Detection and Molecular Characterization of *Babesia canis vogeli* From Naturally Infected Brazilian Dogs

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KEY WORDS: *Babesia canis vogeli*, Brazil, RFLP, PCR, dogs

ABSTRACT

In order to detect and characterize the species and subspecies of *Babesia* spp. infecting dogs in Rio de Janeiro Area, Brazil, the polymerase chain reaction assay (PCR) with genus-specific primers for a portion of the babesial 18S rRNA gene sequence and a restriction fragment length polymorphism analysis (RFLP) were performed. Forty-five samples obtained from dogs naturally infected with *Babesia* spp. were used for this purpose. The protozoan was diagnosed through peripheral blood smear evaluation from November 2002 to December 2004. All samples were PCR positive for babesial 18S rRNA in 2 different protocols. PCR products were digested by the restriction enzymes *Taq* I and *Hinf* I to differentiate the subspecies of *B canis*. Through RFLP, it was possible to assert that the samples studied were from *Babesia canis vogeli*. This is the first characterization of this subspecies in the Rio de Janeiro State, Brazil.

INTRODUCTION

Canine babesiosis is a tick-borne disease caused by the protozoa *Babesia gibsoni* and *Babesia canis*, which affects dogs world-

wide. The acute form is characterized by hemolytic anemia, fever, hemoglobinuria, and may be lethal mainly in puppies.1

Babesia spp. are morphologically classified as large (measuring 3.0-5.0 µm), or small (1.5-2.5 µm) piroplasms. The parasites may be differentiated through the observation of intraerythrocytic merozoite. *Babesia canis* is a large piroplasm and *B gibsoni* is a small piroplasm during blood smear evaluation. There are 3 different subspecies of *B canis*: *B canis canis*, *B canis rossi*, and *B canis vogeli*. These subspecies are morphologically identical but had different vectors and pathogenicity.[1,2]

Molecular methods, such as polymerase chain reaction (PCR), present a higher sensitivity and specificity than the peripheral blood smear evaluation to detect babesial infection in peripheral blood[3] and may differentiate species that cannot be morphologically distinguished by smear method.[4] Combination of PCR and genetic sequence analysis may increase information about subspecies or strains, presenting an advantage in epidemiological studies with molecular methods.[5]

Babesia canis isolates present differences in geographical distribution, immunological aspects, and vector specificity.[1,6] It was suggested that *B canis* might be subdivided into 3 subspecies: *B canis rossi*, transmitted by *Haemophysalis leachi* in South Africa and Asia, *B canis canis*, transmitted by *Dermacentor reticulatus* in Europe, and *B canis vogeli*, transmitted by *Riphicephalus sanguineus* in tropical and subtropical areas. Molecular diagnostic methods are essential to differentiate *B canis* subspecies because these parasites are morphologically indistinguishable.[1,6,7]

Molecular studies confirmed the existence of *B canis* subspecies. *Babesia canis* vogeli was detected and characterized in the United States,[8] Australia,[9] Japan,[5] Africa,[2,10,11] and Europe.[2,10,12] At least one also detected *B canis canis*, while *B canis rossi* was characterized in South Africa.[2,10,11] Recently, a new species of large *Babesia* was characterized in the United States presenting 91.2%-91.6% similarity with *B canis vogeli*, *B canis canis*, and *B canis rossi*.[13]

In Brazil, was reported the first molecular detection of *B canis vogeli* using a PCR assay with a genus-specific primer set and sequencing of its products obtained from 5 dog samples from Minas Gerais and São Paulo States.[14]

The aim of present study was to detect and characterize, by PCR and restriction fragment length polymorphism analysis (RFLP), the species and subspecies of *Babesia* spp. that cause canine babesiosis in Rio de Janeiro State, Brazil.

MATERIALS AND METHODS

Forty-five samples of EDTA-anticoagulated whole blood were obtained from 45 naturally infected dogs with *Babesia* spp. presented at private clinics in Rio de Janeiro, Brazil from November 2002 to December 2004. From each animal one blood sample was collected and sent to Prolab Diagnósticos for a microscopic examination of blood smears. Samples were aliquoted into 1.5 mL micro tubes and stored at -20ºC until DNA extraction.

DNA was extracted from 100 µL of EDTA-anticoagulated whole blood using the GFXTM Genomic Blood Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) according to manufacturer instructions. DNA samples were eluted in 100 µL of UltraPure™ DNase/RNase-free distilled water (Gibco/Invitrogen, Carlsbad, Calif, USA). The extracted DNA was storage at -20ºC until PCR assay.

The amplification of *Babesia* DNA through PCR was performed using a genus-specific set of primers, which amplifies a specific region of the 18S rRNA gene of *Babesia*. The forward primer PIRO A and the reverse primer PIRO B (Table 1) were used to amplify an approximately 400 bp region of the 18S rRNA gene.[10] Briefly, each reaction mixture (50 µL) contained 0.2 mM of each dNTP (Amersham Biosciences,

Piscataway, NJ, USA), 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM of Tris-HCl pH 9.0 (Amersham Biosciences, Piscataway, NJ, USA), 0.25 μM of each primer, 0.5 U of *Taq* DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA) and 5 μL of DNA template. PCR amplification was performed using a programmable thermal cycler (PTC-100 MJ Research TM, INC, Waltham, Mass, USA) with the following program: an initial denaturation at 94ºC for 5 minutes, 30 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, and extension at 72ºC for 1 minute followed by a final extension at 72°C for 5 minutes.[10]

Table 1: Oligonucleotide Sequences of Primers Used to Detect *Babesia* in This Study.

Primer name	Oligonucleotide Sequence (5'→3')
PIRO A1	AGG-GAG-CCT-GAG-AGA-CGG-CTA-CC
PIRO A	AAT-ACC-CAA-TCC-TGA-CAC-AGG-G
PIRO B	TTA-AAT-ACG-AAT-GCC-CCC-AAC

An additional protocol was used to compare the obtained results. As the first one, this set of primers amplify an approximately 450 bp region of the 18S rRNA gene of *Babesia* spp. Primers used were PIRO A1 and the same PIRO B.[9] Mix reaction of this protocol was similar to the first one, except of DNA volume added (2.0 μL). An initial denaturation at 95ºC for 15 minutes, annealing at 62ºC for 1 minute, and extension at 72ºC for 2 minutes followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 62ºC for 20 seconds, and extension at 72ºC for 30 seconds. A final extension step at 72°C for 5 minutes was also used.[9]

PCR results were evaluated by agarose gel (1%) electrophoresis stained with ethidium bromide.

For all amplifications, previously known positive and negative samples for *B canis vogeli* were used as controls. Both controls were tested by PCR assay and the product of the positive one was sequenced. To avoid DNA contamination, all steps, including extraction, PCR setup, amplification, and electrophoresis of the amplified products, was done in different rooms.

The *Hinf* I and *Taq* I restriction enzymes were used for the RFLP of the PCR products, distinguishing the subspecies of *B canis* according to the site of its digestion. For 1/5 of each amplified product, 10 U of *Hinf* I and 10 U of *Taq* I in its appropriated buffer were used, digesting for 3 hours at 37ºC and 65ºC, respectively. Digested products were evaluated by agarose gel (2%) electrophoresis stained with ethidium bromide.

RESULTS

PCR

All of the 45 previously positive samples for *Babesia* spp., found in blood smear evaluation, had shown amplification products compatible to the positive control in both PCR assays (Figure 1).

RFLP

PCR products from positives samples in both protocols were submitted to digestion with Taq I and Hinf I restriction enzymes for evaluating possible sites of cleavage in its genomic chain, being possible to distinguish 3 B canis subspecies. There was no site of cleavage when the Hinf I enzyme was used. However, 2 sites of digestion occurred in all samples with Taq I, originating 3 DNA fragments. One of these fragments was not visualized in agarose gel because of its low number of base pairs (Figures 2-4). Through this cleavage standard it was possible to assert that the studied samples were from B canis vogeli.

DISCUSSION

Forty-five dogs naturally infected with *Babesia* spp. identified during blood smear evaluation were used for molecular studies. Two PCR-RFLP assays with 2 distinct set of primers, which amplify a portion of the 18S rRNA gene from *Babesia* spp., were performed. The results obtained in both pro-

Figure 1: PCR amplification of *Babesia* rDNA from Brazilian dog samples. When the primers PIRO A/PIRO B were used, the products sizes were approximately 400 bp. Using the primer set PIRO A1/PIRO B, the amplified products presented 450 bp. (1) molecular weight marker = 100 bp; (2-4) Amplificated products from set primers PIRO A/PIRO B; (5) *B canis vogeli*-positive control; (6) Negative control; (7) Reaction control; (11-13) Amplificated products from set primers PIRO A1/PIRO B; (14) *B canis vogeli*-positive control; (15) Negative control; (16) Reaction control.

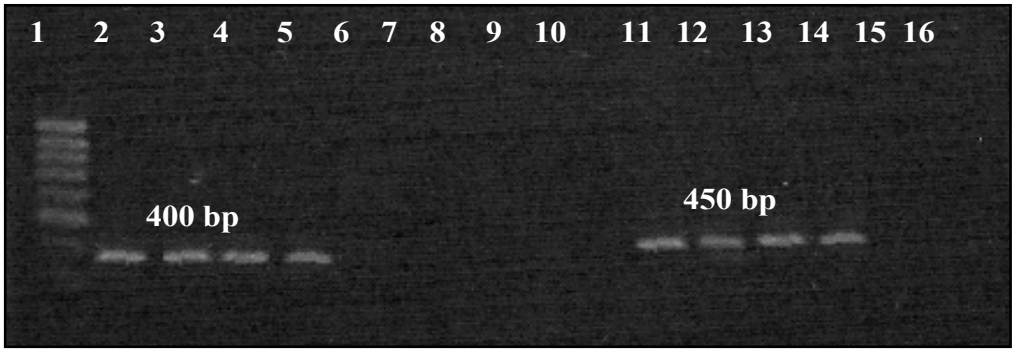

Figure 2: Nucleotide sequence of the PCR-amplified product of *B canis vogeli* between primers PIRO A and PIRO B. The *Taq* I digestion site are outstanding. Two sites of digestion occurs when use *Taq* I, originating 3 DNA fragments. From GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) AJ009796.

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1   AGG TAG TGA CAA GAA ATA ACA ATA CAG GGC TAA TGT CTT GTA ATT GGA ATG ATG GTG ACC
61  CAA ACC CTC ACC AGA GTA GCA ATT GGA GGG CAA GTC TGG TGC CAG CAG CCG CGG TAA TTC
121 CAG CTC CAA TAG CGT ATA TTA AAC TTG TTG CAG TTA AAA AGC TCG TAG TTG AAC TTT AGC
181 GTG TTC GAG TTT GCC ATT CGT TTG GCC TTT TCG AGT TCG CTT TTG GGT TTT CCC TTT TTA
241 CTT TGA GAA AAT TAG AGT GTT TCA AGC AGA CTT TTG TCT TGA ATA CTT CAG CAT GGA ATA
301 ATA GAG TAG GAC TTT GGT TCT ATT TTG TTG GTT ATT GAA CCT TAG TAA TGT TAA TAG GAA
361 CTG
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Figure 3: Nucleotide sequence of the PCR-amplified product of *B canis vogeli* between primers PIRO A1 and PIRO B. The *Taq* I digestion site are outstanding. Two sites of digestion occurs when use *Taq* I, originating 3 DNA fragments. From GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) AY102162.

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1   CAT CTA AGG AAG GCA GCA GGC GCG CAA ATT ACC CAA TCC TGA CAC AGG GAG GTA GTG ACA
61  AGA AAT AAC AAT ACA GGG CTA ATG TCT TGT AAT TGG AAT GAT GGT GAC CCA AAC CCT CAC
121 CAG AGT AGC AAT TGG AGG GCA AGT CTG GTG CCA GCA GCC GCG GTA ATT CCA GCT CCA ATA
181 GCG TAT ATT AAA CTT GTT GCA GTT AAA AAG CTC GTA GTT GAA TTT TAG CGT GTT CGA GTT
241 TGC CAT TCG TTT GGC TTT TTC GAG TTC GCT TTT GGG TTT TCC CTT TTT ACT TTG AGA AAA
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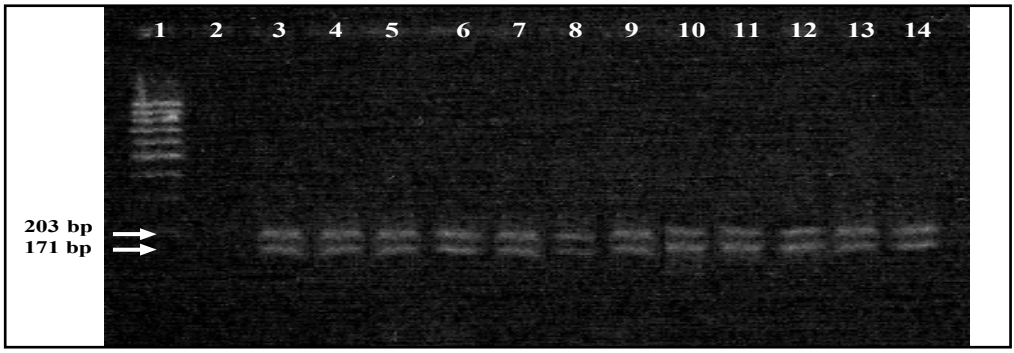
Figure 4: PCR-amplified products of *Babesia* rDNA from Brazilian dog samples digested with *Taq* I restriction enzyme. All samples incubated with *Taq* I originated 3 DNA fragments of 203, 171, and 26 bp. One of these fragments was not visualized in agarose gel electrophoresis, because of its low number of base pairs. (1) molecular weight marker = 100 bp; (2) Negative control; (3) *B canis vogeli*-positive control; (4-14) Samples of *B canis vogeli.*



tocols allowed us to state that all 45 samples were from *B canis vogeli*, in agreement with a previous report performed in Brazil with DNA sequences obtained from PCR products from 5 dogs of 4 different geographic areas. All sequences were characterized as *B canis vogeli*, demonstrating a 100% identity to each other.[14]

Results of this report confirm previous studies based in morphology, pathogenicity, and specific vector, demonstrating that in tropical and subtropical countries like Brazil, the subspecies of *B canis* is *B canis vogeli*, which is considered the lowest pathogenical strain transmitted by *R sanguineus*.[1] According to the same authors, *B canis canis* occurs in Europe and is transmitted by *D reticulatus*, presenting a variable pathogenicity, while *B canis rossi* is considered the most pathogenical, usually fatal in dogs even after treatment, being transmitted by *H leachi* and found in South Africa and Southeast Asia.[1] Molecular studies confirmed the existence of *B canis* subspecies worldwide. *Babesia canis canis* was detected and characterized in Europe,[2,4,10,12] while *B canis rossi* was characterized in South Africa.[2,10,11] DNA sequences of *Babesia canis vogeli* were found in Africa,[2,10,11] Australia,[9] Japan,[5] Europe,[2,10,12] the United States,[8] and Brazil.[14]

Previous studies based on serological methods and microscopic examination demonstrated that canine babesiosis is present in various regions of Brazil. In Belo Horizonte (Minas Gerais State), 127 canine samples were evaluated by indirect fluorescent antibody (IFA) and found 66.9% antibabesial antibodies presence.[15] Also using IFA were observed 42.4% positive dogs in São Paulo (SP).[16] In rural areas of Rio de Janeiro, 41.1% positive animals were found by IFA and 5.2% of these animals presented *B canis* in blood smears.[17] In Londrina, Paraná State, 37.7% of the tested dogs showed a positive serological test.[18] Furthermore, the prevalence of positive dogs through observation of *B canis merozoite* in blood smears in Juiz de Fora, Minas Gerais State was 26.92%.[19] Recently, in Jaboticabal, São Paulo State, 260 dog samples were evaluated by 2 serological tests, IFA and ELISA, showing a prevalence of 67.7% and 94.61%, respectively.[20]

This is the first molecular detection and characterization of *B canis vogeli* in Rio de Janeiro State with a practical, rapid, sensitive, and specific method. In a few hours it is possible to perform the molecular diagnostic by PCR of many samples and differentiate *B canis* subspecies causing infection. The advantage of using genus-specific primers is that various species, including new genotypes, may be detected.

In Brazil, more studies addressing to detection and characterization of *B canis* should be conducted in urban and rural areas in order to better understand the real distribution of this protozoan in the country.

ACKNOWLEDGMENTS

We would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support for this study and the financial support for Dr Alexandre Sá's personal research fellowship. We also would like to express thanks to the Prolab Diagnósticos for sharing positive samples and Dr. Rosângela Zacarias, DVM, MSc, DSc, from Universidade Estadual Paulista - Jaboticabal, for kindly providing positive and negative controls for *Babesia canis vogeli*. This study complies with current laws of Brazil.

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