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Original Article

Molecular detection of vector borne pathogens in anemic and thrombocytopenic dogs in southern Brazil

Detecção molecular de patógenos transmitidos por vetores em cães anemicos e trombocitopenicos no Sul do Brasil

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Abstract

Arthropod-borne pathogens are medically important because of their ability to cause diseases in their hosts. The purpose of this study was to detect the occurrence of *Ehrlichia* spp., piroplasmids and *Hepatozoon* spp. in dogs with anemia and thrombocytopenia in southern Brazil. EDTA-whole blood was collected from 75 domestic dogs presenting anemia or/and thrombocytopenia from Guarapuava, state of Paraná, Brazil. DNA samples were subjected to conventional PCR assays for *Ehrlichia* spp. (*dsb*), piroplasmids (18S rRNA) and *Hepatozoon* spp. (18S rRNA), followed by sequencing and phylogenetic analyses. Among the 75 dogs, one (1.33%) was positive for *Hepatozoon* sp. and six (8%) were positive for piroplasmids in 18S rRNA cPCR assays. None of the dogs showed positive results in *Ehrlichia* spp.-cPCR targeting *dsb* gene. The phylogenetic analyses revealed that three piroplasm sequences were clustered with *Rangellia vitalii*, while one sequence was grouped with *B. vogeli*. The only sequence obtained from *Hepatozoon* spp.-PCR protocol was pooled with *H. canis*. Therefore, there is urgent need for differential molecular diagnosis of the two piroplasm species cited as etiological agents in clinical cases of canine hemoparasitic diseases, given the higher pathogenic potential of *R. vitalii* than of *B. vogeli.*

Keywords: *Babesia vogeli*, *Rangelia vitalii*, *Hepatozoon canis*, phylogenetic analysis, tick borne diseases.

Resumo

Agentes transmitidos por artrópodes têm grande importância na medicina veterinária devido à sua capacidade de causar doenças graves em seus hospedeiros. O presente estudo objetivou investigar a ocorrência de três patógenos transmitidos por vetores, *Ehrlichia canis*, *Rangelia vitalii* e *Hepatozoon canis*, em cães na região sul do Brasil. Foram coletadas amostras de sangue total de 75 cães domésticos que apresentavam anemia e/ou trombocitopenia, em Guarapuava, Paraná, Brasil. As amostras de DNA foram submetidas à técnica de PCR convencional para *E. canis* (*dsb*), piroplasmídeos (18S rRNA) e *Hepatozoon* spp. (18S rRNA), seguida de sequenciamento e análises filogenéticas. Das 75 amostras, uma (1,33%) foi positiva para *Hepatozoon* spp. e seis (8%) foram positivas para *Babesia* spp. Nenhuma amostra mostrou resultados positivos para *Ehrlichia* spp. utilizando a detecção pelo gene *dsb*. As análises filogenéticas revelaram que três sequências obtidas foram agrupadas no mesmo clado que *R. vitalii*, enquanto uma foi agrupada juntamente com *B. vogeli*. A única sequência obtida pelo protocolo de PCR para *Hepatozoon* spp. foi agrupada juntamente com *H. canis*. Assim, é justificada necessidade de diferenciação das espécies de piroplasmas, através do diagnóstico molecular, como agentes etiológicos nos casos clínicos de hemoparasitose canina, considerando o potencial patogênico de *R. vitalii* quando comparado à *B. vogeli.*

Palavras-chave: *Babesia vogeli*, *Rangelia vitalii*, *Hepatozoon canis*, análise filogenética, doenças transmitidas por carrapatos.

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Introduction

Arthropod-borne diseases involve several infectious agents, hosts and vectors, strongly affecting human and animal health (HARRUS & BANETH, 2005). The geographic distribution of arthropods and vector-borne agents is expanding due to climate, ecological and environmental changes (GRAY et al., 2009). In this context, Anaplasmataceae agents, piroplasmids and *Hepatozoon* spp. have emerged as important pathogens among domestic animals worldwide (ANDRÉ et al., 2015).

Ehrlichia canis, a widespread tick-borne pathogen among domestic dogs, is considered the primary pathogen responsible for canine monocytic ehrlichiosis (RIKIHISA, 2011). Clinical manifestations of the disease include anemia, fever, weight loss, anorexia, thrombocytopenia and lymphadenopathy (CASTRO et al., 2004; NAKAGHI et al., 2008). *Ehrlichia canis* has been reported in dogs in several regions in Brazil (DAGNONE et al., 2009; SOUSA et al., 2013; MELO et al., 2016), particularly in tropical and subtropical regions, in accordance with the distribution of its vector, the tropical strain of *R. sanguineus* sensu lato (MORAES-FILHO et al., 2015).

Piroplasmosis, one of the most prevalent vector-borne diseases among animals, is caused by tick-borne protozoan agents that parasitize several wild and domestic vertebrates worldwide (ALVARADO-RYBAK et al., 2016). Piroplasm infections are usually characterized by fever, anemia and hemoglobinuria, and can lead to death in severe cases (KUTTLER, 1988). In Brazil, *B. vogeli* (PASSOS et al., 2005), *Babesia gibsoni* (TRAPP et al., 2006) and *R. vitalii* (SOARES et al., 2011) have been confirmed, by both morphological and molecular methods, to infect dogs. While *R. sanguineus* s.l. is considered the main vector of *B. vogeli*, and a suspected vector of *B. gibsoni* in the country (DANTAS-TORRES, 2008), *Amblyomma aureolatum* is the vector of *R. vitalii* (SOARES & GIROTTO SOARES, 2015).

Canine hepatozoonosis is a disease caused by *H. canis* and by *Hepatozoon americanum* (LAPPIN, 2010). These two agents have distinct clinical, pathological and genetic characteristics. While *H. canis* has a generalized distribution and has been described worldwide, *H. americanum* seems to be narrowly restricted to the United States of America (LAPPIN, 2010; O'DWYER, 2011). In Brazil, *H. canis* and genotypes closely related to *H. americanum* circulate in domestic dogs and wild canids (RUBINI et al., 2005; FORLANO et al., 2007; ANDRÉ et al., 2010; GOMES et al., 2016; MALHEIROS et al., 2016; SOUSA et al., 2017). Although *Rhipicephalus sanguineus* s.l. is considered the main biological vector of canine hepatozoonosis caused by *H. canis* (GIANNELLI et al., 2013), preliminary studies in Brazil indicate that the importance of the tick *R. sanguineus* is negligible or absent in the transmission of *H. canis* (DEMONER et al., 2013). *Amblyomma ovale* has been implicated as the main vector of *H. canis* in rural areas in Brazil (FORLANO et al., 2005; DEMONER et al., 2013).

This study focused on detecting the occurrence of *Ehrlichia* spp., piroplasmids and *Hepatozoon* spp. in dogs showing hematological abnormalities suggestive of hemoparasitic diseases (anemia and thrombocytopenia) in southern Brazil, using molecular methods.

Material and Methods

The blood samples used in this study were collected from 75 dogs presenting anemia (packed cell volume < 37%) or/and thrombocytopenia (platelets < 200 000 cells/mm^3), as indicated by a complete blood count (CBC) performed on a SDH-3 Vet blood counting machine (Labtest®). Anemia was observed in 45 dogs, thrombocytopenia in 12 and anemia and thrombocytopenia in 19 animals. The dogs were treated at the School of Veterinary Medicine (CEVET) of the Universidade Estadual do Centro Oeste (UNICENTRO) in Guarapuava (25°23'43" S 51°27'29" W), state of Paraná, Brazil, between in 2013 and 2014. The blood samples were deposited in the Sample Repository of the Laboratory of Infectious and Parasitic Diseases of CEVET. This study was approved by the Ethics Committee on Animal Use (CEUA) of UNICENTRO, under Protocol no. 011/2015.

DNA was extracted from 200 µL of each whole blood sample, using a commercial kit (QIAmp DNA Blood Mini Kit, Qiagen™), as recommended by the manufacturer. The quality and concentration of extracted DNA was analyzed spectrophotometrically using a Thermo Scientific™ NanoDrop spectrophotometer, based on an evaluation of the absorbance of each sample. The extracted DNA samples were identified and stored at -20 °C until use.

A previously described protocol was performed targeting a 378 pb fragment of *dsb* gene to detect *Ehrlichia* spp. DNA. To this end, primers Dsb-330 and Dsb-728 and thermocycler protocols described in Table 1 are used (DOYLE et al., 2005). The total volume of the reaction was 25 µL, comprising 5 µL of DNA, 0.2 mM dNTPs (Invitrogen, Carlsbad, USA), 2.5 mM $MgCl_2$ (Invitrogen, Carlsbad, USA), 1 pmol of each primer (Invitrogen, Carlsbad, USA), of buffer 10x (Invitrogen, Carlsbad, USA), 1.25 U of Taq DNA Polymerase (Invitrogen, Carlsbad, USA) and ultrapure sterile water (Invitrogen, Carlsbad, USA) q.s.p. *Ehrlichia canis* DNA (SOUSA et al., 2013) and ultrapure sterile water (Promega), respectively, were used as positive and negative controls.

A nested PCR assay protocol targeting 18S rRNA gene was performed in order to detect piroplasm DNA (JEFFERIES et al., 2007). Primers sequences and thermocycler protocol are described in Table 1. The final volume of the reaction was 25 µL containing 5 µL of DNA, 0.2 mM dNTPs (Invitrogen, Carlsbad, USA), 0.5 µM of each primer (Invitrogen, Carlsbad, USA), 1.5 mM $MgCl_2$ (Invitrogen, Carlsbad, USA), 0.75U Taq DNA polymerase (Invitrogen, Carlsbad, USA), buffer and ultrapure sterile water (Invitrogen, Carlsbad, USA) q.s.p. 25µL. *Babesia vogeli* DNA (SOUSA et al., 2013) and sterile ultrapure water (Promega) were used as positive and negative controls, respectively.

Two different protocols based on 18S rRNA were performed in order to detect *Hepatozoon* spp. (PERKINS & KELLER, 2001; UJVARI et al., 2004). Primers sequences and thermocycler protocols are described in Table 1. The reactions involved a total volume of 25 µL, consisting of 5µL of DNA, 0.2 mM dNTP (Invitrogen, Carlsbad, USA), 1µM of each primer (Invitrogen, Carlsbad, USA), 1.0mM $MgCl_2$ (Invitrogen, Carlsbad, USA), 1U Taq DNA Polymerase (Invitrogen, Carlsbad, USA), buffer and ultrapure sterile water (Invitrogen, Carlsbad, USA) q.s.p. 25 µL.

Table 1. Primer sequences, thermocycler conditions and references utilized for each target pathogen.

Pathogen	Primers	Cycles	References
Ehrlichia spp. (*dsb* gene)	Dsb-330 5'- GATGATGTCTGAAGATATGAAACAAAT-3' Dsb-728 5'- CTGCTCGTCTATTTTACTTCTTAAAGT-3'	Initial denaturation at 95 °C; 50 cycles: 94 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min.	Doyle et al. (2005)
Piroplasm DNA (18S rRNA gene)	BTF1 5'-GGCTCATTACAACAGTTATAG-3' BTR1 5'-CCCAAAGACTTTGATTTCTCTC-3' BTF2 5'-CCGTGCTAATTGTAGGGCTAATAC-3' BTR2 5'-GGACTACGACGGTATCTGATCG-3'	94 °C for 3 min; 58 °C for 1 min; 72 °C for 2 min; 45 cycles: 94 °C for 30 sec, 58 °C for 20 sec, 72 °C for 30 sec, 72 °C for 7 min. Annealing temperature 62 °C in the second reaction.	Jefferies et al. (2007)
Hepatozoon spp. (18S rRNA gene)	HEMO1 5'-TATTGGTTTTAAGAACTAATTTTATGATTG-3' HEMO2 5'-CTTCTCCTTCCTTTAAGTGATAAGGTTCAC-3'	94 °C for 3 min, 35 cycles: 94 °C for 45 s, 60 °C for 1 min, 72 °C for 1 m, 72 °C for 7 min.	Perkins & Keller (2001); O'Dwyer et al. (2013)
Hepatozoon spp. (18S rRNA gene)	HepF300 5'-GTTTCTGACCTATCAGCTTTCGACG-3' Hep900 5'-CAAATCTAAGAATTTCACCTCTGAC-3'	94 °C for 3 min, 35 cycles: 94 °C for 45 s, 56 °C for 1 min, 60 °C for 1 m, 72 °C for 7 min.	Ujvari et al. (2004)

These two different PCR protocols were used to amplify different regions of the 18S rRNA gene of *Hepatozoon* spp. in order to obtain a large 18S rRNA fragment (1300pb) to be used in phylogenetic analyses. *Hepatozoon canis* DNA (SOUSA et al., 2017) and sterile ultrapure water (Promega) were used as positive and negative controls, respectively.

The amplified products were subjected to electrophoresis with ethidium bromide (0.5 µL/mL) – 1% agarose gel stain in TBE running buffer, pH 8.0 (44.58 M Tris-base; 0.44 M boric acid; and 12.49 mM EDTA), at 90V/150mA for 60 minutes. The length of the amplified products was determined by means of a 100 pb marker (Life Technologies™). The results were examined and analyzed under an UV light transilluminator (ChemiDoc™ MP Imaging System, Bio Rad™). Amplicons were purified using a Silica Bead DNA Gel Extraction Kit (Fermentas, São Paulo, SP), following the manufacturer's recommendations.

Purified amplicons were sequenced using an automated method based on chain termination by dideoxynucleotides (SANGER et al., 1977) in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems™). After sequencing, the electropherograms were analyzed using BioEdit v. 7.0.5.3 (HALL, 1999) software and then subjected to a screening test using Phred-Phrap-Consed version 23 software (EWING & GREEN, 1998; EWING et al., 1998) to evaluate the quality if the electropherograms and obtain consensus sequences from the alignment of sense and antisense sequences. The sequences were analyzed using the BLAST program (ALTSCHUL et al., 1990) to compare them with sequences retrieved from the GenBank database.

The sequences were aligned using the ClustalW software (THOMPSON et al., 1994) via BioEdit v. 7.0.5.3 (HALL, 1999). Maximum Likelihood (ML) analyses were performed using the W-IQ-Tree tool available online (TRIFINOPOULOS et al., 2016), with the node support evaluated by means of 1000 bootstrap repetitions (FELSENSTEIN, 1985). The Bayesian inference (BI) analysis was performed using the MrBayes 3.2.2 program on XSEDE (RONQUIST & HUELSENBECK, 2003) via the CIPRES Science Gateway (MILLER et al., 2010). Markov Chain Monte Carlo (MCMC) simulations were run for 10^6 generations with a sampling frequency of every 100 generations and a burn-in of 25%. The best model of evolution was selected by means of the jModelTest2 (version 2.1.6) program on XSEDE (DARRIBA et al., 2012), under the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) (POSADA & BUCKLEY, 2004). The phylogenetic trees were edited using TreeGraph 2.0.56-381 beta software (STOVER & MULLER, 2010) and rooting at the outgroup chosen for each parasite. Bootstrap and posterior probability values lower than 50% are not considered relevant by the test, and are therefore not shown.

Results

Among the 75 canine blood samples that were evaluated, 18S rRNA of *Hepatozoon* sp. was detected in one sample (1.33% [1/75]), and 18S rRNA of piroplasmids was detected in six (8.0% [6/75]) samples. None of the samples tested positive in the PCR assay for *Ehrlichia* spp. based on the *dsb* gene. The results of clinical signs, hematological abnormalities, ticks infestation, PCR and sequencing results of each positive dog were in Table 2.

Four of the six samples that tested positive for piroplasmids were selected for sequencing, in view of the higher intensity of bands in agarose gel electrophoresis. In an analysis of these four sequences by nBLAST, three showed 99-100% identity with *R. vitalii* detected in domestic dogs in Passo Fundo, Rio Grande do Sul (GenBank accession number: KT288200) with 100% query coverage. On the other hand, a sequence showed 100% identity with *Babesia vogeli* sequences detected in dogs sampled in the city of Belém, state of Pará (GenBank accession number: KT333456), and a *B. vogeli* sequence obtained from a domestic cat in southern Brazil (GenBank accession number: KT323935). The partial sequence of the 18S rRNA gene from *Hepatozoon* sp.

Table 2. Clinical signs, hematological abnormalities, tick infestation, PCR and sequencing results of each positive dog in PCR assays for piroplasmids and *Hepatozoon* spp. sampled in the city of Guarapuava, state of Paraná, Southern Brazil.

Animal	Clinical signs	Hematological Abnormalities*	Infestation by ticks	PCR results (18S rRNA)	Sequencing results
1	None	anemia	No	*Hepatozoon* sp.	*H. canis*
2	apathy, anorexia	anemia	No	Piroplasmids	Not sequenced
3	apathy, anorexia	anemia and thrombocytopenia	No	Piroplasmids	Not sequenced
4	ear bleeding, apathy	anemia and thrombocytopenia	*A. aureolatum*	Piroplasmids	*R. vitalii*
5	ear bleeding, apathy	anemia and thrombocytopenia	*A. aureolatum*	Piroplasmids	*R. vitalii*
6	apathy	anemia	No	Piroplasmids	*R. vitalii*
7	lymphadenomegaly	anemia	No	Piroplasmids	*B. vogeli*

*All the blood smears of the dogs contained Howell-Jolly bodies, but the presence of *R. vitalii* and *B. vogeli* piroplasms and of *H. canis* gametocytes was not observed.

showed 99% identity with *H. canis* sequences (GenBank accession number: KY026192/KU729737) identified in Brazil.

A similar topology was observed in the cladograms generated by ML and BI analysis. Regarding the analysis of the partial sequences of the 18S rRNA gene of piroplasmids, three samples were found to be allocated in a large clade together with *R. vitalii* sequences, while one of the sequences was positioned in a clade together with *B. vogeli* (Figure 1).

On the other hand, the phylogenetic analysis of the partial sequence of the 18S rRNA gene from *Hepatozoon* sp. indicated that the sequence obtained in this study was positioned in the same clade as *H. canis* sequences. *Hepatozoon americanum* sequences formed a monophyletic group apparently unrelated to the sequence obtained in this study (Figure 2).

Discussion

Given that vector-borne diseases are on the rise worldwide (DANTAS-TORRES et al., 2012), veterinarians should be prepared to diagnose, treat and prevent such infections (DANTAS-TORRES & OTRANTO, 2016). The city of Guarapuava, where this study was conducted, is located in the midwestern region of the state of Paraná, in southern Brazil. No molecular studies had heretofore been conducted to elucidate the occurrence of vector-transmitted agents in the region.

In this study, 8.0% (6/75) of the tested animals were positive in PCR assays for piroplasmids based on the 18S rRNA gene. The occurrence of piroplasmids found in this study was also reported by Lemos et al. (2012) (6.8%) in 103 canine blood samples sent to a clinical laboratory, which, after sequencing of the positive samples, confirmed the presence of *B. vogeli* and *R. vitalii* in Teresópolis, state of Rio de Janeiro, Brazil.

Although rangeliosis in the acute phase is characterized by apathy, anorexia/inappetence and mild to severe jaundice, bloody diarrhea, vomiting and consequent dehydration (FIGHERA et al., 2010), the animals testing positive in this study presented only apathy. The intense ear bleeding observed in two of the positive dogs is well described in cases of rangeliosis. Moreover, bleeding in other sites may also occur, especially in the form of petechiae and suffusions in the skin, oral mucosa and nasal planum (FIGHERA et al., 2010). Anemia with the presence of anisocytosis, polychromasia and Howell-Jolly bodies was found in the CBC of dogs testing positive by PCR for piroplasmids in this study, and such findings are also reported in the literature (FIGHERA, 2007; FIGHERA et al., 2010; FRANÇA et al., 2010; LEMOS et al., 2017). Thrombocytopenia, another frequent finding among dogs positive for *R. vitalii* in this study, has also been reported in dogs with rangeliosis, in response to defects in platelet aggregation associated with impaired release of adenosine diphosphate (ADP) (PAIM et al., 2012; LEMOS et al., 2017). Infestation by *A. aureolatum*, the vector of *R. vitalii* in Brazil (SOARES & GIROTTO SOARES, 2015), was found in two dogs positive for *R. vitalii*.

The differential diagnosis of rangeliosis and babesiosis by means of molecular techniques is important not only for taxonomic purposes. Although piroplasms are genetically related, differences in the pathogenicity of *R. vitalii* and *B. vogeli* have been identified. While acute conditions are described in *R. vitalii* infection in domestic dogs (LORETTI & BARROS, 2004; SOARES et al., 2011; FRANÇA et al., 2014), which require therapeutic intervention (SOARES et al., 2011), *B. vogeli* is usually associated with asymptomatic infections (MALHEIROS et al., 2016). These data corroborate those found in this study, since the animals positive for *R. vitalii* presented acute clinical signs such as anemia, thrombocytopenia, external bleeding and anorexia.

In this study, only one dog (1.33%), which had been run over, tested positive for *H. canis*. The absence of clinical signs has been reported in dogs with hepatozoonosis (SPOLIDORIO et al., 2009). According to Mundim et al. (2008), the clinical presentation of the disease varies according to the level of parasitemia and the immune status of the animal, which, after being run over, would explain the presence of the parasite in the blood. This low occurrence of *H. canis* is similar to that described by Pereira et al. (2011). Upon evaluating dogs from the peri-urban region of Piraí, state of Rio de Janeiro, the authors detected *H. canis* in two canine blood samples out of a total of 88 samples collected by convenience during an anti-rabies vaccination campaign. In addition, a low prevalence of this pathogen in dogs has also been described in an urban area of the state of Rio Grande do Sul (MALHEIROS et al., 2016). It is believed that the agent occurs more frequently in the rural area because of the greater contact of domestic animals with wild hosts and vectors, where the presence of ticks of the genus *Amblyomma* is more evident. In contrast, urban dogs are parasitized mainly by

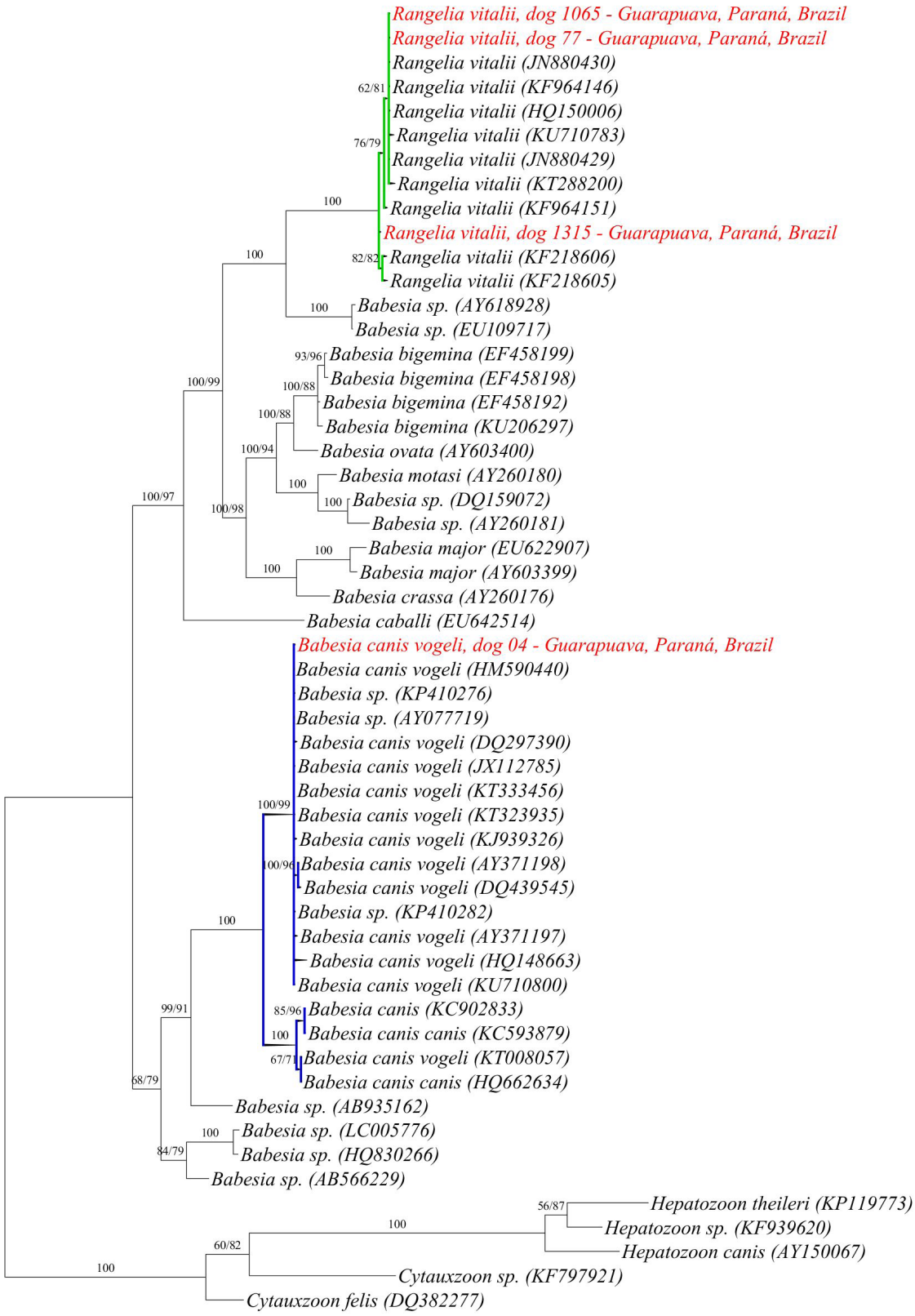

Figure 1. Phylogenetic analysis based on 18S rRNA gene of *Babesia* spp. (1810 bp after alignment). The evolutionary model used here was GTR for the Maximum Likelihood method and TIM2 for Bayesian Inference, both with invariable sites and gamma distribution. The topology presented here is based on Bayesian analysis and shows the posterior probability/bootstrap values in each branch. Monte Carlo Markov Chain (MCMC) simulations were used with 10^6 generations to define the posterior probability and bootstrapping of 1000 replicates. Values above 50% are shown. The sequences of this study are highlighted in red and separated in two different groups, clustering *Rangelia vitalii* (green branches) and *Babesia canis vogeli* (blue branches). *Hepatozoon* spp. and *Cytauxzoon* spp. were used as outgroups.

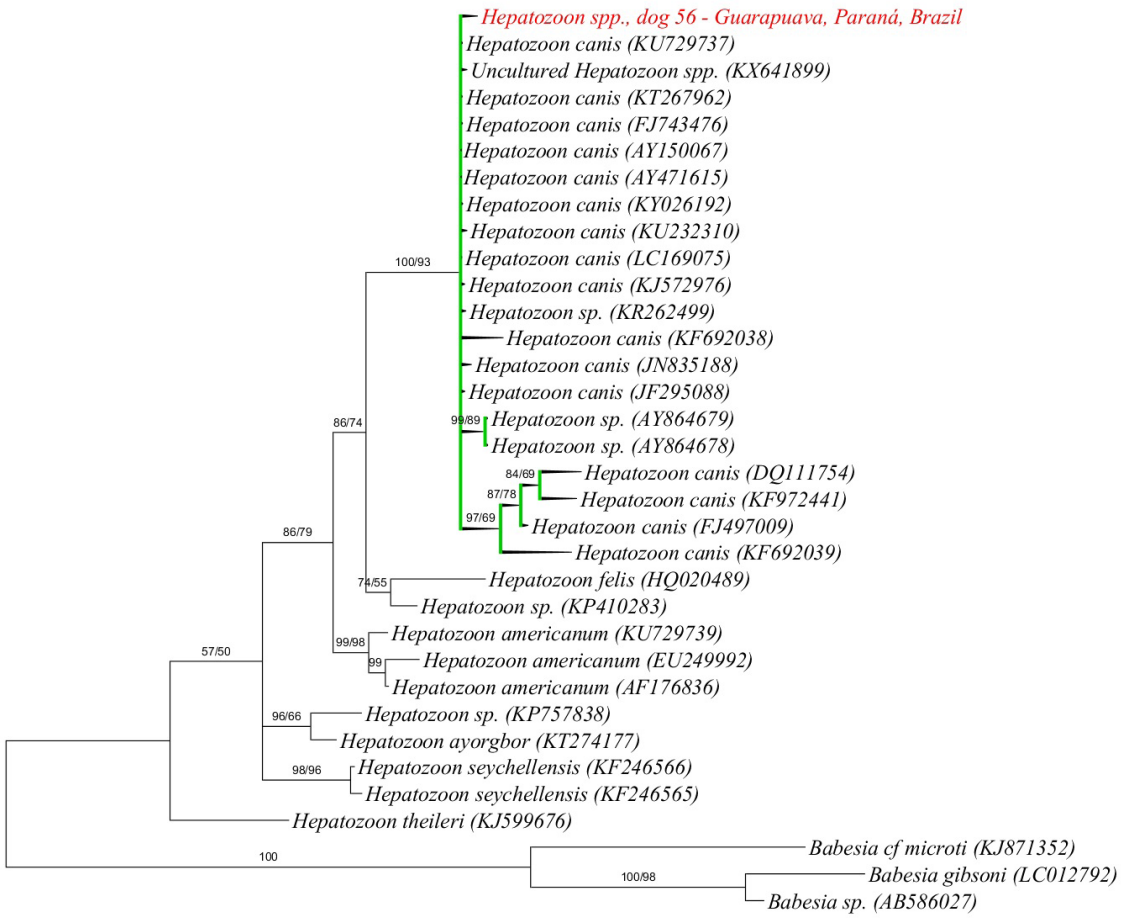

Figure 2. Phylogenetic analysis based on 18S rRNA gene of *Hepatozoon* sp. (1670 bp after alignment). The evolutionary model was TPM2uf with gamma distribution for the Maximum Likelihood method and Bayesian Inference. The topology presented here is based on Bayesian analysis, showing the posterior probability/bootstrap values in each branch. Monte Carlo Markov Chain (MCMC) simulations were used with 10^6 generations to define the posterior probability and bootstrapping of 1000 replicates. Values above 50% are shown. The sequence of the present work is highlighted in red, clustering as a polyatomic branch with *Hepatozoon canis* (green branches) with high support value (100/93). *Babesia* spp. was used as outgroup.

Rhipicephalus sanguineus (O'DWYER et al., 2001), whose vectorial capacity in Brazil still requires further studies (DEMONER et al., 2013). Although *A. ovale* is considered one of the main vectors of *H. canis* in Brazil (FORLANO et al., 2005; DEMONER et al., 2013), the occurrence of this tick species in domestic dogs in the region of Guarapuava has not been reported to date. Future studies are needed to investigate the vectorial competence of other tick species that transmit *H. canis*, as well as other canine hepatozoonosis transmission routes.

The authors of recent studies have proposed that the taxon *Rhipicephalus sanguineus* possibly comprises at least two morphologically and genetically distinct strains in the Neotropical region. In addition to the size of ticks, which tend to be smaller in regions close to the equator than ticks found at higher latitudes (SZABÓ et al., 2005; SANCHES et al., 2016), there are differences in vector competence for *E. canis* between these strains, in which only the tropical strain proved to be a competent vector of *E. canis* (MORAES-FILHO et al., 2015). The absence of anemic and thrombocytopenic animals positive by PCR for *Ehrlichia* sp. in this study may be attributed to the low vector competence of the tick species *R. sanguineus* of the temperate strain that occurs in southern Brazil (NAVA et al., 2015; MORAES-FILHO et al., 2015).

Nakaghi et al. (2010) did not find a significant difference in the results obtained by the 16S rRNA amplification protocol by nested PCR and the dsd gene of *Ehrlichia* by conventional PCR, since a total of 66 (69.7%) positive samples were obtained by the two protocols in their study. However, future studies using more sensitive protocols, such as quantitative real-time PCR, may determine definitively whether or not *Ehrlichia* is circulating in the area under study.

None of the dogs with hematological alterations sampled in this study showed coinfection by the aforementioned infectious agents. These findings differ from those reported by Spolidorio et al. (2009) in the state of Espírito Santo, Brazil. In their study, all the 20 asymptomatic dogs (21.7% [20/92]) that tested positive for *Babesia* sp. were also positive for *Hepatozoon* spp. In addition, the aforementioned authors found a significant correlation between infestation by *R. sanguineus* ticks and the presence of *Hepatozoon* sp.

In the present study, only two animals were infested with *Amblyomma aureolatum* ticks, and both dogs were only positive for *R. vitalii*. In another study by Spolidorio et al. (2011), out of a total of 15 blood samples from dogs diagnosed with *Babesia* sp. and/or *Hepatozoon* sp. infection by direct visualization of parasites in Giemsa-stained blood smears, six tested positive for *Babesia* sp. and nine for *Hepatozoon* sp. by means of conventional PCR, and five animals presented coinfection by the two agents,

The region of Guarapuava is marked by an average temperature varying from 18 °C to 22 °C, fresh summers and the absence of dry seasons, according to the Köppen climate classification (MAAK, 1968). Guarapuava is located at an altitude of 1,098 m.a.s.l. (IPARDES, 2018) and has an annual average rainfall of 1684.7 mm (SALTON et al., 2016). These conditions create a favorable microclimate for the maintenance of the tick *A. aureolatum*, which is typical of the Atlantic Forest and is described in localities at altitudes of 1,000 m.a.s.l., with rainy temperate climates characterized by mean temperatures ranging from 18 °C to 22 °C throughout the year and an annual rainfall of over 1,200 mm (RODRIGUES et al., 2002; PINTER et al., 2004). Although these conditions prove unfavorable for the other tick species that are vectors of pathogens that can infect dogs in Brazil, climate changes may also be responsible for affecting the interactions that result in the transmission of tick-borne agents (GRAY et al., 2009). Furthermore, it should be noted that an increase in tick prevalence and distribution limits depends not only on the absence of climatic stress but also on animal management conditions, especially when it comes to the tick species *R. sanguineus* (GRAY et al., 2013). Thus, studying and monitoring the occurrence of hemoparasites and their possible transmission vectors are essential to shed light on the epidemiology of these diseases that affect dogs in the midwestern region of the state of Paraná, Brazil.

Conclusions

The findings of this study revealed the presence of *Rangelia vitalii*, *Babesia vogeli* and *Hepatozoon canis* in dogs living in the city of Guarapuava, midwestern Paraná, Brazil, based on molecular techniques and phylogenetic analysis. These agents have been detected in other regions of the country, but this is the first description of the study area. These pathogens should be included in the differential diagnosis of dogs with clinical signs and hematologic abnormalities suggestive of tick-borne diseases in the city of Guarapuava, Paraná. Lastly, further studies are needed to pinpoint the main vector of these pathogens in the region.

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