

Study on coinfecting vector-borne pathogens in dogs and ticks in Rio Grande do Norte, Brazil

Estudo da coinfeção por patógenos transmitidos por vetores em cães e carrapatos no Rio Grande do Norte, Brasil

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Abstract

Since dogs presenting several vector borne diseases can show none or nonspecific clinical signs depending on the phase of infection, the assessment of the particular agents involved is mandatory. The present study aimed to investigate the presence of *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Hepatozoon* spp. and *Leishmania* spp. in blood samples and ticks, collected from two dogs from Rio Grande do Norte showing suggestive tick-borne disease by using molecular techniques. DNA of *E. canis*, *H. canis* and *L. infantum* were detected in blood samples and *R. sanguineus* ticks collected from dogs. Among all samples analyzed, two showed the presence of multiple infections with *E. canis*, *H. canis* and *L. infantum chagasi*. Here we highlighted the need for molecular differential diagnosis in dogs showing nonspecific clinical signs.

Keywords: CVBDs, co-infection, *Ehrlichia canis*, *Hepatozoon canis*, *Leishmania infantum*, molecular diagnosis.

Resumo

Cães que apresentam diversas doenças transmitidas por vetores podem mostrar nenhum ou alguns sinais clínicos inespecíficos. Dependendo da fase da infecção, a confirmação dos agentes envolvidos é necessária. O presente estudo teve como objetivo detectar a presença de *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Hepatozoon* spp. e *Leishmania* spp. em amostras de sangue e carrapatos, coletados em dois cães do Rio Grande do Norte. Esses animais apresentavam sinais clínicos sugestivos de doenças transmitidas por carrapatos, quando foram usadas técnicas moleculares. DNA de *E. canis*, *H. canis* e *L. infantum* foram detectados em amostras de sangue e carrapatos *R. sanguineus* coletados dos cães. Entre todas as amostras analisadas, duas mostraram a presença de infecções múltiplas por *E. canis*, *H. canis* e *L. infantum chagasi*. Destaca-se a necessidade de um diagnóstico molecular diferencial em cães com sinais clínicos inespecíficos.

Palavras-chave: DCTVs, coinfeção, *Ehrlichia canis*, *Hepatozoon canis*, *Leishmania infantum*, diagnóstico molecular.

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Introduction

Ticks are important vectors of pathogens that may affect both animals and humans, causing morbidity and mortality in infected hosts (PAROLA; RAOULT, 2001). Dogs and humans are susceptible to infection by tick-borne agents, which include bacteria, protozoa and viruses. Recently, an increased risk of exposure to tick-borne pathogens among dogs has been observed around the world. Because of the close relationship that these pathogens have to humans, they have become a public health concern (BEUGNET; MARIÉ, 2009).

Among tick-borne diseases affecting dogs, canine monocytic ehrlichiosis (CME) is the most widespread illness reported in Brazil (VIEIRA et al., 2011). It is caused by *Ehrlichia canis*, an agent belonging to Anaplasmataceae that mainly parasitizes monocytes and is transmitted by *Rhipicephalus sanguineus* (DUMLER et al., 2001). Canine anaplasmosis is caused by *Anaplasma platys* and *Anaplasma phagocytophilum*, which infect dogs' platelets and neutrophils, respectively (DUMLER et al., 2001). Recently, *A. phagocytophilum* DNA has been detected in blood samples from dogs and in *Amblyomma cajennense* and *R. sanguineus* ticks in Rio de Janeiro (SANTOS et al., 2013). *A. platys* has been molecularly detected in dogs in the states of Mato Grosso do Sul, Paraná, São Paulo and Recife (SOUSA et al., 2013; SILVA et al., 2012; DAGNONE et al., 2009; RAMOS et al., 2010). Although *R. sanguineus* is a potential vector for *A. platys* (INOKUMA et al., 2000), the tick species involved in transmission of *A. phagocytophilum* in Brazil is still unknown.

Canine hepatozoonosis, caused by protozoan parasites belonging to the genus *Hepatozoon*, is transmitted through ingestion of ticks containing mature oocysts in hemocoel (SMITH, 1996). In Brazil, although *Hepatozoon canis* has been found parasitizing domestic dogs (MUNDIM et al., 2008; PALUDO et al., 2005), *Hepatozoon* spp. closed related to *Hepatozoon americanum* has also been molecularly detected in wild canids (CRIADO-FORNELIO et al., 2006; ANDRÉ et al., 2010). *R. sanguineus*, *Amblyomma ovale* and *Rhipicephalus (Boophilus) microplus* are suspected vectors of *H. canis* in Brazil (FORLANO et al., 2005; MIRANDA et al., 2011; DEMONER et al., 2013).

Regarding babesiosis, apart from a single report of *Babesia gibsoni* in a dog from the state of Paraná (TRAPP et al., 2006), dogs are more often affected by *Babesia vogeli* in Brazil (PASSOS et al., 2005; FURUTA et al., 2009; SOUSA et al., 2013).

Leishmania parasites are predominantly transmitted by *Lutzomyia* spp. while feeding on blood. Although recent studies have incriminated ticks as suspected vectors of leishmaniasis (DANTAS-TORRES et al., 2010; COLOMBO et al., 2011), the real role of these arthropods in the transmission of *Leishmania* spp. is still unknown.

Since dogs presenting several vector-borne diseases may only show nonspecific clinical signs, or no signs at all, depending on the phase of infection, it is essential to assess the particular agents involved, given that some of these pathogens (especially *A. phagocytophilum* and *Leishmania* spp.) present potential threats to public health.

The present study aimed to investigate the presence of *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Hepatozoon* spp. and *Leishmania*

spp. in blood samples and ticks collected from two dogs in Rio Grande do Norte that showed signs suggestive of tick-borne disease, by using molecular techniques.

Materials and Methods

Blood samples and ticks collected

Blood samples and ticks were collected from two dogs showing clinical signs of vector-borne diseases that were attended at the teaching hospital of the Federal Rural University of the Semi-Arid Zone (UFERSA), Mossoró, Rio Grande do Norte, Brazil, in February 2013.

A two-month-old mixed-breed male dog (**Dog #1**) showed pale mucous membranes, apathy, dry hair, hepatosplenomegaly and presence of ticks on physical examination. This animal had a history of anorexia, vomiting and diarrhea. Although the owner reported having had the animal dewormed, the history of vaccination was not reported. According to the owner during anamnesis, there were no other animals in the same house where the sampled dog was kept. The dog had been acquired 20 days before the date of presentation to the teaching hospital, from a locality in the rural area of the municipality of Mossoró. Hematological and biochemical analyses showed the presence of hypochromic normocytic anemia, anisocytosis, thrombocytopenia, hyperproteinemia and elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

A three-year-old mixed-breed female dog (**Dog #2**) showed pale mucous membranes, dry thinning hair, onychogryphosis, scaly skin and crusts, lymphadenomegaly, hepatosplenomegaly and ocular mucous secretion on physical examination. This animal had had a history of hyporexia for approximately 2-3 months, with marked anorexia over the past 3 days, and apathy. According to the owner during anamnesis, although the sampled dog had outdoor access, there were no other animals in the same house where it was kept. Also, although the owner reported that the dog had been vaccinated against rabies, there was no history of deworming. Hematological analysis showed the presence of hypochromic normocytic anemia, anisocytosis, polychromasia, neutropenia, lymphopenia and thrombocytopenia.

Ticks

A total of seven ticks (three male and one female *R. sanguineus* ticks from Dog #1; one male and two female *R. sanguineus* ticks from Dog #2) were collected from the sampled dogs. The ticks collected were placed in tubes with 70% ethanol and stored at room temperature. After morphological identification as *R. sanguineus* (WALKER et al., 2000), the ticks were subjected to DNA extraction.

DNA Extraction

DNA was extracted from the dogs' blood samples and from individual ticks using the QIAamp DNA Blood and Tissue Mini Kit (QIAGEN, Valencia, California, USA), in accordance with the manufacturer's instructions.

DNA Amplification of *Ehrlichia* spp., *Babesia* spp., *Hepatozoon* spp., *Anaplasma* spp. and *Leishmania* spp.

Each sample of extracted DNA was used as a template in 25 µL reaction mixtures containing 10x PCR buffer, 1.0 mM of MgCl₂, 0.2 mM of deoxynucleotide triphosphate (dNTPs) mixture, 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, California, USA) with 0.5 µM of genus primers for *Ehrlichia* spp. (16S rRNA gene) (MURPHY et al., 1998), *Hepatozoon* spp. (18S rRNA gene) (INOKUMA et al., 2002), *Babesia* spp. (18S rRNA gene) (JEFFERIES et al., 2007), *Anaplasma* spp. (16S rRNA gene) (MASSUNG et al., 1998), *Leishmania* spp. (kinetoplast DNA) (MICHALSKY et al., 2002) and *L. donovani* complex (kinetoplast DNA) (CORTES et al., 2004). Positive controls for *Ehrlichia canis* and *B. vogeli* DNA were obtained from dogs that had been experimentally infected with Japoticabal strains of *E. canis* (CASTRO et al., 2004) and *B. vogeli* (FURUTA et al., 2009), respectively. *Hepatozoon* sp. and *Anaplasma* spp. DNA samples were obtained from naturally infected wild and domestic dogs (ANDRÉ et al., 2010; SOUSA et al., 2013). A positive control for *Leishmania infantum* DNA was obtained from parasites maintained in culturing medium (GenBank: GQ290460) (OLIVEIRA et al., 2011). Ultra-pure sterile water was used as the negative control. In order to prevent PCR contamination, the DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separate rooms.

Sequencing of PCR products

The reaction products were purified using the Silica Bead DNA Gel Extraction Kit (Fermentas®, São Paulo, SP, Brazil). The purified amplified DNA fragments were submitted for sequence confirmation in an automated sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems/Perkin Elmer), in house, and were used for subsequent phylogenetic analysis. Consensus sequences were obtained through analysis on the sense and antisense sequences using the CAP3 program (<http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py>). Comparisons with sequences

deposited in GenBank were conducted using the basic local alignment search tool (BLAST) (ALTSCHUL et al., 1990). The DNA sequences obtained in the present study were deposited in the GenBank database.

Results

DNA of *E. canis*, *H. canis* and *L. infantum* was detected in the blood samples and *R. sanguineus* ticks collected from these two dogs with nonspecific clinical signs of tick-borne diseases (Table 1). Both of the dogs were coinfecting with at least two pathogens. The *R. sanguineus* ticks collected from both dogs were positive for at least one agent investigated. Among the nine samples analyzed, two of them (Dog #2 and *R. sanguineus* male 1'') showed the presence of multiple infections with *E. canis*, *H. canis*, *L. infantum*. DNA of *Babesia* spp. and *Anaplasma* spp. was not detected in any of the blood and tick samples analyzed. The percentage identicalness and GenBank accession numbers of the DNA sequences amplified from dogs and ticks in the present study are shown in Table 2.

Discussion

In the present study, we reported on the existence of coinfection by vector-transmitted pathogens in blood samples and ticks collected from dogs showing clinical signs suggestive of arthropod-borne diseases. To the authors' knowledge, this is the first report of simultaneous coinfection in dogs and ticks (*R. sanguineus*) by *L. infantum*, *E. canis* and *H. canis*. In addition, this study shows the first molecular detection of single or multiple infection by *L. infantum*, *E. canis* and *H. canis* in dogs and ticks in the state of Rio Grande do Norte, Brazil.

Recently, multiple vector-borne pathogen infection in dogs showing nonspecific clinical signs has been reported around the world. In the Caribbean region, Kelly et al. (2013) reported coinfection by *A. platys*, *E. canis* and *B. vogeli* in 1.1% (3/279) of the dogs that they sampled in St. Kitts, West Indies. Similarly, Yabsley et al. (2008) detected both *A. platys* and *E. canis* DNA in 5.5% (4/73) of the dogs that they sampled in Grenada. Coinfections

Table 1. Vector-borne pathogens detected molecularly in dogs and ticks in this study, RN, Brazil.

Samples	Pathogens				
	<i>Anaplasma</i> spp.	<i>Babesia</i> spp.	<i>Ehrlichia canis</i>	<i>Hepatozoon canis</i>	<i>Leishmania Infantum</i>
Dog#1					
Blood sample	-	-	+	+	-
<i>R. sanguineus</i> male 1'	-	-	+	-	-
<i>R. sanguineus</i> male 2'	-	-	+	-	-
<i>R. sanguineus</i> male 3'	-	-	+	-	-
<i>R. sanguineus</i> female 1'	-	-	+	+	-
Dog#2					
Blood sample	-	-	+	+	+
<i>R. sanguineus</i> male 1''	-	-	+	+	+
<i>R. sanguineus</i> female 1''	-	-	-	+	-
<i>R. sanguineus</i> female 2''	-	-	+	-	+

(') ticks collected in the Dog#1; (") ticks collected in the Dog#2.

Table 2. Identity and GenBank accession numbers of sequences amplified from dogs and ticks in the present study, RN, Brazil.

Samples	Identity (%)				Genbank accession numbers
	<i>E. canis</i>	<i>H. canis</i>	<i>L. infantum</i>		
Dog #1					
Blood sample	100% (JX437966)	99% (KC138535)	-		(KF972446) ; (KF972441) -
<i>R. s.</i> male 1 [*]	99% (JX437966)	-	-		(KF972449) - -
<i>R. s.</i> male 2 [*]	99% (JQ260856)	-	-		(KF972447) - -
<i>R. s.</i> male 3 [*]	100% (JX437966)	-	-		(KF972448) - -
<i>R. s.</i> female 1 [*]	99% (EU781695)	99% (KC138535)	-		(KF972450) ; (KF972442) -
Dog #2					
Blood sample	99% (JQ260856)	99% (AF176835)	99% (HM179995)		(KF972452) ; (KF972443) ; (KF972455)
<i>R. s.</i> male 1 ^{**}	Not sequenced	-	97% (AF169138)		- - (KF972453)
<i>R. s.</i> female 1 ^{**}	-	99% (KC138535)	-		- (KF972444) -
<i>R. s.</i> female 2 ^{**}	99% (JQ260853)	99% (KC138535)	99% (AF169138)		(KF972452) ; (KF972445) ; (KF972454)

(^{*}) ticks collected in Dog#1; (^{**}) ticks collected in Dog#2; *Accession numbers deposited in Genbank from this study; Sample (*R. s.* male 1^{**}) was not sequenced for *Ehrlichia* spp.

by *H. canis* and *B. vogeli* (0.89% [1/89]), *H. canis* and *E. canis* (2.67% [3/89]) and *H. canis* and *A. platys* (3.56% [4/89]) were reported in dogs in Buenos Aires, Argentina (EIRAS et al. 2013). In Alto Tras-os-Montes and Douro, in northern Portugal, coinfection by *B. canis* and *L. infantum* was reported in 13.3% (6/45) of the dogs sampled, while multiple infection by *B. vogeli*, *L. infantum* and *E. canis* was found in one dog (2.2%) (CARDOSO et al., 2010). In Naples, Italy, coinfection by *E. canis* and *L. infantum* was reported in 79% (34/43) of the dogs sampled (MEKUZAS et al., 2009). In Brazil, among 60 *L. infantum*-seropositive dogs sampled in the city of Campo Grande, an endemic area for canine leishmaniasis in the state of Mato Grosso do Sul, Sousa et al. (2013) detected the presence of coinfections between *Leishmania* sp. and *E. canis*; *Leishmania* spp. and *B. vogeli*; *Leishmania* sp. and *A. platys*; and *B. vogeli* and *E. canis* in 22 dogs (33.6%), one (1.66%), one (1.66%) and one (1.66%), respectively.

Although *Anaplasma* spp. and *Babesia* spp. were not molecularly detected in dogs in the present study, these agents have previously been detected in dogs in Brazil. For instance, *A. phagocytophilum* has been detected in dogs and ticks, namely in *R. sanguineus* and *A. cajennense*, in the state of Rio de Janeiro (SANTOS et al., 2013). On the other hand, *A. platys* has been detected in dogs in the states of Mato Grosso do Sul, Paraná, São Paulo and Recife (SOUSA et al., 2013; SILVA et al., 2012; DAGNONE et al., 2009; RAMOS et al., 2010). *B. vogeli* is a widespread tick-borne hemoprotozoan reported in Brazil (DANTAS-TORRES; FIGUEREDO, 2006).

The occurrence and distribution of these pathogens and their respective diseases in both animals and humans can be correlated with the geographical dispersion of ticks and other arthropod vectors (TROTITA et al., 2012). Multiple pathogens showing zoonotic potential have also been detected in the ticks *Ixodes ricinus* (*A. phagocytophilum*, *L. infantum* and *Bartonella henselae*) and *R. sanguineus* (*Rickettsia conorii*, *R. massiliae*, *L. infantum* and *A. phagocytophilum*) collected from domestic dogs in Europe and South America (TROTITA et al., 2012; PODSIADLY et al., 2007; COLOMBO et al., 2011; SANTOS et al., 2013; SMITH et al., 2013). Dogs parasitized

by multiple pathogen-infected ticks may have an unknown clinical outcome that depends on the host-parasite relationship. Studies on interactions between tick-borne agents and *Leishmania* parasites with regard to establishment and progression of the disease are much needed (SOUSA et al., 2013).

Previously, in the state of Rio Grande do Norte, antibodies to *Leishmania* spp. were detected in 28% (39/139) of dogs showing clinical signs of leishmaniasis or asymptomatic dogs that lived in the same area as seropositive dogs (MATOS et al., 2006). Moreover, inclusions suggestive of *Ehrlichia* spp. were detected in 6.5% (13/198) of dogs showing clinical signs suggestive of canine monocytic ehrlichiosis (MEDEIROS; LIMA, 2004).

Since dogs infected by *Anaplasma* spp., *E. canis*, *Leishmania* spp., *H. canis* and/or *Babesia* spp. show nonspecific clinical signs (fever, weight loss, lethargy, splenomegaly, pale mucous membranes, vomiting and anorexia) and hematological abnormalities (anemia, leukopenia and thrombocytopenia) (CARDOSO et al., 2010; KELLY et al., 2013), a differential diagnosis based on identification of the etiological agents is important, in order to assess the zoonotic potential and the best therapy for the pathogens involved.

Since serological assays show cross-reactions (such as those found between *E. canis* and *E. chaffeensis*; *A. platys* and *A. phagocytophilum*; and *Leishmania* spp. and *Trypanosoma cruzi*) and direct detection of these pathogens by means of blood smears shows low sensitivity and specificity, especially in cases of chronic infection or low parasitemia, molecular techniques play a role as an important tool for detection and differentiation of pathogens that infect both animals and humans (LITTLE, 2010; LUCIANO et al., 2009).

Conclusion

The present work showed the presence of coinfection by multiple arthropod-borne pathogens (*L. infantum*, *E. canis* and *H. canis*) in dogs in Mossoró, state of Rio Grande do Norte, and highlighted the need for molecular differential diagnoses among dogs showing nonspecific clinical signs.

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