Molecular characterization of *Babesia vogeli* in dogs from Belém, northern Brazil

P.H.G. Moraes¹, C.P. Rufino¹, A.R.F. Baraúna¹, T. Reis¹, L.T.D. Agnoli¹, A.M.C. Meneses², D.C.F. Aguiar¹, M.R.T. Nunes³ and E.C. Gonçalves¹³

¹Laboratório de Tecnologia Biomolecular, Instituto de Ciências Biológicas, Universidade Federal do Pará, Cidade Universitária, Belém, PA, Brasil
²Instituto da Saúde e Produção Animal, Universidade Federal Rural da Amazônia, Montese, Belém, PA, Brasil
³Centro de Inovações Tecnológicas, Instituto Evandro Chagas, Levilândia, Ananindeua, PA, Brasil

Corresponding author: P.H.G. Moraes
E-mail: pablogmoraes@gmail.com

Received August 9, 2015
Accepted October 23, 2015
Published December 9, 2015
DOI http://dx.doi.org/10.4238/2015.December.9.4

ABSTRACT. Babesiosis is an infectious hemolytic disease that occurs worldwide, and is caused by a protozoan of the *Babesia* genus (Apicomplexa). Little is known about this genus; therefore, this study conducted a molecular characterization of *Babesia* spp in naturally infected dogs in northern Brazil. Blood samples were collected from 172 dogs from metropolitan Belém, and screened for *Babesia* spp using semi-nested polymerase chain reactions. Subsequently, 18S rDNA gene fragments were amplified and sequenced. Alignments of the 27 nucleotide sequences returned fragments measuring 1023 bp in size, which did not include any polymorphic sites (100% identity). This genotype was very similar to the 18S rDNA gene in *B. vogeli*. This study provides an important molecular characterization of the parasite responsible for canine babesiosis in naturally infected dogs in metropolitan Belém. In addition, the 18S rDNA gene in *B. vogeli* may represent the most common genotype occurring in South America.

Key words: *Babesia vogeli*; Canine babesiosis; Molecular characterization; Northern Brazil
INTRODUCTION

Babesiosis, or piroplasmosis, is an infectious hemolytic disease that occurs worldwide, and is caused by a protozoan in the order Piroplasmida (Apicomplexa), which utilizes a variety of tick species as natural vectors (Lobetti, 1998; Boozer and Macintire, 2003; Passos et al., 2005). In addition to causing significant economic losses in the livestock industry, babesiosis causes clinical manifestations in companion animals and humans (Boozer and Macintire, 2003; Adaszek and Winiarczyk, 2008; Irwin, 2010).

Due to the wide range of vertebrate hosts susceptible to infection by tick-transmitted apicomplexan parasites, babesiosis is a disease of great economic, veterinary, and clinical importance, and a major research effort has been exerted in understanding its mechanisms of pathogenicity and drug resistance, and to find a vaccine (Schnittger et al., 2012).

Four protozoan species, *Babesia canis*, *Babesia vogeli*, *Babesia rossi*, and *Babesia gibsoni*, are known to cause canine babesiosis worldwide. The first three species are recognized by their vector (tick) species, virulence, and geographic distributions. *B. canis* is transmitted by *Dermacentor reticulatus*, a tick species found in Europe (Boozer and Macintire, 2003), while the vector of *B. vogeli* is the red tick, *Rhipicephalus sanguineus*, which is found in the United States and in tropical and subtropical regions around the world, including Brazil (Passos et al., 2005). *B. rossi* is the most virulent and is transmitted by *Haemaphysalis* spp, a tick found in Africa (Lobetti, 1998). *B. gibsoni* is transmitted by *Haemaphysalis longicornis* and *R. sanguineus* (Uilenberg, 2006).

In Brazil, canine babesiosis is typically caused by *B. vogeli*, which is known to occur in the south (Trapp et al., 2006; Jojima et al., 2008; Vieira et al., 2013), southeast (Dell-Porto et al., 1990, 1993; Spiewak, 1992; O’Dwyer, 2000; Costa-Junior et al., 2009; Lemos et al., 2012), mid-west (Souza et al., 2004; Duarte et al., 2011; Spolidorio et al., 2011), north-east (Carlos et al., 2006; Lima et al., 2006; Ramos et al., 2010; Silva et al., 2012), and north (Spolidorio et al., 2013; Moraes et al., 2014). In addition, there have been some reports of *B. gibsoni* from the south of the country (Trapp et al., 2006; Jojima et al., 2008).

Regarding canine piroplasms occurring in northern Brazil, their molecular characterization is limited to that conducted by Moraes et al. (2014), who reported a short fragment (394 bp) of the 18S rDNA gene from *B. vogeli* in one dog from the Belém metropolitan area, Pará State. Molecular methods have been used for the description of genotypically novel pathogens by their small subunit rRNA (ssrRNA) gene sequences, which have been applied for the comparison of geographically different strains. Therefore, the present study was conducted to characterize *Babesia* spp. in naturally infected dogs in Belém.

MATERIAL AND METHODS

Samples and DNA extraction

A total of 172 samples were randomly selected from dogs with clinical signs of hemoparasites infection that were treated at the Veterinary Hospital of the Universidade Federal Rural da Amazônia (HOVET-UFRJA), Belém, Pará State between August and October 2011. The blood samples were drawn into tubes containing ethylenediaminetetraacetic acid (EDTA).

Total DNA of each sample was extracted from a 300-μL aliquot of the blood by using a
standard phenol-chloroform procedure, as described by Sambrook et al. (1989). DNA quality was checked by electrophoresis on an agarose gel, and the DNA was then quantified using a Qubit® fluorometer (Invitrogen).

**Polymerase chain reaction (PCR) amplification and DNA sequencing**

The molecular detection of *Babesia* spp was performed in a semi-nested PCR (snPCR), as described by Moraes et al. (2014). Briefly, a primer set targeting a partial sequence (394 bp) of the 18S rDNA gene was used in an initial reaction. A second primer set was then used to amplify an internal fragment (208 bp). The PCRs included a positive control, which was the DNA of a peripheral blood sample from one dog (also treated at HOVET-UFRA) that exhibited large intraerythrocytic piroplasms that were morphologically compatible with *Babesia vogeli*, which was examined by light microscopy after panoptic staining. Sterile, twice-distilled water was used as a negative control.

Positive samples (according to the snPCR) were characterized based on partial small subunit rDNA (ssrDNA) sequence analysis. In addition, two overlapping fragments were amplified using the following primer sets: i) *Babf-fiBabesia* common (Kordick et al., 1999; Martin et al., 2006), which amplified a 394-bp fragment at the 5' end; and ii) RIB-24/RIB-21 (Zahler et al., 2000), which amplified an 834-bp fragment at the 3' end.

All of the PCR products were visualized after electrophoresis on 1.5% agarose gel in a Tris acetate-EDTA (TAE) buffer, using GelRed™ Nucleic Acid stain (Biotium) and an ultraviolet transilluminator (E-BOX VX2, Vilber Lourmat). A 100-bp molecular marker (Invitrogen™ DNA ladder, Life Technologies) was used to estimate the size of each amplified fragment.

The amplicons obtained were purified using a GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and ligated to a pGEM®-T vector plasmid (Promega), which were then inserted into TOP 10 *Escherichia coli* (Invitrogen™, Life Technologies). Cloned fragments were obtained by PCR from the recombinant clones of the colonies using the M13F/M13R primers, and were automatically sequenced in a 3500xL Genetic Analyzer (Applied Biosystems), following the manufacturer protocol.

**Sequence analysis**

BioEdit software (Hall, 1999) was used to assemble the forward and reverse sequences obtained. In order to investigate the occurrence of different genotypes, we generated a database that included, in comparison to the sequences we obtained, all similar sequences of *Babesia* spp. from Brazilian dogs that are currently available in GenBank (accession no.: AY371194-AY371196, DQ469736-DQ469737, EF052623-EF052633, EF636702, EU436750, EU436752, GQ214514, GU386266-GU386282, JF295087, JN36808, KC688693, KC989959, KF753247, and KF970929). We also included sequences of *B. vogeli* strains from Venezuela (DQ297390), USA (AY371198), China (HM590440), Japan (AB083374), Spain (DQ439545), France (AY072925), Taiwan (HQ148663), Romania (HQ662635), and Egypt (AY371197).

**Phylogenetic analysis**

We performed a phylogenetic analysis using the neighbor-joining method in MEGA6 software, using the Kimura-2 parameter (Tamura et al., 2013). Bootstrap consensus was inferred.
from 1000 replicates. The 18S rDNA gene sequence of *Plasmodium falciparum* (GenBank accession No. M19172) was used as an outgroup. MUSCLE software (Edgar, 2004) was used to align and trim the sequences, in order to obtain equal lengths.

**RESULTS**

**Molecular detection of *Babesia* spp.**

The specific 18S rDNA gene fragment of *Babesia* spp. was detected in approximately 15.7% (27/172) of the canine blood samples we analyzed. Alignments of the nucleotide sequences returned 1023-bp fragments (excluding primers), which did not exhibit any polymorphic sites, i.e., they exhibited 100% identity similarity to each other. A BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of this unique sequence (GenBank accession number KT333456) revealed a high similarity to the 18S rDNA gene in *B. vogeli*.

Although the database we generated included sequences ranging from 235 to 1608 bp long (data not shown), a comparison of 75 sequences (27 from this study and 48 retrieved from GenBank) allowed us to generate a consensus sequence that was highly similar to the 18S rDNA gene in *B. vogeli* from elsewhere (Table 1). No polymorphic sites were observed among the 1023 bp of the 18S rDNA gene of *B. vogeli* from Belém and Minas Gerais in Brazil, or from Venezuela, China, or Japan.

**Table 1. Comparison of *Babesia vogeli* (Belém) 18S rDNA sequence with other *B. vogeli* strains.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>GenBank accession No.</th>
<th>Identity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nucleotide position&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 3 4 5 5 5 7 7 8 8 8 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 7 8 0 1 5 8 3 5 7 2 2 3 7 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 7 0 7 8 1 8 0 9 7 4 6 7 1</td>
</tr>
<tr>
<td>Belém</td>
<td>Present study</td>
<td>-</td>
<td>-</td>
<td>T A T A T T T T T A A A C T T</td>
</tr>
<tr>
<td>Brazil</td>
<td>Passos et al. (2005)</td>
<td>QY71196</td>
<td>100</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>Venezuela</td>
<td>Rey-Valeríon et al. (2007)</td>
<td>DQ297390</td>
<td>100</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>Passos et al. (2005)</td>
<td>AY371198</td>
<td>99.9</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>China</td>
<td>Unpublished</td>
<td>HM900440</td>
<td>100</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>Japan</td>
<td>Ari et al. (1998)</td>
<td>AB083374</td>
<td>100</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>Spain</td>
<td>Criado-Moreno et al. (2003)</td>
<td>DQ439545</td>
<td>99.4</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>France</td>
<td>Caccio et al. (2002)</td>
<td>AY072825</td>
<td>99.9</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>Taiwan</td>
<td>Unpublished</td>
<td>HQ148663</td>
<td>99.4</td>
<td>C G C . . . . . . . . . . . .</td>
</tr>
<tr>
<td>Romania</td>
<td>Unpublished</td>
<td>HQ662635</td>
<td>99.8</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>Egypt</td>
<td>Passos et al. (2005)</td>
<td>AY371197</td>
<td>99.8</td>
<td>. . . . . . . . . . . . . .</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are percentages of nucleotide sequence identities for 1023 bp, determined by pairwise alignment. <sup>b</sup>Dots (.) and letters indicate conserved nucleotides and substitutions, respectively.

**Phylogenetic analysis**

All of the sequences were trimmed to the same length (1023 bp), and the results confirmed the close phylogenetic relationships between worldwide *B. vogeli*, which clustered in a branch that was closely related to *B. canis* (Figure 1). A close relationship between *B. vogeli* strains from Venezuela, China, and Japan, in comparison to the Brazilian *B. vogeli* strain, was also found.
Figure 1. Phylogenetic tree based on Babesia 18S rDNA. Sequences were compared using the neighbor-joining method with a distance matrix calculation with Kimura-2 parameters in MEGA6 software, using Plasmodium falciparum as the outgroup. The scale bar indicates the number of mutations per sequence position. The numbers at the nodes represent the percentages of 1000 bootstrap re-samplings.

DISCUSSION

The molecular method we used allowed for the detection of four protozoan species (B. canis, B. vogeli, B. rossi, and B. gibsoni), which are known to cause canine babesiosis worldwide, and revealed the presence of B. vogeli in dogs from Belém.

Despite the fact that several studies have been conducted on Babesia spp., those by Spolidorio et al. (2013) and Moraes et al. (2014) appear to be the only published reports of the occurrence of the genus in northern Brazil. Spolidorio et al. (2013) conducted a serosurvey and an indirect immunofluorescence assay with the antigens B. vogeli, Ehrlichia canis, and six Rickettsia species, while Moraes et al. (2014) used the same molecular method as used in the present study. Whereas Spolidorio et al. (2013) detected anti-B. vogeli antibodies in 59.6% of urban dogs in Santarém, Pará State and in 29.1% of rural dogs, Moraes et al. (2014) reported that only 22% (22/100) of dogs tested positive for Babesia spp. DNA. The results of these two studies and of the present report suggest that B. vogeli is the most common, if not the only, species of Babesia in dogs east of the Amazon in northern Brazil.

Molecular phylogeny has made major contributions towards revealing evolutionary lineages and their relationships (Schnittger et al., 2012). The present study is, to the best of our knowledge, the first time that the molecular analysis of a large fragment of 18S rDNA gene sequence of B. vogeli from Belém has been conducted. Our results, i.e., the sequence alignments and phylogenetic tree, confirm those of previous studies (Passos et al., 2005; Duarte et al., 2011), which suggest, based on the 18S rDNA sequences, that there is low genetic diversity among B. vogeli strains in Brazil.

Indeed, our analysis returned only one 18S rDNA genotype of B. vogeli occurring in Belém, which was identical to those from Minas Gerais, Brazil (Passos et al., 2005) and Venezuela (Rey-
Valeiron et al., 2007). It is noteworthy that the genotype obtained here was the fourth-largest 18S rRNA gene fragment of B. vogeli ever obtained in Brazil.

Duarte et al. (2011), based on a short B. vogeli 18S rDNA gene fragment from Goiânia, Goiás (mid-west Brazil), identified two genotypes: GO 1 and GO 4, which differed by one substitution (A to G) at position 128 of their alignment. GO 1 was 100% identical to three sequences from Minas Gerais, Brazil, as well as to the sequences from Belém obtained in the present study.

Most Brazilian studies that have characterized the 18S rDNA gene sequences of B. vogeli have been based on the direct sequencing of enzymatically amplified DNA, which has a higher error rate than sequencing based on molecular cloning (Clark and Whittman, 1992). Further evidence of sequencing errors in the database we generated is that most nucleotide differences (indels and/or substitutions) were located at the ends of the sequences. In addition, some nucleotide sequences were not curated before submission to GenBank. For example, in the sequence KC989959 (not published), the first 183 of the 584 nucleotides did not clearly align with any other 18S rDNA gene sequences of Babesia. Therefore, we cannot conclusively state that only one B. vogeli strain is in Brazil. However, based on the genetic similarities that we observed, we suggest that the B. vogeli 18S rDNA genotype detected in dogs from Belém may represent the most common one on the South American continent.

Based on partial ssrRNA sequences, Carret et al. (1999) questioned the phylogenetic position of B. vogeli; however, the findings of the present study are similar to those obtained by Passos et al. (2005), who reported 100% homology between B. vogeli from Brazil, Venezuela, Japan, and China. B. vogeli is transmitted by the red (brown) dog tick (R. sanguineus), which is found in tropical and subtropical regions (Irwin, 2010). Genetic similarities between the B. vogeli described here and isolates from tropical (Venezuela) and subtropical (Japan) regions suggest that the development, establishment, and permanence of the same vectors and pathogens was possible, because there are similar climatic conditions between these countries and northern Brazil.

Based on vector specificity, pathogenicity, and morphology, it has been assumed that B. vogeli causes almost all cases of babesiosis in Brazil (Passos et al., 2005), and only recently have a few cases of infection by B. gibsoni been detected in dogs from southeast Brazil (Trapp et al., 2006). However, there have been no studies conducted on the characterization of Babesia species in dogs in northern Brazil. Therefore, this study provides an important molecular characterization of the protozoan parasite responsible for canine babesiosis in naturally infected dogs in northern Brazil.

The high genetic homology demonstrated here between B. vogeli isolates, even on different continents (ranging from 99.4 to 100%), corroborates the results of Passos et al. (2005), who found that these protozoa belong to the same taxonomic entity. The question of whether small sequence variations between B. vogeli isolates affect the virulence of the parasite remains unanswered, and requires further study.

**Conflicts of interest**

The authors declare no conflict of interest.

**ACKNOWLEDGMENTS**

We are grateful to FAPESPA (#ICAAF 012/2012) for financial support, and to CAPES, CNPQ, and PROEX/UFPA for scholarships to P. Moraes, C.P. Rufino, A.R. Baraúna, L.D. Agnol, and T. Reis.
REFERENCES


Phylogeny of Babesia vogeli in northern Brazil


