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Molecular survey of *Babesia vogeli* and *Hepatozoon* species in dogs from urban area of Midwestern Brazil

Levantamento molecular de *Babesia vogeli* e *Hepatozoon* spp. em cães da área urbana do Centro-Oeste do Brasil

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Abstract

In Brazil, the most important tickborne pathogens affecting dogs include *Babesia vogeli*, *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis*, and *Mycoplasma haemocanis*. *Babesia* spp. and *Hepatozoon* spp., transmitted by ixodid ticks, have been reported to naturally infect dogs and are widespread. The authors aimed to investigate the incidence of *B. vogeli* and *Hepatozoon* spp. infection using molecular methods to identify factors associated with the infection in dogs from urban areas of Cuiabá municipality, Midwestern Brazil. Polymerase chain reaction (PCR) assay revealed a prevalence of 9.36% (Confidence Interval-CI 95%; 2.72%; 6.79%) and 9.61% (CI 95%; 7.0%; 13.0%) among dogs for *B. vogeli* and *Hepatozoon,* respectively. DNA sequences obtained from 10 *Hepatozoon* PCR positive samples were sequenced and were identical to one another and, moreover, were 100% (541/541 base of pairs-bp) homologous to the corresponding 18S rDNA sequences of *H. canis*. Twenty-five dogs (6.02%) generated amplicons using PCR protocols for both organisms, indicating co-infection by these two protozoans. To the best of our knowledge, our study was the first molecular survey to consider the entire population of dogs from the study area. Moreover, young dogs (0-12 months of age), as well as animals living in walled houses—without access to the street—were more susceptible to infection with *B. vogeli* and *H. canis*, respectively.

Key words: Canine babesiosis. Canine hepatozoonosis. PCR. Tick-borne diseases.

Resumo

No Brasil, os mais importantes patógenos transmitidos por carrapatos que acometem cães incluem *Babesia vogeli*, *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis* e *Mycoplasma haemocanis*. *Babesia* spp.

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e *Hepatozoon* spp., transmitidas por carrapatos ixodídeos, têm sido relatadas como capazes de infectar naturalmente cães e são amplamente distribuídos. Os autores objetivaram investigar a incidência de *B. vogeli* e *Hepatozoon* spp. por métodos moleculares para identificar fatores associados à infecção em cães da área urbana do município de Cuiabá, centro-oeste do Brasil. A reação em cadeia da polimerase (PCR) revelou uma prevalência de 9,36% (Intervalo de Confiança-IC 95%; 2,72%; 6,79%) e 9,61% (IC 95%; 7,0%; 13,0%) entre os cães para *B. vogeli* e *Hepatozoon*, respectivamente. Sequências de DNA obtidas de 10 amostras positivas para PCR de *Hepatozoon* foram sequenciados e eram idênticos entre si e, além disso, foram 100% (541/541 pares de base-pb) homólogos com a correspondente sequência de 18S rDNA de *H. canis*. Vinte e cinco (6,02%) cães geraram amplificados usando protocolos de PCR para ambos os organismos, indicando coinfecção por esses dois protozoários. Até onde sabemos, nosso estudo foi o primeiro levantamento molecular a considerar toda a população de cães da área de estudo na amostragem. Além disso, cães jovens (0-12 meses de idade), bem como animais que viviam em casas muradas - sem acesso à rua - foram mais suscetíveis à infecção por *B. vogeli* e *H. canis*, respectivamente.
Palavras-chave: Babesiose canina. Hepatozoonose canina. PCR. Doenças transmitidas por carrapatos.

Babesia species (spp.) are intraerythrocytic protozoan parasites of the phylum Apicomplexa, causing disease of worldwide importance. In Brazil, at least two species - *B. vogeli* and *Babesia gibsoni* - have been reported to naturally infect dogs and are transmitted by the tick *Rhipicephalus sanguineus* sensu lato (s.l). Parasitological and serological surveys conducted in Brazil have revealed that canine babesiosis is widespread. Moreover, molecular-based techniques, such as polymerase chain reaction (PCR), have been shown to be useful for the detection of *Babesia* DNA in canine blood samples (DANTAS-TORRES; FIGUEREDO, 2006).

Canine hepatozoonosis is caused by an apicomplexan protozoa transmitted by ingestion of oocyst-infected ticks (VINCENT-JOHNSON, 2014). In Brazilian domestic dogs, both *Hepatozoon canis* (O'DWYER, 2011) and, recently, *Hepatozoon americanum* (GOMES et al., 2016), have been detected. In Brazil, although *R. sanguineus* s.l. has been incriminated as vector of *H. canis* (O'DWYER, 2011), Demoner et al. (2013) failure to demonstrate the vectoral capacity of *R. sanguineus* s.l. since nymphs of this tick species failed to develop infection after feeding on dogs naturally infected by *H. canis* under controlled conditions. Otherwise, several studies have found evidence that *Amblyomma ovale* is a natural vector (RUBINI et al., 2009; DEMONER et al., 2013).

Molecular diagnosis based on conventional PCR techniques is a sensitive and specific alternative to other methods for the direct diagnosis of *Hepatozoon* infections in blood (GOMES et al., 2016). Moreover, molecular-based studies investigating *H. canis* in dogs from Brazil have reported variable rates of prevalence (O'DWYER, 2011).

In the present study, we evaluated infection by *B. vogeli* and *Hepatozoon* spp. using molecular detection techniques to identify associated risk factors in dogs from urban area. This study was performed in the municipality of Cuiabá (15°35′56″S, 56°06′01″W), located in the State of Mato Grosso, midwestern Brazil.

The animals were sampled primarily for another project investigating the epidemiology of neosporosis in dogs and, thereafter, the stored samples were made available for the present study. Blood samples were obtained from 415 domiciled dogs in the urban area from December 2008 to December 2009. Owned and apparently healthy dogs were randomly sampled at their residences, according to accessibility.

At the time of blood collection, a questionnaire focusing on characteristics of the dogs (sex and age), and behavior and environment (contact with other animals, walled house [without access to the street]) was given to each dog owner.

To calculate the number of samples, the canine population of the Cuiabá municipality (according to Control Center of Zoonoses in 2007) of 96,504 dogs was used. An expected prevalence of 50% (given the absence of previous studies, and that the maximum incidence with normal distribution would be 50%), statistical error of 5%, and 95% confidence interval (CI), and with the aid of the Epi info 3.5.3 program, corresponded to a minimum number of 383 animals sampled. The sample size was adjusted from 383 dogs to 415 to account for potential losses. Collection of blood samples was performed randomly involving the four administrative regions (north, south, east and west regions) of Cuiabá municipality, Mato Grosso state.

Blood samples were obtained by cephalic or jugular venipuncture and collected into EDTA anticoagulant tubes and kept frozen at -20 °C until DNA extraction. The samples were then processed individually for DNA extraction using the phenol-chloroform method (SAMBROOK; RUSSELL, 2001). In order to verify the presence of amplifiable DNA, 42 randomly selected samples were submitted to internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as previously described (BIRKENHEUER et al., 2003). Furthermore, DNA was quantified in a Nanodrop® ND1000 (Thermo Scientific, USA). DNA from blood samples was tested using PCR and primer pairs BAB1 (5′-GTGAACCTTATCACTTAAAGG-3′) and BAB4 (5′-CAACTCCTCCACGCAATCG-3′), which amplified a 590 base pair (bp) region between the 18S rRNA and the 28S rRNA genes of *B. vogeli* (DUARTE et al., 2008). The second protocol used primers HEPF (5′-GGTAATTCTAGAGCTAATACATGAGC-3’) and HEPR (5′-ACAATAAAGTAAAAAACAYTTCAAAG-3’), targeting a 574 bp region of the 18S rDNA gene from *Hepatozoon* spp. (ALMEIDA et al., 2012). For all PCR assay, *B. vogeli* and *H. canis* DNA samples obtained from naturally infected dogs (SPOLIDORIO et al., 2011) and ultra-pure sterile water were used as positive and negative controls, respectively. PCR products of the expected size for both reactions were analyzed on a 1.5% agarose gel stained with nucleic acid gel stain (GelRed, Biotium Inc, Freemaont, CA, USA) and visualized using an image capture system (ChemiDoc XRS, BioRad, Temecula, CA, USA). DNA from positive samples for *Hepatozoon* spp. were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to manufacturer's instructions and sequenced using an automated sequencer (ABI-PRISM 3500 Genetic Analyzer, ThermoFisher, USA). In order to evaluate the electropherogram quality of sequences obtained from positive blood samples, they were subjected to a screening test using the CLC Genomics Workbench software (Quiagen®). After analysis of the sense and antisense sequences, we obtained a consensus sequences that were then submitted to Basic Local Alignment Search Tool (Blast) analysis to determine similarity with other *Hepatozoon* species.

Prevalence were expressed in the form of confidence intervals. Statistical analysis was performed using a generalized linear model through R software (www.cran.r-project.org). Only a binomial distribution (presence or absence of the characteristic) was considered, with the aid of the logistic link function as a linear predictor.

This study was approved by the Committee on Ethics in the Use of Animals (CEUA-UFMT) # 23108.040913/08-2.

Overall, 39 of 415 blood samples were positive for *B. vogeli*, corresponding to a prevalence of 9.36% (CI 95%; 2.72%; 6.79%). Putative risk factors, including sex, access to the street and a walled house, were not associated with *B. vogeli* infection, as shown in Table 1. However, animals 0-12 months of age were more susceptible to infection ($P = <0.01$), increasing the probability of being positive by 1.57-fold. A total of 40 samples

were positive for *Hepatozoon*, corresponding to a prevalence of 9.61% (CI 95%; 7.0%; 13.0%). DNA sequences obtained from 10 *Hepatozoon* PCR positive samples were identical to one another and matched (541/541bp) to the corresponding 18S rDNA sequences of *H. canis* (KX712129, KX712128). The GenBank nucleotide sequence accession number for the partial sequence generated in the present study is MG496257. In the analysis of risk factors, only dogs living in walled houses without access to the street—were associated with the presence of protozoa ($P = 0.02$), increasing the probability of being positive by 2.29-fold (Table 1). Moreover, 6.02% (25/415) dogs generated amplicons using both PCR protocols, indicating co-infection by *B. vogeli* and *H. canis*. All 42 selected DNA samples amplified the predicted product for GAPDH gene, with mean of DNA concentration = 25.42 ng/µl, and mean 260/280 ratio = 1.67.

Table 1. Cross-sectional analysis of risk factors for *Babesia vogeli* e *Hepatozoon canis* of 415 dogs from Cuiabá municipality, state of Mato Grosso, during December 2008 to December 2009.

Characteristic	*Babesia vogeli*		*Hepatozoon canis*	
	p-value	OR*	p-value	OR*
Sex	0.09	-	0.33	-
Age (months)				
0-12	<0.01	2.57	0.51	-
12-24	0.12	-	0.52	-
24-48	0.44	-	0.35	-
48-72	0.40	-	0.31	-
>72	0.32	-	0.54	-
Contact with other animals	0.15	-	0.32	-
Dogs living in walled houses—without access to the street	0.24	-	0.02	3.29

*Odds Ratio: values marked as - are not applied (*P*-value>0,05).

Cases of canine babesiosis and hepatozoonosis have been reported throughout Brazilian states (DANTAS-TORRES; FIGUEREDO, 2006; O'DWYER, 2011; MIRANDA et al., 2014; ROTONDANO et al., 2015; MORAES et al., 2015; DEMONER et al., 2016; SOUSA et al., 2017). In Brazil, molecular detection up to 15.7% and 84.3% of *B. vogeli* and *H. canis* in dogs has been previously described, respectively (MORAES et al., 2015; MIRANDA et al., 2014). In the present study, 9.36% and 9.61% of dogs from the urban area of the Cuiabá municipality, state of Mato Grosso, were infected with *B. vogeli* and *H. canis,* respectively. Although these agents have already been reported to infect dogs in Cuiabá (SPOLIDORIO et al., 2011), to the best of our knowledge, our study was the first molecular survey to consider the whole canine population of the study area in the sampling.

Most dogs acquire subclinical babesiosis, which hinders control programs and therapeutic intervention (DANTAS-TORRES; FIGUEREDO, 2006). In endemic areas, the environment where dogs are raised has implications for vector-borne diseases because environmental conditions are related to tick biology (SINGLA et al., 2016). Therefore, it is important to study environmental risk factors, although none were shown to be statistically significant for *Babesia* incidence in the present study. Regardless, related epidemiological factors should be assessed for a better understanding of the disease.

No statistical difference was observed between sexes and the incidence of *B. vogeli* infection, revealing that both sexes have the same susceptibility to infection by *B. vogeli*. However, age was found to be a risk factor for infection by *B. vogeli*, with higher frequency observed in dogs 0-12 months of age. A study conducted by Rotondano et al. (2015) reported age as a risk factor for babesiosis in northeastern Brazil. Notably, young dogs were more likely to present severe babesiosis when infected by *Babesia*. Irwin and Hutchinson (1991) reported clinical and pathological findings of *Babesia* infection in 32 dogs in northern Australia and, despite a wide range of clinical signs, the greatest severity was observed in younger dogs, which usually presented in a state of shock.

The published literature reports that, in Brazil, *H. canis* is present in all regions, but that its prevalence is quite variable, depending on the state, the origin of the animals (i.e., whether the dogs are from rural or urban areas), and the diagnostic methodology (i.e., blood smears or PCR). In fact, our results revealed a low prevalence (9.61%), despite the use of sensitive molecular methods but, nevertheless, are in accordance with other studies from many others Brazilian regions. In addition, our results were corroborated by earlier data that demonstrated lower rates of infection in dogs from urban areas (O'DWYER, 2011). This lower rate of infection for *H. canis* observed in the present study was expected, since high prevalence of *H. canis*, among apparently asymptomatic dogs, through molecular methods, appears to be common mainly in rural areas of Brazil (MIRANDA et al., 2014; DEMONER et al., 2016; SOUSA et al., 2017). Probably, because in rural areas, where dogs often share areas with wild carnivores and other mammals, domestic dogs can be infested by endem*ic Amblyomma* species, in addition to *R. sanguineus* s.l., depending on the environmental area as well as on the wild and domestic hosts (LABRUNA et al., 2000), especially *A. ovale*, a tick species incriminated as possible vector of *H. canis* (RUBINI et al., 2009; DEMONER et al., 2013).

As observed in the present study, *H. canis* does not discriminate between the sexes or age groups; nonetheless, dogs living in walled houses—precluding access to the street—were associated with the presence of the protozoan. According to Labruna et al. (2000), ticks infecting dogs in Brazil can be found in two distinct scenarios, which are strictly dependent on dog habitats. Dogs that are reared in urban environments, or in small, enclosed confinement, similar to those observed in most dogs in this study, may allow the establishment of *R. sanguineus* s.l. populations. Although not observed in the present study, the occurrence of *R. sanguineus* s.l. has already been registered on dogs in Cuiabá municipality (personal communication). It is possible that the way dogs are reared here allow for the establishment of this nidicolous tick—a known vector associated with *H. canis* infection (O'DWYER, 2011).

However, additional studies should be performed to analyze transmission of *R. sanguineus* s.l., because another routes of infection among dogs, as predation of paratenic hosts, such as rodents infected with cystozoites have been suggested for *Hepatozoon americanum*, the causative agent of canine hepatozoonosis in the USA, or infested with the tick vector (JOHNSON et al., 2009), as well as vertical transmission (i.e., to puppies born from an infected bitch) described to *H. canis* (MURATA et al., 1993).

Finally, *Hepatozoon* spp. is a potential pathogen and an opportunistic parasite in immunocompromised animals, and may occur in concomitant infections (O'DWYER, 2011). In the present study, 6.02% of dogs generated amplicons using both PCR protocols, indicating co-infection with *B. vogeli* and *H. canis*. Dogs infected with *H. canis* have no or only mild signs of illness; however, immunosuppression or concurrent disease is an important factor in expression of illness in *H. canis* infections, despite healthy appearance during

collection of samples. Furthermore, co-infections with other pathogens, such as *Ehrlichia*, *Babesia*, *Anaplasma*, *Leishmania*, parvovirus, or canine distemper virus, can enable the establishment of a new *H. canis* infection, as well as progression or reactivation of an existing infection (VINCENT-JOHNSON, 2014).

To our knowledge, this was the first molecular-based survey of *B. vogeli* and *H. canis* that considered the entire population of dogs from Cuiabá municipality, midwestern Brazil. We observed a similar prevalence for both protozoans, with 9.36% and 9.61% of dogs infected with *B. vogeli* and *H. canis,* respectively; in addition, 6.02% of animals presented with co-infection. Moreover, young dogs (0-12 months of age), as well as animals living in walled houses (without access to the street), were more susceptible to infection by *B. vogeli* and *H. canis,* respectively.

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