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Full Article

Babesia spp. infection in dogs from rural areas of São Paulo State, Brazil

Infecção por *Babesia* spp. em cães de áreas rurais do estado de São Paulo, Brasil

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Abstract

The status of *Babesia* spp. infection in dogs from rural areas of São Paulo State, Brazil was studied. For this, 150 animals were examined by blood smears and by PCR; the presence of tick infestation was also investigated. By the blood smear examination, 3 animals (2%) were detected positive and by PCR for *Babesia* spp. 12 (8%) were positive, with bands visualized in 450 bp. *Rhipicephalus sanguineus* or *Amblyomma* spp. were found on 36 (24%) of the 150 dogs. *Amblyomma* species found were *A. cajennense* (9/36-25%) and *A. ovale* (9/36-25%). It was not possible to correlate the presence of *R. sanguineus* and the infection with *Babesia* spp. The sequencing of four positive samples demonstrated close identity with *B. canis vogeli* already characterized in Brazil.

Keywords: *Babesia canis vogeli*, dogs, rural areas, blood smears, PCR.

Resumo

A presença de infecção por *Babesia* spp. em cães de áreas rurais do Estado de São Paulo, Brasil foi investigada. Para tanto, 150 cães foram examinados por técnicas parasitológicas de esfregaços sanguíneos e moleculares (PCR), e também, foi verificada a presença de carrapatos nestes animais. Pela análise de esfregaços sanguíneos, 3 (2%) dos cães estavam infectados, enquanto pela PCR, 12 (8%) dos animais foram positivos com bandas aproximadas de 450 pares de base (pb). Foram observados 36 (24%) cães infestados com *Rhipicephalus sanguineus* ou com *Amblyomma* spp. As espécies de *Amblyomma* observadas foram *A. cajennense* (25%) e *A. ovale* (25%). Não foi possível correlacionar a presença de *R. sanguineus* com a infecção por *Babesia* spp. O seqüenciamento de quatro amostras positivas demonstrou alta identidade com *B. canis vogeli*, já caracterizada no Brasil.

Palavras-chave: *Babesia canis vogeli*, cães, áreas rurais, esfregaço sanguíneo, PCR.

Introduction

Babesia species are intraerythrocytic tick-transmitted Apicomplexa protozoan parasites that infect a variety of vertebrate hosts. In dogs, there are two described species: *Babesia canis*, a large species, and *B. gibsoni*, a small one. The large babesias of dogs are divided into three subspecies: *B. canis canis*, *B. c. vogeli*, and *B. c. rossi*, depending on vector specificity, pathogenicity, antigenic properties, and molecular biological methods (UILENBERG et al., 1989; CITARD et al., 1995; HAUSCHILD et al., 1995; SCHETTERS et al., 1997; ZAHLER et al., 1998). It has been suggested that each of these subspecies is distinctive enough to assume species status on the basis of genetic characterization (CARRET et al., 1999). Recently, Birkenheuer et al. (2004) described a new species of large *Babesia* that was detected in a dog from North America with clinical and hematological abnormalities consistent with babesiosis. This species presented 91.2%-91.6% sequence identity with the three already described subspecies of large *Babesia* that infect dogs, and was placed in the *Babesia* spp. *sensu stricto* clade, representing a genetically unique large *Babesia* sp. The species remains with no name.

The diagnosis of *Babesia* spp. infection is usually based on the detection of merozoites in the peripheral blood smear under a microscope.

However, such a technique is limited because of low sensitivity and the impossibility to distinguish morphologically similar strains and species (KRAUSE et al., 1996).

The use of polymerase chain reaction (PCR) has become necessary to detect and identify *Babesia* spp. infection effectively and has provided important taxonomic information (SCHETTERS et al., 1997; ZAHLER et al., 1998; JEFFERIES et al., 2003; BIRKENHEUER et al., 2003; BIRKENHEUER et al., 2004; PASSOS et al., 2005).

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The molecular characterization of *B. c. vogeli* in naturally infected dogs in Brazil was recently reported in isolates from Minas Gerais and São Paulo (PASSOS et al., 2005) and from Rio de Janeiro (SÁ et al., 2006). Nevertheless, in Brazil only a few epidemiological studies have been carried out and little is known about *Babesia* infection, especially in dogs from rural areas where the vector *Rhipicephalus sanguineus* is not the most prevalent dog-infesting tick.

In this study we aimed to verify the *Babesia* spp. infection in dogs from rural areas of São Paulo State, Brazil, using blood smear examination and PCR.

Material and Methods

To detect *Babesia* infection, 150 dogs from rural areas of three counties in São Paulo State (50 from Botucatu, 50 from Rio Claro, and 50 from Presidente Prudente) were examined between November 2004 and January 2005. There were 86 males and 64 females; 32 of them were under one year old and 118 were older than one year.

The smears were made with blood taken from the ear margin capillary bed, were air dried, fixed with methanol, and stained with Giemsa. For PCR, EDTA-anticoagulant blood samples were collected by puncturing the cephalic vein and stored at –20 °C until used.

All dogs were examined for the presence of ticks, mainly on the ears, along the nape of the neck, and between the toes. The specimens found were collected and classified using the morphologic key by Aragão and Fonseca (1961).

DNA was extracted from 100 µL of EDTA blood with GFX™ Genomic Blood Purification Kit (Amersham Biosciences, Piscataway, USA) according to the manufacturer's instructions. DNA samples were eluted in 100 µL of "UltraPure™ DNase/RNase-Free Distilled Water" (Gibco/Invitrogen, Carlsbad, USA). The primers Piro A1 forward (5'- AGG GAG CCT GAG AGA CGG CTA CC - 3') and Piro B reverse (5'- TTA AAT ACG AAT GCC CCC AAC - 3') were used to amplify an approximately 450 bp region of the 18 sRNA gene, as described by Jefferies et al. (2003). Briefly, each reaction mixture (50 µL) contained 0.2 mM of each dNTP (Amersham Biosciences, Piscataway, USA), 10 mM of Tris-HCl pH 9.0, 1.5 mM of $MgCl_2$, 50 mM of KCl (GE Healthcare, Piscataway, USA), 0.25 µM of each primer, 0.5 U of Taq DNA polimerase (GE Healthcare, Piscataway, USA), and 5 µL of DNA template. PCR amplification was performed using a programmable thermal cycler (PTC-100 MJ Research™, INC, Waltham, USA) with the following program: an initial denaturation at 95 °C for 15 minutes, annealing at 62 °C for 1 minute, and extension at 72 °C for 2 minutes followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 62 °C for 20 seconds, and extension at 72 °C for 30 seconds. A final extension step at 72 °C for 7 minutes was used.

Aliquots of amplified products (8 µL) were analyzed in ethidium bromide-stained 1% agarose gel by electrophoresis at 100 V for 30 minutes in TAE buffer and visualized under UV transluminator. The total remaining reaction products were purified by purification Kit Montage™ PCR Centrifugal Filter Devices (Millipore, Billerica, USA). The purified products were dissolved in 20 µL of TE prior to sequencing. Selected product results were confirmed by sequencing.

Sequencing of PCR products amplified from five dog blood samples was carried out in both directions using the "ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit" (PE Applied Biosystems, Foster City, USA). Briefly, for each sequencing reaction 10 ng of purified DNA was combined with 3.2 pmol of primer (sense and/or reverse) used in the amplification reaction. Nucleic acid sequence analysis was performed on an automated Applied Biosystems 377 DNA sequencer.

The computer analysis of nucleic acid sequence data was performed using MERGER package software. The multiple sequence alignment method and a neighbor-joining phylogenetic tree (SAITOU and NEI, 1987) was constructed using the Clustal W program (THOMPSON et al., 1994). The bootstrap test was applied to estimate the confidence of branching patterns of the neighbor-joining tree (FELSENSTEIN, 1985). Divergences were estimated by the two-parameter method using the MEGA "Molecular Evolutionary Genetics" software (KUMAR et al., 2004) in the final documentation.

Results of microscopy were compared using the Kappa test (COHEN, 1960) and results of PCR and the proportion of positive tests were compared using the Chi-square test. A value of $p < 0.05$ was considered significantly different. The studied variables were analyzed statistically using the Goodman contrast test between and within multinomial proportions (GOODMAN, 1965).

Results

Babesia spp. infection was detected in the blood smear of three (2%) dogs: two of the dogs examined in Botucatu and one from Presidente Prudente. By PCR, 12 (8%) samples were positive by showing the 450 bp product amplification (Figure 1). Four positive samples were from the dogs from Botucatu, six from the dogs from Rio Claro, and two from the dogs from Presidente Prudente, without statistically significant difference ($p > 0.05$). Also, there were no differences when gender or age were considered.

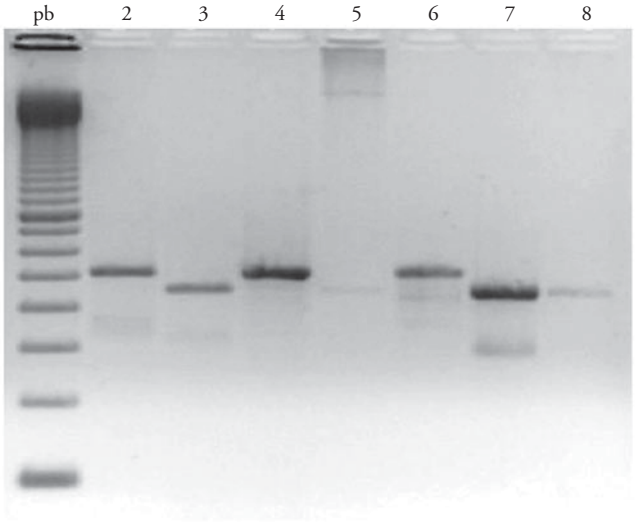

Figure 1. Ethidium bromide-stained 2% agarose gel showing amplification of a 450 bp product for *Babesia*-positive samples (lanes 3, 5, 7, and 8) and 520 bp product for *Hepatozoon*-positive samples (lanes 2, 4, and 6). (pb) Molecular marker.

Four samples of the 12 dogs PCR positive for *Babesia* infections were sequenced. The sequences were compared with other sequences from GenBank and had 100% identity with *B. c. vogeli* from urban areas of Brazil (GenBank accession number AY371196) (PASSOS et al., 2005) and from Japan (GenBank accession number AB083374) (Figure 2).

The primers Piro A1 and Piro B were designed to amplify *Babesia* species (JEFFERIES et al., 2003). Nevertheless, some animals showed a band with about 500 bp, higher than the one corresponding to the *Babesia* band (Figure 1). Sequencing of four of these samples showed that they were of *Hepatozoon canis*, with close identity with an already reported sample found in Brazil (RUBINI et al., 2005).

Ticks were collected from 36 (24%) of the dogs; 19 infested with *R. sanguineus*, nine with *A. cajennense*, two with both *R. sanguineus* and *A. cajennense*, and nine with *A. ovale*. One specimen of *Amblyomma* was at nymph stage and could not be identified. The distribution of the ticks within the counties is shown in Table 1. There was not a positive correlation with the *Babesia* infection and the infestation by *R. sanguineus* (Table 2).

Discussion

Babesia spp. are important tick-borne protozoa of wild and domestic canidae. Since the geographic range of specific piroplasms appears to be expanding (CRIADO-FORNELIO et al., 2004), location should not be used as the sole criterion for species or subspecies identification. It is diagnostically important to determine the species, subspecies, and genotypes that cause canine babesiosis, as the virulence, prognosis, and response to antibabesial drugs may be different for each organism (BIRKENHEUER et al., 2003). Besides, in Spain 12 asymptomatic dogs were analyzed by seminested PCR and sequencing, and three healthy dogs were positive for *B. equi* (*Theileria equi*) (CRIADO-FORNELIO et al., 2003). Also, a new species of large *Babesia* was described in the USA (GenBank accession number AY618928) (BIRKENHEUER et al., 2004). These findings demonstrate the importance of molecular studies and molecular characterization of canine piroplasm species, since new species and new hosts have been described.

In Brazil, few studies have been done in the country and little is known about the epidemiological aspects in rural dogs (PASSOS et al., 2005). Characterization of *Babesia* isolates on a molecular basis was performed by Passos et al. (2005) and Sá et al. (2006) in urban dogs, and *B. c. vogeli* was found to be the only infecting species. However, those studies did not analyze samples from dogs from rural areas. Our study shows that *Babesia* infection is not as prevalent in rural areas as in urban areas, where *B. canis* is endemic (PASSOS et al., 2005).

Ribeiro et al. (1990) detected 66.9% of anti-*B. canis* (sensu lato) – antibodies in dogs from an urban area. O'Dwyer et al. (2001) observed 5.2% of infected dogs by blood smear examination in a rural area. In the present study, 2% of the dogs were diagnosed by microscopic examination, and 12% of the dogs by PCR. As PCR is a sensitive test, we believe that this is a low prevalence. As far as we know, no other studies have been done with dogs from the same areas, to which our results can be compared.

Rhipicephalus sanguineus is not as frequent in rural areas as in urban areas but it may occur when the rural dogs are maintained within a kennel (LABRUNA and CAMPOS PEREIRA, 2001). In our study we observed 19 dogs infested with *R. sanguineus* (25%). Nevertheless, *R. sanguineus* infestation was more common in *B. c. vogeli* negative dogs than in positive ones.

These results could be explained by the low number of infected dogs that may hinder the correlation between infection and tick infestation. The number of infected dogs may be insufficient to assure infection by *R. sanguineus* and this could explain the number of infested dogs that are negative for *B. c. vogeli*. We did not observe differences among the three counties in relation to the number of infected dogs either; neither there was a difference when gender and age were considered. We expected the younger dogs to be more infected.

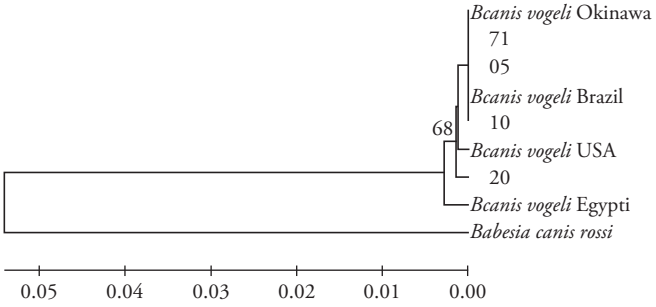

Figure 2. Neighbor-joining tree based on *Babesia* rDNA gene. Phylogenetic relationships among *Babesia canis vogeli* isolate detected in rural area (5, 10, 71) and sequences registered in GenBank based on partial nucleotide sequences of the 18S rRNA. The number at the node is the proportion of 50 bootstrap. GenBank accession numbers from the isolates were: *B. canis* from Okinawa - AY077719; *B. canis vogeli* from Brazil - AY371196; *B. canis vogeli* from USA - AY371198; *B. canis vogeli* from Egypt - AY371197.

Table 1. Ticks species found infesting dogs from rural areas of three counties of São Paulo State, Brazil.

Ticks	Botucatu	Rio Claro	Presidente Prudente
	n (%)	n (%)	n (%)
Rhipicephalus sanguineus	5 (29.4)^{A,a}	10 (76.9)^{A,a}	4 (80.0)^{A,a}
Amblyomma cajennense	7 (41.1)^{A,a}	2 (15.3)^{B,ab}	0^{B,b}
A. ovale	6 (35.2)^{A,a}	2 (15.3)^{B,a}	1 (20.0)^{AB,a}
Total	18 (100)	14 (100)	5 (100)

The lower case letters in the columns and the capital letters in the rows indicate statistically significant differences ($p < 0.05$) by the Goodman test.

Table 2. Correlation between *Babesia* spp. infection, diagnosed by PCR, and the presence of *Rhipicephalus sanguineus* on dogs in rural areas of São Paulo State, Brazil.

	***Babesia* spp. infection**		
Rhipicephalus sanguineus	**Positive**	**Negative**	**Total**
Infected	5^{A,a}	14^{B,b}	19
Not infected	7^{A,b}	124^{A,a}	131
	12 (100%)	138 (100%)	150

The lower case letters in the columns and the capital letters in the rows indicate statistically significant differences ($p < 0.05$) by the Goodman test.

In rural areas in Brazil, the low prevalence of the vector *R. sanguineus* may lead to the low prevalence of *B. c. vogeli* infection. Therefore, the dogs do not acquire immunity to *Babesia* infection and may be predisposed to show clinical signs if infected.

The primers Piro A1 and Piro B, which were described as being specific to *Babesia* spp, could also detect *Hepatozoon* species. PCR cross reaction between *Babesia* and *Hepatozoon* were also obtained by Oyamada et al. (2005) using the primers *Babesia F.* and *Babesia R.* Oyamada et al. (2005) discussed that these primers could be successfully used to detect *Babesia* and *Hepatozoon* simultaneously and this provided an easy screening method to detect both parasites in a single PCR. But specific primers for both species should be designed to evaluate with more accuracy each infection when both parasites are present.

It has been suggested that other PCR protocols could be tested to differentiate canine piroplasm species (MARTIN et al., 2006; JEFFERIES et al., 2007). The subspecies that was detected infecting the dogs of rural areas is *B. c. vogeli*, with 100% of identity with the *B. c. vogeli* already described in Brazil (PASSOS et al., 2005). As eight samples were not sequenced, we could not confirm that these samples were of *B. c. vogeli* or *B. gibsoni* since there is only a small difference between the amplification products of these two species. Trapp et al. (2006) described the presence of genotype Asia in dogs from Brazil. The studies on this subject must be expanded to amplify the knowledge on the canine piroplasm infection in Brazil.

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