E. canis, Anaplasma* spp., *Babesia* spp., *Leishmania* spp. and *L. donovani* complex.

Agent and Primers	Oligonucleotide Sequence	Product PCR Size	References
Ehrlichia spp.			
-ECC	5'- GAACGAACGCTGGCGGCAAGC-3'	478	MURPHY et al., 1998
-ECB	5'- CGTATTACCGCGGCTGCTGGCA -3'		
Nested *E. canis*			
-ECAN-5	5'-CAA TTATTTATAGCCTCTGGCTATAGGA-3'	358	MURPHY et al., 1998
-HE3	5'-TATAGGTACCGTCATTATCTTCCCTAT-3'		
Anaplasma spp.			
-gE3a	5′-CACATGCAAGTCGAACGGATTATTC-3′	932	MASSUNG et al., 1998
-gE10R	5′- TTCCGTTAAGAAGGATCTAATCTCC′-3′		
Nested			
Anaplasma spp.			
-gE2	5′- GGCAGTATTAAAAGCAGCTCCAGG-3′	546	MASSUNG et al., 1998
-gE9f	5′-AACGGATTATTCTTTATAGCTTGCT-3′		
Babesia spp.			
-Piro A	5'-AATACCCAATCCTGACACAGGG-3'	400	CARRET et al., 1999
-Piro B	5'-TTAAATACGAATGCCCCCAAC-3'		
Leishmania spp.			
-L1	5'-GGGGAGGGGCGTTCTGCGAA-3'	120	MICHALSKY et al., 2002
-L2	5'-GGCCCACTATATTACACCAACCCC-3'		
L. donovani complex			
-MC1	5'-GTTAGCCGATGGTGGTCTTG-3'	447	CORTES et al., 2004
-MC2	5'-CACCCATTT TTCCGA TTT TG-3'		

Kit (Fermentas®, São Paulo, SP, Brazil). Purified amplified DNA fragments were subjected in-house to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyzer - Applied Biosystem/ Perkin Elmer) and used for subsequent phylogenetic analysis. Phylogenetic reconstructions were based on DNA sequence alignment of positive samples. Consensus sequences were obtained by analyzing the sense and antisense sequences using the CAP3 program (http://mobyle.pasteur.fr/cgibin/MobylePortal/portal.py). Comparisons were made with sequences deposited in GenBank using the basic local alignment search tool (BLAST).

Results

IgG antibodies to *Ehrlichia* spp., *B. vogeli* and *L. infantum* were found, respectively, in 39 (65%), 49 (81.6%) and 60 (100%) sampled dogs. IFAT titers ranged from 40 to 5120 for *B. vogeli*, 64 to 2048 for *Ehrlichia* spp. and 40 to 20480 for *L. infantum*. Thirty-four dogs (56.6%) showed antibodies to *Ehrlichia* spp., *B. vogeli*, and *L. infantum*; 6 (10%) dogs were seronegative for *Ehrlichia* spp. and *B. vogeli*, but seropositive for *L. infantum*. Five (8.3%) dogs were seropositive only for *Ehrlichia* spp. and *L. infantum*; 15 (25%) were seropositive only for *B. vogeli* and *L. infantum*, and six (10%) dogs were only seropositive for *L. infantum* (Table 2).

Twenty-seven (45%) dogs were positive for *E. canis* nPCR, 54 (90%) for *Leishmania* spp. PCR, 53 (88.3%) for *L. donovani* complex PCR, two (3.3%) for *Babesia* spp. PCR and only one dog (1.6%) for *Anaplasma* spp. nPCR. All 27 *E. canis*-nPCR positive dogs showed antibodies to the agent; 12 (30.7%) out of 39 *Ehrlichia* spp -seropositive dogs did not test positive in the nPCR assay. Twenty-one (35%) out of 60 sampled dogs were negative for both *Ehrlichia* spp -IFAT and nPCR. Twenty-three (38.3%) dogs were positive for both *E. canis* and *Leishmania* spp. PCR assays, and 22 (36.6%) for *E. canis* and *L. donovani* complex PCR assays (Table 2).

Only one dog was positive for both *B. vogeli*-IFAT and PCR, showing a low titer (40) in IFAT. One dog was positive in PCR but negative in IFAT for *B. vogeli*. Forty-eight (80%) dogs tested positive for *B. vogeli* only in IFAT. Ten (16.6%) were negative for both *B. vogeli*-IFAT and PCR. One dog (1.6%) was

positive for *E. canis* nPCR and *Babesia* spp. PCR, respectively, and one animal (1.6%) tested positive in the *Leishmania* spp., *L. donovani* complex and *Babesia* spp. PCR assays. One (1.6%) dog tested positive in all *Anaplasma* spp. nPCR, *Leishmania* spp. and *L. donovani* complex PCR assays, and showed antibodies for both *B. vogeli* and *L. infantum* (Table 2).

Fifty-three dogs (88.3%) tested positive in *L. infantum* IFAT and *Leishmania* spp. and *L. donovani* complex PCR assays. Only one dog was positive for *Leishmania* spp. PCR and negative for *L. donovani* complex PCR. All 54 *Leishmania*-PCR positive dogs showed antibodies to this agent; 6 (10%) and 7 (11.6%) out of 60 *L. infantum*-seropositive dogs did not test positive in *Leishmania* spp. and *L. donovani* complex PCR assays, respectively (Table 2).

After sequencing, amplicons obtained in the *E. canis* nPCR (358 pb) showed 99% of identity with sequences of *E. canis* found in dogs from the Philippines (JX893523), Japan (AB723712) and Taiwan (GU810149) by BLAST analysis. An amplicon obtained after *Anaplasma* spp. nPCR (546 pb) showed 99% of identity with sequences of *A. platys* found in dogs from the Philippines (JQ894779), Croatia (JQ396431) and Malaysia (JF683610). Amplicons obtained in *Babesia* spp. PCR (400 pb) showed 99% of identity with sequences of *B. vogeli* found in dogs from Colombia (JN368081), China (HM590440) and Venezuela (DQ297390). Amplicons obtained in *L. donovani* complex PCR (447pb) showed 99% of identity with the complete sequence of *Leishmania infantum* (syn. *L. chagasi*) (AF169138, AF103741, AF169139). *Ehrlichia canis* and *A. platys* strain Campo Grande-MS 16S rRNA partial sequence, *B. vogeli* strain Campo Grande-MS 18S rRNA partial sequences, and *L. infantum* strain Campo Grande-MS kinetoplast DNA partial sequence were deposited in GenBank database under access numbers KC989958, KC989957, KC989959 and KF695386 respectively.

Discussion

This study demonstrated the occurrence of *E. canis, A. platys* and *B. vogeli* in *L. infantum*-seropositive dogs from Campo Grande, MS, by molecular and serological techniques. *L. infantum* IFAT confirmed the results of the ELISA test conducted at the CZC. On the other hand, only 54 (90%) of the animals

Table 2. Results of serology for *E. canis*, *B. vogeli* and *L. infantum* and PCR for *Anaplasma* spp, *Babesia* spp, *E. canis, Leishmania* spp. and *L. donovani* complex in 60 dogs from Campo Grande, MS, Brazil.

Serology	PCR									
	Babesia spp.		*E. canis*		*Anaplasma* spp.		*Leishmania* spp..		*L. donovani* complex	
	+	-	+	-	+	-	+	-	+	-
Seropositive to *B. vogeli* (n = 49)	1	48	24	25	1	48	45	4	44	5
Seronegative to *B. vogeli* (n = 11)	1	10	3	8	0	11	9	2	9	2
Seropositive to *E.canis* (n = 39)	1	38	27	12	0	39	34	20	33	6
Seronegative to *E.canis* (n = 21)	1	20	0	21	0	21	5	1	20	1
Seropositive to *L. infantum* (n = 60)	2	58	27	33	1	59	54	6	53	7
Seronegative to *L. infantum* (n = 0)	0	0	0	0	0	0	0	0	0	0
Total (n = 60)	2	58	27	33	1	59	54	6	53	7

tested positive in the *Leishmania* spp. PCR assay and 53 in the *L. donovani* complex PCR assay. Although Brazil's Ministry of Health recommends serological assays for the diagnosis of *L. infantum* (BRASIL, 2003), cross-reactivity between *Trypanosoma cruzi* and *Leishmania braziliensis*-infected patients is well documented in the serodiagnosis of leishmaniasis (VEXENAT et al., 1996; LUCIANO et al., 2009). In our study, cross-reactivity between dog serum samples positive to *T. cruzi* and *L. braziliensis* could not be discarded, since the occurrence of *T. cruzi* and *L. braziliensis* among dogs is documented in the state of Mato Grosso do Sul (POMPILIO et al., 2005; SAVANI et al., 2009; SOUZA et al., 2009; CORVALAN et al., 2011). One dog that tested positive in the *Leishmania* spp. PCR assay but negative in the PCR assay for *L. donovani* complex could be infected by other *Leishmania* spp. species, i.e., *L. braziliensis* or *L. amazonensis,* which have been reported previously in the state of Mato Grosso do Sul (DORVAL et al., 2006; CORVALAN et al., 2011).

There are a few reports about the occurrence of *Babesia* spp. in dogs from Campo Grande, MS, which are based only on the observation of piroplasms in blood smears (SALGADO, 2006). Serology is a useful tool for the diagnosis of reservoir hosts for *Babesia* spp., because the parasitemia in dogs naturally infected with *B. vogeli* is usually low (FURUTA et al., 2009). Although the molecular detection of *B. vogeli* in the present study was low (3.3%), the overall seroprevalence was 81.6%, suggesting a previous exposure to this agent. PCR is considered a highly sensitive and specific technique, but its sensitivity is low when samples are collected from naturally asymptomatic *Babesia*-infected dogs in the chronic phase of the disease (BOOZER; MACINTIRE, 2003), when low fluctuations in parasitemia are observed (IRWIN, 2009). In our study, the presence of dogs seropositive to *B. vogeli* showing negative results for *Babesia* spp. PCR suggests the occurrence of subclinical or chronic phase of the disease in these animals. On the other hand, these same dogs could have been exposed to *B. vogeli* previously, maintaining detectable antibody levels. After sequencing, the *Babesia* spp. found in dogs from Campo Grande showed 99% of identity with *B. vogeli*, which has already been found in dogs in the Brazilian states of Minas Gerais (PASSOS et al., 2005; COSTA-JÚNIOR et al., 2009), Pernambuco (RAMOS et al., 2010), Goiás (DUARTE et al., 2011) and Mato Grosso (SPOLIDORIO et al., 2011). Serological surveys revealed that the prevalence of antibodies to *B. vogeli* in dogs in Brazil ranged from 28.7% (COSTA-JÚNIOR et al., 2009) to 46.4% (VIEIRA et al., 2013). Also, *B. gibsoni* has been detected in dogs in the state of Paraná (TRAPP et al., 2006).

Although the presence of *E. canis* DNA has already been detected in dogs in Campo Grande (DAGNONE et al., 2009), the present work showed a high occurrence of seropositive (65%) and PCR-positive animals (45%), indicating that this agent is widespread among dogs in this city. Thirty percent of dogs seropositive to *E. canis* were negative in the PCR assay, suggesting that these animals could be in the subclinical or chronic phase of the disease or that they were previously exposed to *E. canis*. *Ehrlichia canis* is a widespread tick-borne agent of dogs in Brazil and has been molecularly detected in several states, e.g., São Paulo (NAKAGHI et al., 2008; DAGNONE et al., 2009), Rio de Janeiro (MACIEIRA et al., 2005), Paraná (DAGNONE et al.,

2003), Bahia (CARVALHO et al., 2008), Mato Grosso do Sul (DAGNONE et al., 2009) and Paraíba (TANIKAWA et al., 2013). Antibodies to *E. canis* have also been detected in almost all Brazilian states, with the prevalence of exposure in dogs ranging from 4.8% (SAITO et al., 2008) to 73% (NAKAGHI et al., 2008). Although *E. canis* DNA has been detected in humans in Venezuela (PEREZ et al., 2006; UNVER et al., 2001), the importance of the parasite as a zoonotic agent remains unknown.

Anaplasma platys has been molecularly detected in dogs in the states of from Mato Grosso do Sul (DAGNONE et al., 2009), São Paulo (DAGNONE et al., 2009), Pernambuco (RAMOS et al., 2010) and Paraná (SILVA et al., 2012). The percentage of *A. platys* positive samples by PCR assay in our study (1.6%) was lower than that found by Dagnone et al. (2009) (45%) in dogs of Campo Grande, MS, probably because all the dogs sampled in the earlier study showed clinical signs of tick-borne diseases and the presence of intracytoplasmic inclusion bodies in leucocytes and platelets. PCR has shown higher sensitivity in dogs sampled in the acute phase of the disease caused by *A. platys* (DAGNONE et al., 2009).

The occurrence of co-positivity in PCR for *B. vogeli* and *E. canis* in one dog, and the evidence of exposure to both agents (seropositivity for *E. canis* and *B. vogeli* in 56.6% of the sampled dogs) suggest the transmission of both agents by the same tick vector. Cases of co-infection by *B. vogeli*, *E. canis* and *A. platys* are frequently found due to the common tick vector of Brazilian dogs, *R. sanguineus* (DAGNONE et al., 2003; NAKAGHI et al., 2008; OLIVEIRA et al., 2008), which can transmit all three agents (DANTAS-TORRES, 2008). Moreover, the environmental conditions may have contributed to the animals' exposure to the agents, since they lived in the same location (CZC). Studies are much needed on the interaction of tick-borne agents and *Leishmania* parasites in the establishment and course of the disease.

In conclusion, this paper described a study of the molecular detection of *E. canis*, *A. platys* and *B. vogeli* in *L. infantum*-seropositive dogs from Campo Grande, MS. These pathogens should be included in the differential diagnosis of dogs with compatible clinical signs and hematological abnormalities suggestive of tick-borne diseases in Campo Grande, MS.

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