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SEROLOGICAL AND MOLECULAR SURVEY OF TICK-BORNE PATHOGENS AMONG DOGS IN NORTHERN BRAZIL

(*Levantamento sorológico e molecular de patógenos transmitidos por carrapatos em cães do Norte do Brasil*)

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ABSTRACT - This study aimed to evaluate the occurrence of anti-*Rickettsia* spp. and anti-*Ehrlichia canis* antibodies, as well as the molecular detection of *Babesia vogeli*, *Ehrlichia* spp. and *Hepatozoon* spp. DNA in dogs from Ji-Paraná municipality in the state of Rondônia, northern Brazil. For this purpose, we analyzed 177 serum and blood samples using indirect immunofluorescence assay (IFA) and polymerase chain reaction (PCR), respectively. A total of 8.47% (15/177) of dogs were seroreactive to at least one *Rickettsia* species, with endpoint titers ranging from 64 to 1,024. The highest endpoint titers were observed for *Rickettsia parkeri* and *Rickettsia rhipicephali.* Furthermore, anti-*E. canis* antibodies were detected in 24.29% (43/177) of the tested serum samples, with endpoint titers ranging from 80 to 2,560. Among the blood samples analyzed molecularly, 5.65% (10/177), 22.03% (39/177), and 48.02% (85/177) were infected with *B. vogeli*, *E. canis*, and *Hepatozoon canis*, respectively. Herein, we provide serological evidence of *Rickettsia* spp. and *E. canis* infection, and confirmed the occurrence of *B. vogeli, E. canis*, and *H. canis* DNA in dogs from the rural area of the state of Rondônia, in the Western Amazon.

Key words - Indirect immunofluorescence assay; PCR; Rondônia, Tick borne diseases.

RESUMO - O estudo teve como objetivo avaliar a ocorrência de anticorpos anti-*Rickettsia* spp. e anti-*Ehrlichia canis*, bem como a detecção molecular de DNA de *Babesia vogeli*, *Ehrlichia* spp. e *Hepatozoon* spp. em cães do município de Ji-Paraná, estado de Rondônia, norte do Brasil. Para tanto, foram analisadas 177 amostras de soro e sangue por meio reação de imunofluorescência indireta (RIFI) e reação em cadeia pela polimerase (PCR), respectivamente. Um total de 8,47 (15/177) cães foram sororreativos para pelo menos uma espécie de *Rickettsia*, com títulos finais variando de 64 a 1.024. Os títulos finais mais elevados foram observados para *Rickettsia parkeri* e *Rickettsia rhipicephali*. Além disso, anticorpos anti-*E. canis* foram detectados em 24,29% (43/177) das amostras de soro testadas, com títulos finais variando de 80 a 2.560. Dentre as amostras de sangue analisadas molecularmente, 5,65% (10/177), 22,03% (39/177) e 48,02% (85/177) estavam infectados por *B. vogeli*, *E. canis* e *Hepatozoon canis*, respectivamente. Neste trabalho, fornecemos evidências sorológicas de *Rickettsia* spp. e *E. canis*, além disso, confirmamos a ocorrência de DNA de *B. vogeli*, *E.*

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canis e *H. canis* em cães da área rural do estado de Rondônia, na Amazônia Ocidental.

Palavras-chave - Reação de imunofluorescência indireta; PCR; Rondônia; Doenças transmitidas por carrapatos.

INTRODUCTION

Vector-borne pathogens affect domestic animals and wildlife and pose a potential zoonotic risk (Jurković et al., 2019). In this regard, *Rickettsia* (Rickettsialles: Rickettsiaceae) is an intracellular obligate gram-negative alpha proteobacterium that infects vertebrate and invertebrate hosts worldwide (Dumler et al., 2001). In Brazil, several studies have identified circulation of different *Rickettsia* species, classified in different groups, namely: "*Candidatus* Rickettsia paranaensis", "*Candidatus* Rickettsia andeanae", "*Candidatus* Rickettsia colombianensi", "*Candidatus* Rickettsia wissemanii", *Rickettsia rickettsii*, *Rickettsia parkeri*, *Rickettsia amblyommatis*, and *Rickettsia rhipicephali* of spotted fever group (SFG); *Rickettsia asembonensis* and *Rickettsia felis* of transitional group *Rickettsia* (TRG); *Rickettsia bellii* of the bellii group (BG); *Rickettssia typhi* of typhus group (TG); and *Rickettsia monteiroi* of the canadensis group (CG) (Parola et al., 2013; Nieri-Bastos et al., 2014; Dall'agnol et al., 2017; Luz et al., 2019; Peckle et al., 2019), and domestic dogs have been considered an important sentinel for tick-borne spotted fever (Sangioni et al., 2005; Pinter et al., 2008).

Ehrlichia canis is a tick-transmitted gram-negative obligate intracellular bacterium, grouped within the family Anaplasmataceae, a causative agent of canine monocytic ehrlichiosis, an important infectious disease in domestic dogs in many parts of the world (Moraes-Filho et al., 2015). Canine ehrlichiosis is widely prevalent in most regions of Brazil (Rotondano et al., 2015; Soares et al., 2017; Costa et al., 2019), however studies conducted in dogs from the northern region of the Brazilian Amazon are scarce (Aguiar et al., 2007a; Labruna et al., 2007a; Rufino et al., 2013; Spolidorio et al., 2013).

Babesia and *Hepatozoon* are vector-borne protozoa of the phylum Apicomplexa, order Piroplasmorida, and suborder Adelorina, respectively. These protozoans represent a group of blood parasites with significant economic, veterinary, and medical impacts (Modrý et al., 2017; Jalovecka et al., 2018). In Brazil, *Babesia vogeli* and *Babesia gibsoni* have been reported to naturally infect dogs (Jojima et al., 2008), however, parasitological and serological surveys have revealed that canine babesiosis caused by *B. vogeli* is

predominant and widespread across the country (Spolidorio et al., 2013; Costa et al., 2015; Rotondano et al., 2015; Paulino et al., 2018; Maia et al., 2019). In Brazilian domestic dogs, canine hepatozoonosis is caused by *Hepatozoon canis* (O'Dwyer, 2011). Epidemiological reports have described the occurrence of *H. canis* in dogs throughout the country (Rotondano et al., 2015; Sousa et al., 2017; Lopes et al., 2019; Maia et al., 2019), mainly in animals from rural areas (O'Dwyer, 2011; Miranda et al., 2014).

Knowledge of the epidemiology of tick-borne diseases among domestic dogs in the Brazilian Amazon region is limited (Spolidorio et al., 2013). Thus, we aimed to evaluate the occurrence of anti-*Rickettsia* spp. and anti-*E. canis* antibodies, as well as to detect *B. vogeli*, *Ehrlichia*, and *Hepatozoon* infections in the blood of dogs from rural areas in the Western Amazon.

MATERIALS AND METHODS

Study site and collections samples

This study was conducted in the municipality of Ji-Paraná (10º 52' 42" S, 61º 56' 41 W), Rondônia, in the Western Amazon (Figure 1). Blood samples from 177 apparently healthy dogs were collected by convenience, together with another study on the seroprevalence of *Neospora caninum* in sampled farms (Boas et al., 2015). Blood samples were collected from the jugular vein, and the whole blood and serum from each dog were stored individually in microtubes at -20°C until laboratory analyses were performed.

Serologic analyses

Canine sera were tested by indirect immunofluorescence assay (IFA) to detect anti-*Rickettsia* spp. and anti-*E. canis* antibodies, as described by Labruna et al. (2007b) and Aguiar et al. (2007b), respectively. IFA for *Rickettsia* was performed using the following four *Rickettsia* isolates of the SFG from Brazil as crude antigens: *R. rickettsii* (strain Taiaçu), *R. parkeri* (strain At24), *R. amblyommatis* (strain Ac37), and *R. rhipicephali* (strain HJ#5). Dog sera were diluted in two-fold increments with PBS, and titers ≥ 64 were considered positive. Serum samples that reacted at the screening dilution (1:64) were then titrated using serial two-fold dilution to determine endpoint titers. Serum showing a *Rickettsia* species titer at least four times higher than those observed for the other *Rickettsia* species was considered to be homologous to the first *Rickettsia* species or to a very closely related genotype (Piranda et al., 2008).

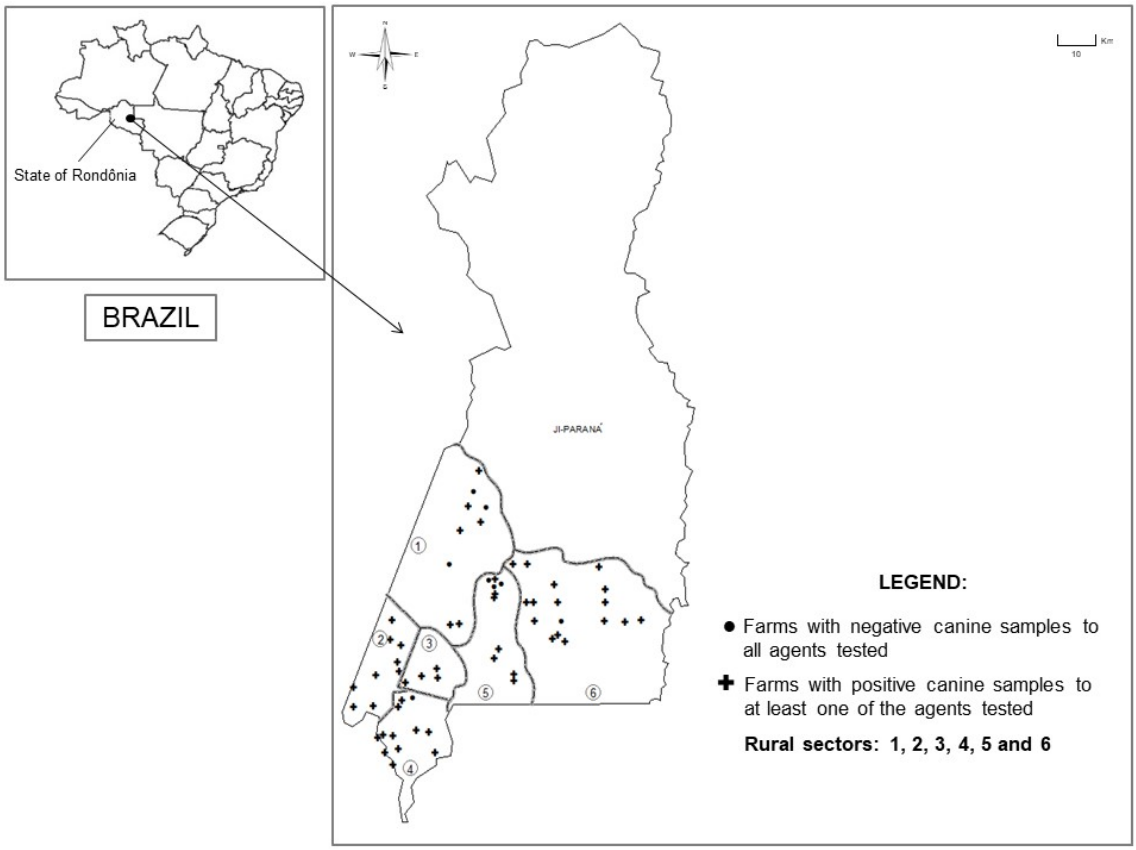

Figure 1 - Distribution of farms into six strata (rural sectors) where dogs were sampled in the Ji-Paraná municipality, State of Rondônia, from September 2012 to November 2013.

For serological survey of *Ehrlichia* by means of IFA, we used DH82 cells infected with *E. canis* (São Paulo strain) as a crude antigen. Serum was considered to contain antibodies reactive if it displayed a reaction at a dilution of 1:40. Endpoint titers were determined in positive samples (dilution ≥ 1:40) using serial two-fold dilution. For both serological tests, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were tested at dilutions of 1:64 and 1:40 for *Rickettsia* spp. and *E. canis*, respectively, as previously reported (Melo et al., 2011).

Molecular analyses

Blood samples were processed for DNA extraction by the phenol-chloroform method (Sambrook and Russell, 2001). To evaluate the presence of amplifiable DNA, samples were tested using conventional polymerase chain reaction (cPCR) assay targeting fragments of the mammalian glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene as an internal control (Birkenheuer et al., 2003). DNA of all 177 samples subjected to

gapdh internal control amplified the predicted product, with an average concentration of DNA of 25.35 ng/µL and a 260/280 ratio of 1.67, according to the DNA concentration measurements made using the Nanodrop ND1000 instrument (Thermo Scientific, Waltham, MA USA).

Each DNA sample was tested using cPCR to amplify specific fragments of 590 base pairs (bp), located between the *18S* small subunit ribosomal RNA (*18S* rRNA) and 28S large subunit ribosomal RNA (*28S* rRNA) genes of *B. vogeli* (Duarte et al., 2008), and specific 574 bp fragments of the *18S* rRNA gene of *Hepatozoon* and Piroplasmorida members (*Babesia*, *Cytauxzoon*, and *Theileria*), according to Almeida et al. (2012). Furthermore, a heminested (hnPCR) was performed to detect ehrlichial DNA, focusing on amplification of the disulfide bond formation protein gene (*dsb*) of *Ehrlichia* spp. (Aguiar et al., 2014). For each PCR assay, blank controls (water), an appropriate positive control, and DNA samples obtained from dogs naturally infected with *B. vogeli* (Maia et al., 2019), *H. canis* (Maia et al., 2019), and *E. canis* (Melo et al., 2016) were run together with the canine clinical DNA samples.

PCR products of the expected sizes were analyzed on 1.5% agarose gels stained with GelRed™ (Biotium Inc, Freemaont, CA, USA) and visualized using the ChemiDoc XRS System (BioRad, Temecula, CA, USA). Amplicons from six positive samples for both *Hepatozoon* and *Ehrlichia* were purified using the illustra GFX PCR DNA and Gel Band Purification Kit GE (GE Healthcare Life Sciences, Pittsburgh, PA, USA.), according to the manufacturer's instructions, and sequenced using an automated DNA sequencer (ABI DNA Model 3500 Series Genetic Analyzer, Applied Biosystems, Inc., Foster City, CA, USA). Partial sequences obtained were submitted for BLAST analysis (Altschul et al., 1990) to determine the closest sequence similarities in GenBank.

Concurrent with sampling, each farmer was asked to fill out a questionnaire for subsequent used for risk factor analysis. Thereafter, the following variables were analyzed: presence of free-ranging dogs (yes or no), presence of wild canids nearby (yes or no), dogs feeding on commercial food or meals, gender, and age. Then, statistical analyses of prevalence values were calculated with a confidence interval (CI) of 95% using EpiInfo v.7.2.4 software. Possible associations between the variables and positivity by PCR and IFA were performed using the Chi-square test or Fisher's exact test, with a statistical significance of 5%.

RESULTS

A total of 15 (8.47%; CI 95%; 4.82-13.59%) canine serum samples were seroreactive (titers ≥ 64) against at least one *Rickettsia* species. Seroreactive serum showed titers for anti-*Rickettsia* antibodies as follows: *R. amblyommatis,* 64–256; *R. rhipicephali,* 128–1,024; *R. parkeri,* 256–1,024; and *R. rickettsii,* 64–256, with endpoint titers of 1,024 found for both *R. rhipicephali* and *R. parkeri*. The total numbers of positive samples for each rickettsial antigen tested are shown in Table 1.

Table 1 - Results of indirect immunofluorescence assay (IFA) for four *Rickettsia* species in 177 dogs from 61 farms of Ji-Paraná municipality, an Amazonian area of the State of Rondônia, Brazil.

Sector (no. of farms)	No. of tested dogs	No. of seroreactive dogs to each of the *Rickettsia* species (% seroreactivity)				
		R. rickettsii	***R. parkeri***	***R. amblyommatis***	***R. rhipicephali***	**No. of positive dogs (PAIHR*)**
1 (9)	26	1 (3.8)	2 (7.7)	0	2 (7.7)	0
2 (9)	23	1 (4.3)	0	1 (4.3)	0	0
3 (4)	14	2 (14.3)	4 (28.6)	2 (14.3)	4 (28.6)	
4 (12)	35	1 (2.9)	2 (5.7)	1 (2.9)	1 (2.9)	0
5 (10)	24	0	0	0	0	0
6 (17)	55	3 (5.4)	3 (5.4)	4 (7.3)	6 (10.9)	3 (*R. rhipicephali*)
Total	177	8 (4.5)	11 (6.2)	8 (4.5)	13 (7.3)	3 (*R. rhipicephali*)

*PAIHR= Possible antigen involved in a homologous reaction.

In addition, in three canine sera, anti-*R. rhipicephali* antibody endpoint titers were at least fourfold higher than the endpoint titers to the remaining rickettsial antigens, suggesting homologous reactions to *R. rhipicephali* or a closely related organism (Table 1). For the remaining seroreactive dogs, it was not possible to discriminate the infective agent because they displayed similar titers (< 4-fold difference) for two or more *Rickettsia* species or had a single titer of 64 for one *Rickettsia* species.

For *E. canis*, serological surveys revealed that 43 (24.29%; CI 95%; 18.17-31.30%) dogs were seropositive for anti-*E. canis* antibodies (titers ≥ 40), as shown in Table 2. The following endpoints were detected among seropositive dogs: 80 (1 dog; 2.32%), 160 (9 dogs; 20.93%), 320 (12 dogs; 27.91%), 640 (9 dogs; 20.93%), 1,280 (7 dogs; 16.28%), and 2,560 (5 dogs; 11.63%). Anti-*Rickettsia* spp. and anti-*E. canis* antibodies were detected concomitantly in three dogs.

A total of 10 (5.65%; CI 95%; 2.74-10.14%) samples yielded cPCR amplicons for *B. vogeli*, and 85 (48.02%, 95% CI 40.47-55.64%) samples were positive for *Hepatozoon*. Six *Hepatozoon* cPCR-positive samples sequences obtained were identical to one another

and 99% (568/569 bp) identical to the corresponding *18S* rDNA sequences of *H. canis* (MK238384), which has been reported in dogs from South Korea. Finally, 39 (22.03%, CI 95%; 16.16-28.87%) samples were positive for ehrlichial DNA, and six sequences generated from a fragment of the *dsb* gene of *Ehrlichia* spp. exhibited 100% (324/324 bp) of identity with *E. canis* (MG772657) detected in dogs from Rio Grande do Norte, Brazil. Nineteen (10.73%) dogs were co-infected with both *H. canis* and *E. canis*, while only one dog (0.56%; 1/177) demonstrated co-infection with *B. vogeli*, *H. canis*, and *E. canis*. Finally, regarding the ehrlichial results, 12 dogs showed positive results concomitantly with hnPCR and IFA for *E. canis*. Among the DNA samples, coinfections involving the three studied pathogens were detected in 0.56% (1/177) of animals; 3.98% (7/177) of dogs were coinfected with *B. vogeli* and *H. canis;* and 11.30% (20/177) with *E. canis* and *H. canis*. All PCR results are shown in Table 2. DNA sequences generated in this study have been deposited in GenBank under the accession numbers MT081049 for the *E. canis dsb* partial sequence and MT081050 for the partial *H. canis 18S* rDNA sequence.

Table 2 - Results of indirect immunofluorescence assay (IFA) for *Ehrlichia canis*, and molecular survey for *Babesia vogeli*, *Ehrlichia* spp., and *Hepatozoon* spp. by Polymerase Chain Reaction (PCR) in 177 dogs from 61 farms of Ji-Paraná municipality, an Amazonian area of the State of Rondônia, Brazil.

Sector (no. of farms)	No. of tested dogs	No. of seroreactive dogs to *E. canis* (% seroreactivity)	No. of PCR positive (%)		
		E. canis	***B. vogeli***	***Ehrlichia* spp.**	***Hepatozoon* spp.**
1 (9)	26	3 (11.5)	1 (3.8)	2 (7.7)	12 (46.1)
2 (9)	23	6 (26.1)	1 (4.3)	5 (21.7)	15 (65.2)
3 (4)	14	4 (28.6)	1 (7.1)	6 (42.8)	8 (54.1)
4 (12)	35	7 (20)	1 (2.9)	11 (31.4)	22 (62.9)
5 (10)	24	4 (16.7)	2 (8.3)	3 (12.5)	5 (20.8)
6 (17)	55	19 (34.5)	4 (7.3)	12 (21.8)	23 (41.8)
Total	**177**	**43 (24.3)**	**10 (5.6)**	**39 (22.0)**	**85 (48.2)**

The risk factors evaluated in dogs that tested positive for any of the agents showed a statistically significant difference ($p < 0.05$) only for animals positive for the *Ehrlichia* spp. PCR (*dsb* gene) and gender, and for IFA of *Ehrlichia* spp. and age between 12 and 24 months old. The statistical results are presented in Table 3 (Supplementary document).

DISCUSSION

Anti-*Rickettsia* spp. antibodies were found in 8.47% of the dogs sampled indicating that these animals have been exposed to *Rickettsia* spp. in the rural region of

Ji-Paraná municipality, and this seroprevalence is within the values already published in other serological surveys conducted in dogs from rural areas of the Brazilian Amazon region, which ranged from 7.6-15.4% (Labruna et al., 2007b; Soares et al., 2014; Da Costa et al., 2021).

In the present study, we provided serological evidence for infection by *R. rhipicephali* or a very closely related organism in dogs. However, there are no reports on the pathogenicity of *R. rhipicephali* in humans and animals yet (Burgdorfer et al., 1975). Other studies have also reported evidence for infection by *R. rhipicephali* in dogs, based on the same serological criteria (Labruna et al., 2007b; Melo et al., 2011; Spolidorio et al., 2013). *Rickettsia rhipicephali* DNA has already been detected in free-living *Haemaphysalis juxtakochi* ticks in the state of Rondônia (Labruna et al., 2005a), and although species of the family Cervidae are the most common hosts for *H. juxtakochi* adults, they have also been found on a variety of other animals groups such as rodents, lagomorphs, non-human primates, birds, tapirs (Jones et al., 1972, Beldomenico et al., 2003, Nava et al., 2017; Pacheco et al., 2020; Labruna et al., 2021), including *H. juxtakochi* adults and nymphs on domestic dogs in the state of Rondônia (Labruna et al., 2005b).

Since Vieira et al. (2018) have proposed that an area is considered to be at high risk for Brazilian Spotted Fever (BSF) transmission when 50% of sentinel hosts are serum-reactive, and dogs are suitable sentinels for SFG rickettsiosis (Sangioni et al., 2005; Pinter et al., 2008), in this sense, it is possible to infer that the human population in the rural area of Ji-Paraná municipality is at low risk of acquiring tick-borne spotted fever rickettsiosis.

The results obtained by means of IFA for *E. canis* (24.29%) were in accordance with values detected in dogs from rural areas of the country, ranging from 13.84% to 67.5% (Aguiar et al., 2007a; Melo et al., 2011; Spolidorio et al., 2013; Costa et al., 2015). Regarding the detection of ehrlichial DNA in blood samples of dogs, despite the rate of infection being variable among different states in Brazil (Costa et al., 2015; Guedes et al., 2015; Soares et al., 2017; Costa et al., 2019; Lopes et al., 2019), considering solely studies conducted in rural areas in the country, our rate of infection (22.03%) found in dogs was higher compared to those of other studies (Santos et al., 2013; Dantas-Torres et al., 2018) which have reported results ranging from 4.17% to 14.4%. In general, these variations occur because of characteristics related to the dogs' exposure to infected vectors (Guedes et al., 2015; Sainz et al., 2015).

Both ehrlichial DNA and anti-*E. canis* antibodies were detected in 6.77% of the analyzed samples, confirming the circulation of *E. canis,* the main *Ehrlichia* species

infecting dogs in the country (Vieira et al., 2011). Regarding differences between the hnPCR and IFA results, the positive hnPCR results (22.03%) were closely related to the serological results (24.29%), which may indicate that the number of dogs previously exposed to *E. canis* was similar to the number of cases with active infections. The overall proportion (17.51%) of serum samples reactive by IFA, but not observed by molecular analysis, reinforces that the detection of antibodies does not discriminate an active infection from a prior, resolved one (Vieira et al., 2011). On the other hand, such differences in results should be anticipated given the sensitivity of PCR in order to detect *Ehrlichia* DNA in seropositive dogs and may vary according to the sample sources and target genes involved (Harrus et al., 2004).

The molecular detection of *B. vogeli* in the present study was consistent with values reported in other surveys in rural areas of the country, ranging from 0.9% to 11% (O'Dwyer et al., 2009; Costa et al., 2015; Castro et al., 2020). In this sense, future studies are needed, including epidemiological and management data reporting, as well as the characteristics of dogs, to obtain more in-depth knowledge of the epidemiology of tick-borne diseases in the state of Rondônia. In addition, data regarding *Babesia* spp. in dogs in the Amazon region are scarce, with a limited number of serological and molecular studies conducted in the state of Pará (Spolidorio et al., 2013; Moraes et al., 2014) and Amazonas (Soares et al., 2014). Thus, this is the first study to report the molecular detection of *Babesia* genus infections in dogs from Rondônia state in the Western Amazon.

This is the first molecular study of *H. canis* in the state of Rondônia, and the rate of infection of *H. canis* found in the present study was within previously reported infection rates among healthy rural dogs identified in other studies in the country, which observed infection rates ranging from 41.2% to 66.45% (Rubini et al., 2009; Miranda et al., 2014; Demoner et al., 2016; Sousa et al., 2017). Studies encompassing the molecular detection of *H. canis* in rural areas have demonstrated higher values compared with data from dogs resident in urban areas (Miranda et al., 2014; Demoner et al., 2016; Sousa et al., 2017). It is likely that in rural areas, where dogs often share areas with wild carnivores and other mammals, domestic dogs can be infested by endemi*c Amblyomma* species, in addition to *Rhipicephalus sanguineus* sensu lato, 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 a possible vector of *H. canis* (Rubini et al., 2009).

Regarding statistical analyses, only two statistically significant associations were identified. First association, among molecular detection of *E. canis* DNA and age, we

observed that dogs aged 12 to 24 months were more likely to have the ehrlichial agent is circulating. One possible explanation is that this vulnerability of *E. canis* is related to the young age and high activity of the animals, increasing the level of exposure to infected tick vectors. Gender was also considered a risk factor for the presence of anti-*E. canis* antibodies, and the higher frequency observed in female dogs in this study was not expected, since no correlation has been reported regarding gender and the presence of antibodies in dogs (Rotondano et al., 2015), or higher seropositivity in males, due to behavioral characteristics that increase the chance of exposure to *E. canis* (Sainz et al., 2015). Therefore, the circumstances that influence exposure and, consequently, the rates of seropositivity to *E. canis* throughout canine life require more detailed analysis of parasite-host interactions.

CONCLUSIONS

Our results revealed the presence of antibodies and DNA of pathogens responsible for tick-borne diseases in dogs from rural areas of Western Amazon, in the Ji-Paraná municipality, state of Rondônia, Brazil. Thus, there is a need for further studies regarding the natural transmission and maintenance of these agents in neglected regions of the country.

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ETHICS COMMITTEE

The Bioethical Committee for Animal Research of the Federal University of Mato Grosso approved the present study, under the protocol n. 23108.015662/12-5.

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