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

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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 blood3 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 *Rhipicephalus 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 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 -20°C 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 stored at -20°C 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 MgCl₂, 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 72ºC for 5 minutes.¹⁰

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 72ºC 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 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* vogelli-positive control; (6) Negative control; (7) Reaction control; (11-13) Amplificated products from set primers PIRO A1/PIRO B; (14) *B. canis* vogelli-positive control; (15) Negative control; (16) Reaction control.

Figure 2: Nucleotide sequence of the PCR-amplified product of *B canis* vogelli 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 CAG GCC TAA TGT CTT GTA ATT GGA ATG ATG GTG ACC
61     CAA ACC CTC ACC AGA GTA GCA ATT GGA GGG CAA GTC 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 TAC
181   GTG TGG TTT GCC ATT GGT TGG TAC TTT TGC AGT CTT GTG ATT TTA TCC TTT TAA
241   CTG GAA AAT TAG AGT GTG TCA AGC AGA CTT TTG TCT TGA ATC CTT CAG CAT GGA ATA
301   ATA GAG TAG GAC TTT GTG ATT TTG TTG GCC AGT TAG TAA TGT TAA TAG GAA
361   CTG
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Figure 3: Nucleotide sequence of the PCR-amplified product of *B canis* vogelli 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 GCA ATA GCA
61     AGA AAT AAT ACA GGG CTA ATG TCT TGT TAA ATG GAT GGT GGT CTA TTT TCA CTA CTC CAC
121   CAG AGT AGC AAT TGG AGG GCA AGT CTG ATT CCA GTA GCA GCC CGG GCA GTG ATT CCA GCT CCA ATA
181   GGG TAT ATT AAA CTT GGT GCA GGT AAA AAG CTC GTA GGT GAA TTT TAG CGT AAG CTT
241   TGT CAG CTT TGG GCC GTC TTC TGG TTT GCC TTT TTT TTT TTT TTT TTA
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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* vogelli-positive control; (4-14) Samples of *B canis* vogelli.

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1       2     3      4      5       6      7      8      9     10    11    12    13    14
203 bp
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15     16
171 bp
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protocols 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.16

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 pathogical, 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% anti-babesial 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.

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