

Rangelia vitalii, *Babesia* spp. and *Ehrlichia* spp. in dogs in Passo Fundo, state of Rio Grande do Sul, Brazil

Rangelia vitalii, *Babesia* spp. e *Ehrlichia* spp. em cães de Passo Fundo, estado do Rio Grande do Sul, Brasil

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Abstract

Pathogens transmitted by ticks are an emerging problem worldwide, this study aimed to diagnose the causal agents of infection in dogs presenting suspected hemoparasitoses. Fifty-eight dogs with clinical signs such as depression, hemorrhagic diathesis and fever were evaluated regarding clinical presentation, hemogram, blood smears and serological tests, using the indirect immunofluorescence method for the agents *Babesia vogeli* and *Ehrlichia canis* and conventional PCR for *Babesia* spp. (gene 18S rRNA), *Rangelia vitalii* (gene 18S rRNA) and *Ehrlichia* spp. (gene dsb). Five (8.6%) of the 58 dogs were serologically positive for *Babesia* spp. and three (5.1%) for *E. canis*. Four dogs (6.8%) were positive for *R. vitalii* through the molecular diagnosis. The PCR products were sequenced and the DNA from *R. vitalii* was found to be 99% genetically identical to samples of *R. vitalii* that had been isolated in Brazil. No presence of *Babesia* spp. or *E. canis* was observed through PCR on the dogs evaluated here. The results indicate the presence of *R. vitalii* and exposure to *Babesia* spp. and *Ehrlichia* spp. among the dogs analyzed.

Keywords: Hemoparasites, piroplasmids, sequencing, dogs, nambiuú, indirect immunofluorescence.

Resumo

Patógenos transmitidos por carrapatos são um problema emergente em todo o mundo, o trabalho objetivou diagnosticar os agentes causais da infecção em cães com suspeita de hemoparasitoses. Cinquenta e oito caninos com sinais clínicos como depressão, diáteses hemorrágicas e febre foram avaliados quanto à apresentação clínica, hemograma, esfregaço sanguíneo, sorologia pelo método de Imunofluorescência Indireta para os agentes *Babesia vogeli* e *Ehrlichia canis* e na PCR convencional para *Babesia* spp. (gene 18S rRNA), *Rangelia vitalii* (gene 18S rRNA) e *Ehrlichia* spp. (gene dsb). Cinco (8,6%) dos 58 cães apresentaram sorologia positiva para *Babesia* spp. e três (5,1%) para *E. canis*. Quatro (6,8%) animais mostraram-se positivos para *R. vitalii* no diagnóstico molecular. Os produtos da PCR foram sequenciados e o DNA encontrado de *R. vitalii* mostrou 99% de identidade genética com amostras de *R. vitalii* isoladas no Brasil. Não foi observada a presença de *Babesia* spp. e *E. canis* na PCR dos cães avaliados. Os resultados indicaram a presença de *R. vitalii* e exposição a *Babesia* spp. e *Ehrlichia* spp. entre os cães analisados.

Palavras-chave: Hemoparasitas, piroplasmídeos, sequenciamento, cães, nambiuú, imunofluorescência indireta.

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Introduction

Diseases transmitted through the bites of arthropod vectors, such as rangeliosis, ehrlichiosis, babesiosis and anaplasmosis, affect domestic and wild animals around the world, with potential zoonotic risk (BEUGNET & MARIÉ, 2009; OTRANTO & DANTAS TORRES, 2010). In this regard, *Babesia vogeli* and *Ehrlichia canis* are transmitted to vertebrate hosts by the tick *Rhipicephalus sanguineus* sensu lato (s.l.) (DANTAS TORRES & OTRANTO, 2014).

In South America, *R. vitalii* has been reported in domestic dogs in southern and southeastern Brazil (SOARES et al., 2011; MOREIRA et al., 2013), Argentina (EIRAS et al., 2014) and Uruguay (SOARES et al., 2015).

B. vogeli (PASSOS et al., 2005) and *Babesia gibsoni* (TRAPP et al., 2006) have been detected molecularly in Brazil. *B. vogeli* is the canine babesiosis agent most commonly reported, with seroprevalence ranging from 18.8% to 73.3% (TRAPP et al., 2006; FURUTA et al., 2009; SPOLIDORIO et al., 2010). A serological study involving 316 dogs that was conducted in the central region of the state of Rio Grande do Sul, Brazil, showed that only 4.43% of the animals presented antibodies to *E. canis* according to the indirect fluorescence antibody test (IFAT), although infestation by the tick *R. sanguineus* is common in this region (KRAWCZAK et al., 2012). Seropositivity for the causative agent of canine monocytic ehrlichiosis has been found to range from 0.7% to 86.2% among several regions of Brazil (VIEIRA et al., 2013).

Regenerative anemia is frequently observed on hemograms, caused by intravascular and extravascular hemolysis (babesiosis) and exclusively extravascular hemolysis (rangeliosis) (FURLANELLO et al., 2005; FIGHERA, 2007). Thrombocytopenia is a blood abnormality commonly identified in dogs infected with *B. canis*, *E. canis* and *R. vitalii* (FIGHERA, 2007; M'GHIRBI et al., 2009).

Blood smear examinations are useful during the acute phase of the disease, which coincides with the peak level of parasitemia (NEVES, 2000). However, their sensitivity is low because of the morphological similarity seen between agents of the family Anaplasmataceae and piroplasms.

Serological tests form a useful diagnostic method during the chronic phase of hemoparasitosis and in epidemiological surveys. However, they have low specificity, given that they do not distinguish current infection from previous infection (SHAW et al., 2001).

PCR and sequencing of positive samples have proved to be highly sensitive and specific, and are important in establishing a definitive diagnostic method, since the clinical signs involved in hemoparasite infections are clinically very similar (CRIADO-FORNELIO et al., 2003).

The present study aimed to investigate occurrences of diagnoses of infection by *Babesia* spp., *E. canis* and *R. vitalii* in dogs with clinical signs compatible with tick-borne diseases in the Passo Fundo, state of Rio Grande do Sul, Brazil, using hematological, parasitological, serological and molecular methods.

Materials and Methods

Location and animals of the study

The study was conducted in the municipality of Passo Fundo, in the northern region of the state of Rio Grande do Sul. The municipality has a total area of 783.4 km², with approximately 200,000 inhabitants, at a density of 0.26 inhabitants/km² (IBGE, 2010).

Fifty-eight dogs that were routinely attended at the veterinary hospital of the University of Passo Fundo (UPF) between January and December 2013, with clinical signs such as depression, hemorrhagic diathesis and fever, were screened so that a clinical evaluation questionnaire could be filled out. The dogs were shaved and, after antisepsis using 70% alcohol, two samples of venous blood were collected from the jugular vein with the aid of a hypodermic needle coupled to a sterile syringe. One sample was used for a complete hemogram and for performing PCR (1-3 mL, in a sterile flask, with the anticoagulant ethylenediaminetetraacetic acid, EDTA) and the other was used without anticoagulant to perform serological testing by means of indirect immunofluorescence.

Serological and molecular tests and blood-smear examination

Indirect immunofluorescence kits for the agents *B. vogeli* (dilution 1:40) and *E. canis* (dilution 1:64), supplied by the Imunodot immunoparasitology laboratory, Jaboticabal, São Paulo, Brazil, were used for the serological diagnosis. The samples were stored in a freezer in the veterinary parasitology laboratory of the UPF veterinary hospital, at a temperature of -20 °C.

Babesia spp. antigen was prepared by intravenously inoculation of *B. vogeli* (Jaboticabal strain) into a splenectomized three month-old dog, negative for hemoparasites by PCR and serology (ANDRÉ et al., 2011). Blood smears were performed twice a day to check for the presence of parasites in microscopic examination of Giemsa-stained blood smears. After observing parasitaemia peak on the fifth day after inoculation, infected blood was collected with Alsever solution (113.7 mM glucose, 27.2 mM sodium citrate, 71.8 mM sodium chloride). *E. canis* antigen was obtained from *E. canis* (Jaboticabal strain)-infected DH82 cells maintained in culture in the Immunoparasitology Laboratory, UNESP, Jaboticabal, São Paulo (ANDRÉ et al., 2010).

Slides containing air-dried fixed *B. vogeli* trophozoite-infected blood and *E. canis*-infected DH82 cells were used in IFAT as previously described (FURUTA et al., 2009; NAKAGHI et al., 2008). Briefly, antigen slides were removed from storage (-20 °C) and allowed to thaw at room temperature for 30 min. Ten microliters of two fold dilutions of sera (cut-off of 1:40 for *B. vogeli* and 1:64 for *E. canis*) were placed in wells on antigen slides. Known positive and negative serum samples for the studied agents were obtained from naturally-infected dogs and felids (ANDRÉ et al., 2010, 2011) from Brazil. 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 (1:32) or anti-cat (1:32) conjugates (Sigma, St. Louis, Missouri) were added to each well. These slides were incubated again at 37 °C, washed 3 times in PBS, once more in distilled water, and air dried at room

temperature. Next, slides were coverslipped, and examined under a fluorescence microscope.

Using sterile hypodermic needles, punctures were made in the internal face of the pinnae of the dogs sampled, in order to obtain peripheral capillary blood. These samples were used to produce four blood smears per dog. The slides were fixed in methanol for two minutes and were then stained using the Giemsa method for 30 minutes. The smears were examined under an optical microscope at a magnification of 1000x.

The blood samples were subjected to DNA extraction using the commercial DNeasy blood and tissue kit (Qiagen®, Hilden, Germany), in accordance with the manufacturer's recommendations. The DNA was subjected to a nested PCR assay for *Babesia* spp., as proposed by Jefferies et al. (2007), in which the oligonucleotide primers BTF1 (5-GGCTCATTACAACAGTTATAG-3), BTR1 (5-CCCAAAGACTTTGATTTCTCTC-3), BTF2 (5-CCGTGCTAATTGTAGGGCTAATAC-3) and BTR2 (GGACTACGACGGTATCTGATCG-3) flanked a fragment of 800 base pairs of the 18S rRNA gene. The oligonucleotide primers dsb-330 (5-GATGATGTCTGAAGATATGAAACAAAT-3) and dsb-728 (5-CTGCTCGTCTATTTTACTTCTTAAAGT-3), which flanked a fragment of 409 base pairs of the dsb gene (DOYLE et al., 2005), were used to amplify the DNA of the genus *Ehrlichia*. The molecular analysis on the microorganisms *Babesia* spp. and *Ehrlichia* spp. was performed in the immunoparasitology laboratory at UNESP, Jabcabal, SP, Brazil. The samples of *R. vitalii* were subjected to PCR using the primer pair BAB143-167 (5-CCG TGC TAA TTG TAG GGCTAA TAC A-3) and BAB694-667 (5-GCT TGA AAC ACT CTARTT TTC TCA AAG-3), which amplify a fragment of approximately 500 base pairs of the 18S rRNA gene of piroplasms. The molecular analysis on *R. vitalii* was performed as described by Soares et al. (2011), at the parasitic diseases laboratory of the School of Veterinary Medicine and Zootechnics (FMVZ), University of São Paulo, São Paulo, SP, Brazil. The same reagent concentrations and cycling as described by Jefferies et al. (2007), Doyle et al. (2005) and Smith et al. (2011) were used for the PCR analysis. The PCR products were then transferred onto to 1.5% agarose gel containing ethidium bromide for electrophoresis. The product was examined under a UV transilluminator (ChemIDoc™ MP Imaging System, BIO RAD®).

Phylogenetic analysis

The amplicons were purified using commercial silica bead DNA gel extraction kits (Fermentas®, Thermo Fisher Scientific Inc., USA) and were sequenced in an automated sequencer (Applied Biosystems ABI PRISM 3700 DNA Analyzer), following the instructions of the technical manual. The sequences thus obtained were analyzed using BLAST (ALTSCHUL et al., 1990) to determine genetic similarities with the data deposited in GenBank. Partial sequences (541 nucleotides) of the 18S rRNA gene derived from the positive samples were aligned with genotypes of the genera *Babesia*, *Rangelia*, *Theileria*, *Cytauxzoon* and *Hepatozoon* (obtained from GenBank), using the ClustalW v. 1.8.1 software (THOMPSON et al., 1994). Using the nucleotide substitution model, a maximum likelihood phylogenetic tree was generated by means of the Mega 6.0.6 software (TAMURA et al., 2013) with 100 bootstrap replicates. The substitution model Tamura-Nei+G+I, was selected using the

Mega 6.0.6 software (TAMURA et al., 2013), based on the lowest score of the BIC parameter (Bayesian information criterion). The sequence of *Hepatozoon canis* was used as an outgroup.

This study was approved by the Ethics Committee for Animal Use of the University of Passo Fundo (CEUA-UPF), under permit no. 003/2013.

Results

Among the 58 dogs, 49 (84.5%) presented ticks at the time of the clinical evaluation. Depression (81.0%), anorexia (56.9%) and emaciation (43.1%) were the clinical signs most observed among the dogs evaluated (Table 1).

Thrombocytopenia and reduced hematocrit were found in 14 (24.1%) and 28 (48.2%) of the dogs, respectively.

One dog (1.7%) presented structures suggestive of free piroplasms in the plasma and inside mononuclear cells in the blood smears (Figure 1).

Table 1. Observed frequencies of clinical signs and findings from hemograms, blood smears, serological tests (indirect immunofluorescence test) and PCR among the dogs evaluated.

Variables	Positive	(%)	Negative	(%)
Presence of ectoparasites	49	84.5	09	15.5
Depression	47	81.0	11	19.0
Anorexia	33	56.9	25	43.1
Emaciation	25	43.1	33	56.9
Thrombocytopenia	14	24.1	44	75.8
Reduced hematocrit	28	48.2	30	51.7
Blood smear (<i>B. canis</i>)	1	1.7	57	98.3
Blood smear (<i>E. canis</i>)	-	-	58	100
Blood smear (<i>R. vitalii</i>)	-	-	58	100
Serological test (<i>B. canis</i>)	05	8.6	53	91.3
Serological test (<i>E. canis</i>)	03	5.1	55	94.8
PCR (<i>R. vitalii</i>)	04	6.8	54	93.1
PCR (<i>E. canis</i>)	-	-	58	100.0
PCR (<i>B. canis</i>)	-	-	58	100.0

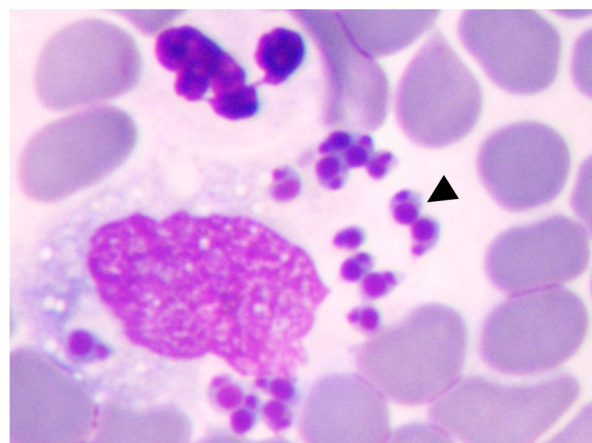


Figure 1. Free structures in the plasma (arrow) and inside a mononuclear cell that were compatible with piroplasms, observed in a Giemsa stained blood smear from a dog.

Five dogs (8.6%) presented anti-*B. vogeli* antibodies and three (5.1%) presented anti-*E. canis* antibodies in the indirect immunofluorescence test.

Among the 58 dogs sampled, four (6.8%) were positive for *R. vitalii* according to molecular analysis. These four dogs yielded PCR amplicons that generated DNA sequences that were 99% genetically identical to samples of *R. vitalii* previously isolated in Brazil, by means of BLAST analysis. Initially, four dogs (6.8%) tested positive for *Babesia* spp. through molecular diagnosis based on the protocol of Jefferies et al. (2007). However, after sequencing of these samples, the amplicons showed identicalness to *R. vitalii* (Figure 2). No other piroplasm organisms were detected in our study. The blood of these four dogs (6.8%) that were positive for *R. vitalii* was subjected to PCR and sequencing using two different protocols based on 18S rRNA, as described by Jefferies et al. (2007) and Soares et al. (2011).

Discussion

Out of the 58 dogs evaluated, 84.5% presented ectoparasites at the time of the clinical examination, presenting clinical signs such as depression, anorexia, emaciation, mucopurulent oculonasal discharge, and hemorrhagic diathesis during evaluation. According to Moraes-Filho et al. (2011), it is possible that more than one species of the *R. sanguineus* complex may be occurring in the Americas. One species might resemble ticks of African origin, with distribution in tropical and subtropical regions of the Americas, while another might resemble European samples, with distribution in temperate regions of southern South America. This hypothesis might explain the absence of dogs positive for *E. canis* through PCR, among those evaluated here. According to Moraes-Filho et al. (2015), the tick *R. sanguineus* does not have vector capacity for transmitting *E. canis*.

One of the dogs that showed ectoparasites lived in an urban area but was also kept in the countryside of Rio Grande do Sul during certain times of the year. The findings from this survey may indicate that urban dogs have greater contact with rural settings and wild animals, as a result of processes such as deforestation and the expansion of urban centers, and therefore have greater exposure to the arthropod *A. aureolatum*. It has been speculated that in rural areas of Brazil, wildlife could play an important role as reservoirs of *R. vitalii* (LORETTI & BARROS, 2005). This parasite has been recently detected through molecular diagnosis in the blood and tissues of the crab-eating fox (*Cerdocyon thous*) in the northern region of Rio Grande do Sul and in the central and northern regions of the state of São Paulo (SOARES et al., 2014). This suggests that this wild animal may be involved in the lifecycle of *R. vitalii* in the study region (LORETTI & BARROS, 2005; SOARES et al., 2014). This information corroborates the hypothesis that *R. vitalii* circulates between vertebrate animals and the tick *A. aureolatum*, thus corroborating the results presented here.

Clinical signs such as depression (81.0%), anorexia (56.9%) and emaciation (43.1%) were identified among the dogs evaluated. However, these signs are nonspecific and common to other diseases of dogs. One common clinical manifestation in animals with *R. vitalii* is hemorrhagic diathesis (FRANÇA et al., 2014), but this

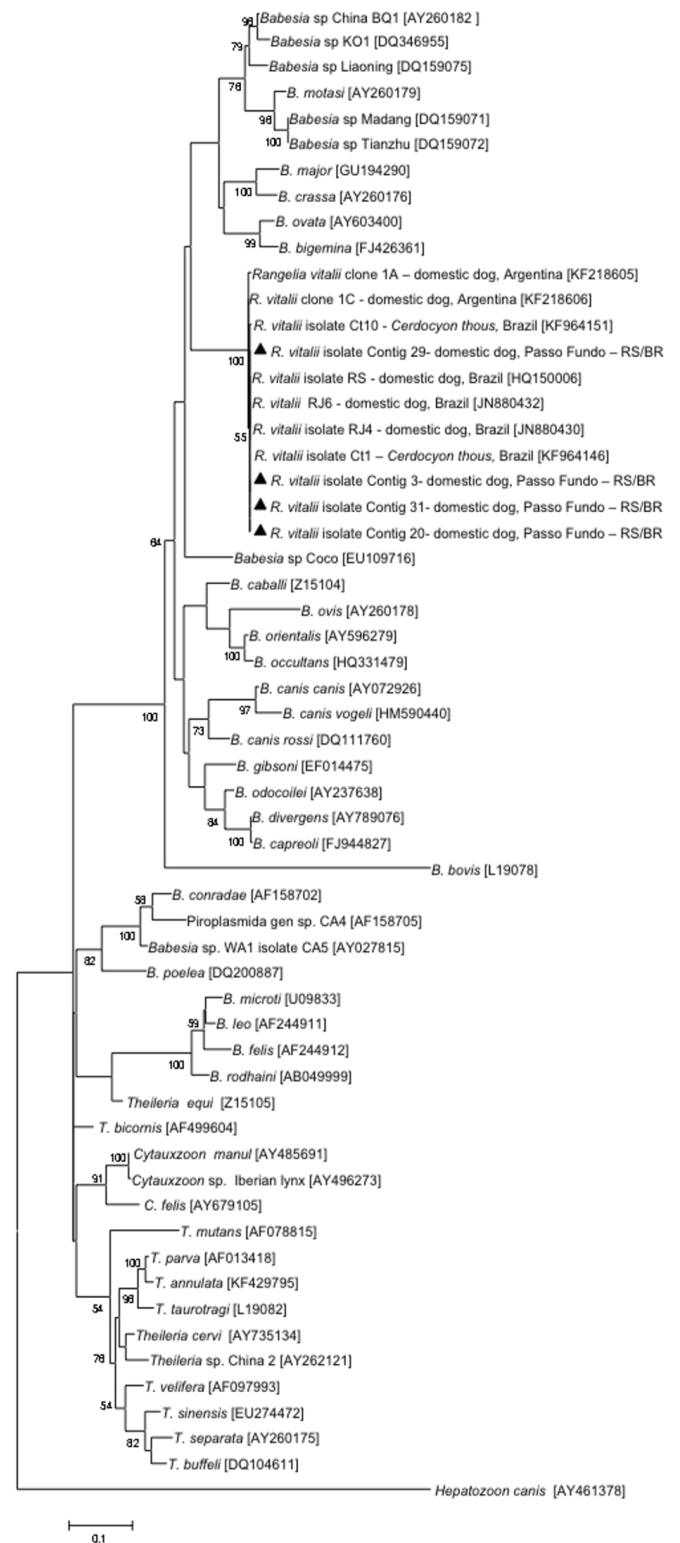


Figure 2. Maximum likelihood phylogenetic tree of 18S rRNA partial sequences (541 nucleotides) of *Rangelia vitalii* and other piroplasmids. The *R. vitalii* sequences generated in the present study are indicated by a black arrow head. Numbers on the nodes indicate bootstrap values from 100 replicates. Only bootstrap values > 50 are shown. Numbers in brackets are GenBank accession numbers. *Hepatozoon canis* was used as an outgroup.

was not identified among the dogs samples here. The importance of clinical, epidemiological and laboratory test findings for reaching a definitive diagnosis can be highlighted.

Thrombocytopenia is commonly observed among dogs with hemoparasitosis. Some of the mechanisms involved are splenic sequestration, platelet dysfunction, immune-mediated thrombocytopenia, consumptive thrombocytopenia and decreased platelet aggregation. Regenerative anemia of macrocytic hypochromic type, secondary to intravascular or extravascular hemolytic disorders, along with the presence of thrombocytopenia, aids in making a presumptive diagnosis of hemoparasitosis (PADDOCK & CHILDS, 2003; FIGHERA, 2007; PAIM et al., 2012). This corroborates the results from the present study, in which 24.1% of the dogs presented thrombocytopenia and 48.2% presented reduced hematocrit.

Out of the 6.8% of the dogs that were positive through PCR for *R. vitalii*, only 1.7% presented structures compatible with piroplasm in the blood smears (Table 2). These results show that although direct detection by means of parasitological examination of blood smears is a rapid screening technique, it has low sensitivity and specificity. It is therefore only useful in acute cases of hemoparasitosis, at the time of the peak level of parasitemia. The low number of cells parasitized, the level of experience of the microscopist and the small number of blood smears affect the sensitivity of the test (NEVES, 2000; PASSOS et al., 2005).

Out of the 8.6% of the dogs that presented anti-*B. vogeli* antibodies, only 1.7% were positive through PCR for *R. vitalii*. Among the 5.1% of the dogs with anti-*E. canis* antibodies, none of them were found to be positive through PCR. Although serological tests are frequently used as a diagnostic method, it is known that several hemoparasite species have antigens in common, thereby giving rise to cross-reactions. This may have occurred in the present study or, furthermore, the dogs may have previously been in contact with agents of the genus *Babesia* or the family Anaplasmataceae. The serological results for *E. canis* were similar to those described by Krawczak et al. (2012), who found that only 4.43% of the dogs were seropositive for this agent in the central region of Rio Grande do Sul. This demonstrated that there was low prevalence of anti-*E. canis* antibodies, despite the presence of *R. sanguineus* ticks in the dogs. Therefore, interpretations of the serological tests need to be made cautiously, in conjunction with evaluation of the clinical parameters (clinical signs and physical examination) and laboratory parameters, such as the platelet count

Table 2. Blood smears, indirect immunofluorescence tests and PCR on the dogs that were positive for *R. vitalii* through the molecular diagnosis.

Animals	Blood smears	Diagnosis	
		Indirect immunofluorescence test for <i>B. vogeli</i> and <i>E. canis</i>	PCR
Dog 7	N	N	<i>R. vitalii</i>
Dog 17	Piroplasm	1	<i>R. vitalii</i>
Dog 51	N	N	<i>R. vitalii</i>
Dog 54	N	N	<i>R. vitalii</i>

N: negative. 1: *B. vogeli*. 2: *E. canis*.

(TABOADA, 1998). Currently, no commercial tests that would allow determination of serum antibody levels specifically against *R. vitalii* are available in Brazil (FIGHERA, 2007).

The agent most frequently found in the dogs evaluated was *R. vitalii*. The validity of the species *R. vitalii* was frequently confirmed by Soares et al. (2011) through molecular analysis, and the results from their study indicated that this is a reemerging disease. The results highlight the importance of sequencing to determine the definitive diagnosis. The protocol proposed by Jefferies et al. (2007) amplifies a 18S rRNA gene segment, which is common to the species *R. vitalii* and *Babesia* spp, thus demonstrating that these organisms are phylogenetically related.

The agents found through the molecular diagnosis help to guide clinical management strategies and control measures, and to define the diagnosis. We suggest that further research on this topic should be conducted, particularly with regard to the epidemiology of hemoparasitic diseases, given that the data reported here relate to a portion of the resident dog population that, in theory, receives care and attention from its owners. The results from such evaluations might be different if they involved strays and animals from shelters and/or from animal welfare non-governmental organizations.

R. vitalii was identified as infecting domestic dogs in this study, through the molecular diagnosis. This tick-borne pathogen causes disease that often exhibits nonspecific signs and, therefore, molecular diagnostics is a very important tool for conducting specific treatments. These help reduce the adverse effects of some medications, as well as the possible resistance associated with their unnecessary use.

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