


Molecular detection of *Cercopithifilaria bainae* and other tick-borne pathogens in *Rhipicephalus sanguineus* s.l. isolated from dogs in Midwest Brazil

Detecção molecular de Cercopithifilaria bainae e outros patógenos transmitidos por carrapatos em Rhipicephalus sanguineus s.l. isolados de cães no Centro-Oeste do Brasil

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Abstract

The aim of the present study was to detect *Cercopithifilaria bainae* and other tick-borne pathogens and to perform molecular characterization of the tick *Rhipicephalus sanguineus* s.l. collected from dogs. Ticks (n = 432, including 8 larvae, 59 nymphs, and 365 adults) were sampled from domiciled dogs (n = 73) living in Campo Grande, Mato Grosso do Sul (Midwest Brazil). All ticks were morphologically identified as *R. sanguineus*. Genomic DNA was extracted in pools (three to five ticks per animal) and was used for definition of *R. sanguineus* haplotypes (based on 16S rRNA analysis) and pathogen identification (*Cercopithifilaria* sp., *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis*, *Babesia vogeli* and *Rickettsia* spp.). *Rhipicephalus sanguineus* specimens were identified as haplotypes A and B. DNA of *Cercopithifilaria bainae* (43.83%; 32/73), *Ehrlichia canis* (24.65%; 18/73), *Anaplasma platys* (19.17%; 14/73), and *Hepatozoon canis* (5.47%; 4/73) was detected. The identity of pathogens was confirmed by DNA sequence analysis. The present study confirms the presence of haplotypes A and B of *R. sanguineus* in the state of Mato Grosso do Sul and its importance as a vector of several pathogens of veterinary concern. Finally, this is the first report to identify *C. bainae* in ticks in the Midwestern region of Brazil.

Keywords: Ticks, genetic characterization, dog, central western region, Brazil.

Resumo

O objetivo do presente estudo foi detectar *Cercopithifilaria bainae* e outros patógenos transmitidos por carrapatos e realizar a caracterização molecular do carrapato *Rhipicephalus sanguineus* s.l. coletado em cães. Carrapatos (n = 432, incluindo 8 larvas, 59 ninfas e 365 adultos) foram amostrados de cães domiciliados (n = 73) residentes no município de Campo Grande, Mato Grosso do Sul (centro-oeste do Brasil). Todos os carrapatos foram identificados morfologicamente como *R. sanguineus*. O DNA genômico foi extraído em pools (três a cinco carrapatos por animal), seguido pela definição de haplótipos (com base no gene 16S rRNA) e pela investigação de patógenos

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(*Cercopithifilaria* sp., *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis*, *Babesia vogeli* e *Rickettsia* spp.). Os espécimes coletados foram identificados como haplótipos A e B de *R. sanguineus*. Foram detectados DNA de *Cercopithifilaria binae* (43,83%; 32/73), *Ehrlichia canis* (24,65%; 18/73), *Anaplasma platys* (19,17%; 14/73) e *Hepatozoon canis* (5,47%; 4/73). A identidade dos patógenos foi confirmada por análise de sequência de DNA. O presente estudo confirma a circulação dos haplótipos A e B de *R. sanguineus* no estado de Mato Grosso do Sul e sua importância como vetor de vários patógenos de interesse veterinário. Finalmente, este é o primeiro relato de *C. binae* em carrapatos na região centro-oeste do Brasil.

Palavras-chave: Carrapato, caracterização genética, cão, Centro-Oeste, Brasil.

Introduction

Ticks and tick-borne pathogens constitute an important problem of medical and veterinary concern around the world. Several microorganisms (e.g., *Rickettsia rickettsii*, *Borrelia burgdorferi* and *Ehrlichia canis*) transmitted by ticks may cause infection in humans and animals (Almeida et al., 2013; Schreiber et al., 2014). Therefore, studies on the detection of ticks and their associated pathogens provide valuable information to better understand their epidemiology (Schreiber et al., 2014).

Among the tick species infesting dogs, *Rhipicephalus sanguineus* sensu lato is considered the most important and is widespread throughout the world. Two genetic lineages are currently recognized within this species (i.e., the Southern or temperate lineage and the Northern or tropical lineage) (Moraes-Filho et al., 2011). The vector competence of ticks belonging to these different lineages has been subject of speculation. For example, differences have been observed in the transmission of distinct Brazilian strains of *E. canis* to dogs (Moraes-Filho et al., 2015) and it is likely that this variation in vector competence also holds true for other pathogens. In the state of Mato Grosso do Sul (central western Brazil), little is known about the occurrence of different lineages of *R. sanguineus*, although there is evidence of genetic similarity between local strains and those recognized as tropical lineages and reported in the States of Rio de Janeiro and São Paulo (Burlini et al., 2010; Moraes-Filho et al., 2011).

Ehrlichia canis and *Anaplasma platys* are among the most prevalent and disease-causing pathogens in dogs in Brazil (Ramos et al., 2010; Soares et al., 2017). On the other hand, the distribution and epidemiological impact of other pathogens are poorly documented. For instance, although the filarid *Cercopithifilaria binae* was first reported in Brazil in 1984 (Almeida & Vicente, 1984), the parasite has only gained attention in the last four years, especially in the North-Eastern regions of the country (Ramos et al., 2016; Santos et al., 2017).

Researchers have placed increasing importance on investigating the occurrence of pathogens in ticks (Schreiber et al., 2014; Melo et al., 2016), which is an interesting alternative to surveys carried out in dogs, especially for pathogens that present with low parasitemia. Therefore, the present study aimed to characterize *R. sanguineus* strains infesting dogs in Campo Grande, State of Mato Grosso do Sul, Brazil, as well as to identify the main pathogens carried by this tick.

Materials and Methods

The present study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Mato Grosso do Sul (protocol number: 961/2018).

Ticks were collected from 73 naturally infested domiciled dogs at the Veterinary Teaching Hospital of the Federal University of Mato Grosso do Sul and the Zoonosis Control Center, municipality of Campo Grande (20°30'32.0"S 54°37'15.2"W), Mato Grosso do Sul (MS), Brazil.

Ticks were manually and randomly collected from each dog during a period of 5 min, for a total of 432 ticks. The specimens were placed in glass vials containing 70% ethanol

and subsequently identified based on morphological characteristics, according to Barros-Battesti et al. (2006).

Adult ticks were pooled (three to five per animal) and DNA was extracted from them. The ticks were first washed with autoclaved distilled water and dried. The selected parasites were macerated in microtubes; this was followed by addition of 500 µl of 20% sodium dodecyl sulfate and 10 µl of proteinase K (20 mg/ml), incubation for 24 hours at 56°C, addition of 400 µl of chloroform and vigorous homogenization by vortexing. After the addition of 300 µl of protein precipitation solution (0.8 M potassium acetate and 11% glacial acetic acid), the mixture was centrifuged at 10,000 × g for 10 minutes. The aqueous phase was transferred to a microtube and DNA was precipitated by the addition of 1 ml of 100% ethanol. After centrifugation at 10,000 × g for 5 minutes, the pellet was washed with 1 ml of 70% ethanol and dried at room temperature (approximately 25°C) for 30 minutes. The pellet was suspended in 1x TE buffer. DNA integrity and quantity were evaluated by electrophoresis in 0.8% agarose gels and spectrophotometry (A260/A280), respectively.

Samples were submitted to polymerase chain reaction (PCR) using the primers described in Table 1. A single PCR was used for the detection of *Cercopithifilaria* sp., *B. vogeli*, *Hepatozoon* sp., and *Rickettsia* sp., and a nested PCR was used for *E. canis* and *A. platys*. The PCR products were visualized in an UV transilluminator after 1.5% agarose gel electrophoresis and staining with Gel Red (Biotium, Fremont, CA, USA).

Table 1. Polymerase chain reaction primers used to test DNA extracts of ticks sampled from dogs in Campo Grande, Mato Grosso do Sul, Brazil.

Target organism	Primers (5'-3')	Gene	Amplicon (bp)	Reference
Ticks	16S Tick F- CCGGTCTGAACTCAGATCAAGT 16S Tick R- GCTCAATGATTTTTTAAATTGCTGT	16S rRNA	460	Mangold et al. (1998)
<i>Babesia vogeli</i>	Bab1- TGAACCTTATCACTTAAAGG Bab4- CAACTCCTCCACGCAATCG	18S rRNA	590	Duarte et al. (2008)
<i>Hepatozoon</i> sp.	HepF- ATACATGAGCAAAATCTCAAC HepR- CTTATTATCCATGCTGCAG	18S rRNA	666	Inokuma et al. (2002)
<i>Ehrlichia canis</i>	ECC - AGAACGAACGCTGGCGGCAAGCC ECB - CGTATTACCGCGGCTGCTGGC HE- ATAGGTACCGTCATTATCTTCCTAT* ECA-CAATTATTTATAGCCTCTGGCTATAGGAA*	16S rRNA	389	Wen et al. (1997)
<i>Anaplasma platys</i>	8F- AGTTTGATCATGGCTCAG 1448R- CCATGGCGTGACGGGCAGTGT PLATYS- GATTTTTGTCGTAGCTTGCTATG* EHR16SR- TAGCACTCATCGTTTACAGC*	16S rRNA	678	Martin et al. (2005)
<i>Cercopithifilaria</i> sp.	Fila12SF- CGGGAGTAAAGTTTTGTTTAAACCG Fila12SR- CATTGACGGATGGTTTGACCAC	12S rRNA	330	Otranto et al. (2011)
<i>Rickettsia</i> spp.	CS78- GCAAGTATCGGTGAGGATGTAAT Cs323- GCTTCCTTAAAATTCATAAATCAGGAT	<i>gltA</i>	401	Labruna et al. (2004)

*Used in a nested-PCR reaction.

Amplicons were purified using Clean Sweep PCR Purification reagent (Thermo Fisher Scientific Carlsbad, CA, USA) and sequenced in both directions by Sanger sequencing in an ABI-3130 automated sequencer (Applied Biosystems). Sequences were analyzed with the aid of Contig editor version 2.2.0 (Gene Studio Inc), and the identity of DNA sequences was determined by comparison with sequences available in the GenBank database using the BLASTn search tool.

Ten tick-positive samples were subjected to 16S rRNA sequencing. A phylogenetic tree was constructed using the UPGMA method (Sneath & Sokal, 1973). DNA sequences of ticks available in the GenBank database were used for phylogenetic analysis. Bootstrap resampling (1000 replicates) was performed for statistical support of reliability of tree nodes (Felsenstein, 1985) using the MEGA software version 6.0 (Tamura et al., 2013).

Moreover, five samples positive for *Cercopithifilaria* sp., four positive for *E. canis*, four positive for *A. platys*, and two positive for *Hepatozoon* sp., were similarly submitted for DNA sequencing. The DNA sequences were deposited in Genbank under the accession numbers shown in Table 2.

Table 2. Genbank accession numbers for the DNA sequences amplified from ticks sampled from dogs in Campo Grande, Mato Grosso do Sul, Brazil.

Sequence identification	Genbank accession number
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 10	MG793426
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 12	MG793427
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 15	MG793428
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 18	MG793429
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 20	MG793430
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 22	MG793431
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 24	MG793432
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 25	MG793433
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 30	MG793434
<i>Rhipicephalus sanguineus</i> Campo Grande/MS 38	MG793435
<i>Cercopithifilaria bainae</i> Campo Grande/MS 01	MG793436
<i>Cercopithifilaria bainae</i> Campo Grande/MS 02	MG793437
<i>Cercopithifilaria bainae</i> Campo Grande/MS 03	MG793438
<i>Cercopithifilaria bainae</i> Campo Grande/MS 04	MG793439
<i>Cercopithifilaria bainae</i> Campo Grande/MS 05	MG793440
<i>Ehrlichia canis</i> Campo Grande/MS 01	MG793441
<i>Ehrlichia canis</i> Campo Grande/MS 02	MG793442
<i>Ehrlichia canis</i> Campo Grande/MS 03	MG793443
<i>Ehrlichia canis</i> Campo Grande/MS 04	MG793444
<i>Anaplasma platys</i> Campo Grande/MS 01	MG793445
<i>Anaplasma platys</i> Campo Grande/MS 02	MG793446
<i>Anaplasma platys</i> Campo Grande/MS 03	MG793447
<i>Anaplasma platys</i> Campo Grande/MS 04	MG793448
<i>Hepatozoon canis</i> Campo Grande/MS 01	MG793449
<i>Hepatozoon canis</i> Campo Grande/MS 02	MG793450

Results and Discussion

All ticks analyzed in the present study were morphologically identified as *R. sanguineus* partially or completely engorged (n=432, including 8 larvae, 59 nymphs and 365 adults). The 16S rRNA gene was amplified from all 73 samples (pools); the 10 samples sequenced revealed ≥ 99% identity (100% coverage) with sequences of *R. sanguineus* available in the Genbank database.

In the phylogenetic analysis, the Campo Grande (CG) strains clustered with haplotypes A and B (Figure 1), previously identified by Moraes-Filho et al. (2011) and defined as tropical haplotypes.

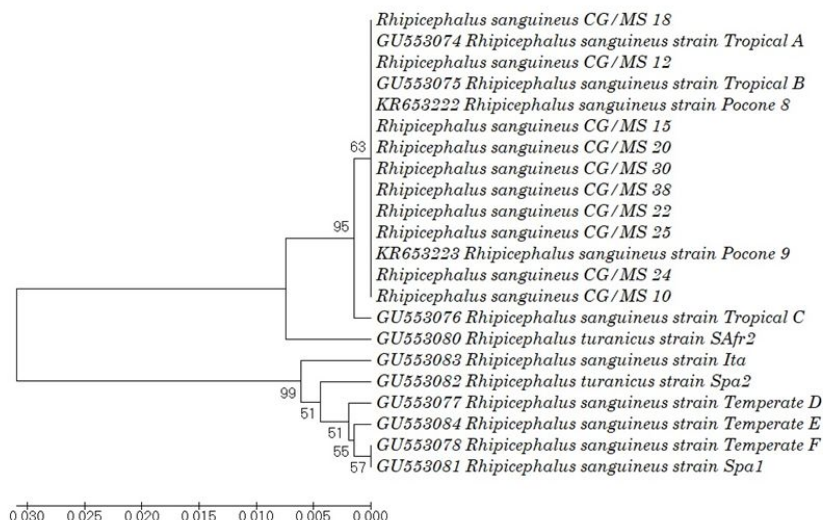


Figure 1. Phylogenetic tree constructed with the UPGMA method showing the relationship between strains of *Rhipicephalus sanguineus* from Campo Grande, MS, Brazil, and other isolates available in Genbank.

The present results confirmed the presence of these *R. sanguineus* haplotypes in the region and corroborate the observations made by Melo et al. (2016), who found only the A and B haplotypes in the Pantanal region of the state of Mato Grosso, Brazil. The C haplotype described in the Northern regions of South America (e.g., Colombia) (Moraes-Filho et al., 2011) segregated into a separate branch from the strains from Mato Grosso do Sul, but with all these haplotypes having a common ancestry. In contrast, the D, E, and F haplotypes (temperate haplotypes) formed a separate clade, supported by a high bootstrap value. These haplotypes have been reported in Chile, Argentina, Uruguay, and Southern Brazil (state of Rio Grande do Sul) (Moraes-Filho et al., 2011) and have greater genetic proximity to strains from Europe (Szabó et al., 2005).

Regarding the pathogens analyzed from ticks (divided in 73 pools) by PCR, *Cercopithifilaria* sp., (43.83%; 32/73), *E. canis* (24.65%; 18/73), *A. platys* (19.17%; 14/73), and *Hepatozoon* sp. (5.47%; 4/73) were identified. DNA from *Babesia vogeli* and *Rickettsia* spp. was not detected in any sample. The DNA sequencing results confirmed the presence of *C. binae*, *E. canis*, *A. platys*, and *H. canis* in ticks from Campo Grande, and the sequences were deposited in Genbank under the accession numbers shown in Table 2.

A part from *C. binae*, all other pathogens have been frequently reported in dogs in the region of interest (Ramos et al., 2015; Soares et al., 2017), among which *E. canis* stands out as the most prevalent agent and is often associated with veterinary health problems. An *E. canis* infection rate of 55.75% has been described in pet dogs from Campo Grande- MS (Soares et al., 2017), as well as a rate of 8.5% in stray cats in the same region (André et al., 2015).

The high prevalence of *E. canis* in dogs in the region may also be associated with the presence of A and B haplotypes of *R. sanguineus* observed in the present study, corroborating the findings of a vector competence study by Moraes-Filho et al. (2015), who observed in that the tropical haplotype of *R. sanguineus* is more effective at transmitting *E. canis* to dogs compared to temperate haplotypes.

It should be highlighted that *A. platys* was one of the most frequent pathogens found in the studied ticks (19.17%), a finding which is compatible with the *A. platys* prevalence rate described in dogs in the region. According to Soares et al. (2017), the infection frequency of dogs by *A. platys* in Campo Grande was 16.96% between 2007 and 2009. The high prevalence of *A. platys* and *E. canis* in dogs and ticks in the region provides evidence that the A and B haplotypes of *R. sanguineus* are competent vectors for both pathogens, which can be found in the same geographic areas where the tropical haplotypes (A and B) of *R. sanguineus* circulate.

Another important result of the present study was the identification, for the first time, of *C. bainae* in ticks from the central western region of Brazil. *Rhipicephalus sanguineus* is a vector for this filarid, which parasitizes the dermis of dogs (Brianti et al., 2012). It has been recently described in dogs and *R. sanguineus* in North-Eastern Brazil (Ramos et al., 2016; Santos et al., 2017). However, its clinical importance remains unknown. In the present study, *R. sanguineus* (haplotypes A and B) was considered a competent vector for this pathogen in the region, since *C. bainae* was detected in 43.83% of tick samples. Despite the small number of collected ticks and the analyses being carried out in pools, this prevalence was very high, since Santos et al. (2017) found *C. bainae* in only 51 (2.67%) out of 1906 ticks.

Moreover, Otranto et al. (2012) found the frequency of ticks positive for *C. bainae* (determined through molecular analysis) to be lower than 3.9% in the studied regions of Italy, Spain, and Greece, although the frequencies of infection in dogs in these regions ranged from 0 to 45.4%. This indicates that the frequency of dogs infected by *C. bainae* in the city of Campo Grande may be high as well. Further studies are needed to clarify this issue.

In the present study, *Hepatozoon canis* was detected in only 5.47% (4/73) of the *R. sanguineus* samples, but this prevalence is compatible with this pathogen infecting dogs (3.63%; 6/165) from the same region (Ramos et al., 2015). Although *R. sanguineus* has been implicated as the main vector of *H. canis* pathogens in urban areas of Brazil, low infection rates have been reported in dogs, despite high tick infestation rates (Ramos et al., 2010; Ramos et al., 2015). The prevalence observed in dogs and ticks from Campo Grande- MS points to the low competence of *R. sanguineus* (A and B haplotypes) in acquiring and transmitting these specific isolates of the protozoan.

Previous studies have reported unsuccessful attempts at describing the development of *H. canis* in ticks, including *R. sanguineus* from Brazil (Forlano et al., 2005; Gomes et al., 2010; Demoner et al., 2013). On the other hand, the development of *H. canis* in populations of *R. sanguineus* in Europe has been reported frequently (Baneth et al., 2001; Giannelli et al., 2013). Therefore, genetic and morphological variability in *R. sanguineus* populations can probably be attributed to the tick's ability to transmit this pathogen in different regions.

Although no DNA from *Babesia vogeli* and *Rickettsia* spp. was found in the tick pools analyzed, both pathogens occur in the region, albeit at a low frequency. DNA from *Babesia vogeli* was found in dogs from the city of Campo Grande at a frequency of only 3.3% (2/60) (Sousa et al., 2013). *Rickettsia rickettsii* was identified in only one tick of a total of 2015 specimens collected from dogs in the city of Campo Grande (Almeida et al., 2013).

The results of the present study confirm the presence of *C. bainae*, *E. canis*, *A. platys*, and *H. canis* in dogs in the municipality of Campo Grande, MS, Brazil and demonstrate the importance of the tick *R. sanguineus* (haplotypes A and B) as a host and potential vector of a set of pathogens in the region.

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