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Short communication

First molecular detection of *Babesia vogeli* in dogs from Brazil

Lygia Maria Friche Passos[a,*], Stefan M. Geiger[b], Múcio Flávio Barbosa Ribeiro[c], Kurt Pfister[b], Monika Zahler-Rinder[b,d]

[a]*Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), C.P. 567, Belo Horizonte 30123-970, Minas Gerais, Brazil*
[b]*Institute for Comparative Tropical Medicine and Parasitology, Ludwig-Maximilians-University Munich, Leopoldstr. 5, D-80802 Munich, Germany*
[c]*Departamento de Parasitologia-ICB, Universidade Federal de Minas Gerais (UFMG), C.P. 486, Belo Horizonte 31270-901, Minas Gerais, Brazil*
[d]*National Research Center for Protozoal Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro shi, Inada-cho, Hokkaido 080-8555, Japan*

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Abstract

The present work describes the detection and first molecular characterization of *Babesia vogeli* in dogs, naturally infected in Brazil and even in South America. Microscopic examination of Giemsa-stained peripheral blood smears collected from dogs originating from four different locations in Brazil revealed the presence of large *Babesia* merozoites and trophozoites (>2.5 μm). DNA was extracted from infected blood samples and PCR amplifications of the 18S rDNA were carried out. As a reference, DNA from an isolate of *B. vogeli* originated from Egypt was used. PCR products were purified and sequenced. The DNA sequences demonstrated 100% identity among the Brazilian isolates. Comparisons with the 18S rDNA sequence of the *B. vogeli* isolate from Egypt and with other *B. vogeli* sequences from Spain, France, Japan, Australia and South Africa confirmed the affiliation of all Brazilian isolates to the species *B. vogeli*.
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1. Introduction

Canine Babesiosis is a tick-borne disease of domestic and wild canidae characterized by fever, depression and anaemia. In dogs, large and small babesias were described, which generally were attributed to two species, *Babesia canis* and *Babesia gibsoni*, respectively. Usually, the diagnosis is made upon size and morphological appearance of the intra-erythrocytic forms in peripheral blood smears. However, taxonomy of canine *Babesia* species is under great discussion and the question arises whether

* Corresponding author. Tel.: +55 31 3499 2092; fax: +55 31 3499 2080.
E-mail address: lygia@dedalus.lcc.ufmg.br (L.M.F. Passos).

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morphological features are sufficient for differentiation of these pathogens. Previous studies have suggested that large Babesias should be divided into three subspecies: *Babesia canis canis*, *Babesia canis rossi*, and *Babesia canis vogeli*, depending on vector specificity, pathogenicity and antigenic properties (Uilenberg et al., 1989; Hauschild et al., 1995). The existence of three taxonomic entities was confirmed recently with molecular biological methods (Zahler et al., 1998; Carret et al., 1999; Cacciò et al., 2002). Since, at least for *B. canis canis* and *B. canis rossi*, differences in main pathogenicity mechanisms have also been detected (Schetters et al., 1997), the differentiation of species is not only of mere academic interest but has also implications for vaccination and therapy. However, the concept of three subspecies based on the characterization of isolates from distinct geographic regions is still unclear. Besides, molecular biological techniques and comparisons of the 18S rDNA of isolates from different areas of the world have shown that small Babesia, generally attributed to *B. gibsoni*, separated in genetically distantly related species (Zahler et al., 2000a; Kjemtrup et al., 2000). Furthermore, a small *Babesia microti*-like piroplasm has been described most recently in north-west Spain (Zahler et al., 2000b; Camacho et al., 2001), which has initiated new considerations and discussions about distribution and epidemiology of Babesioses in dogs. Since the discussion about classification of canine *Babesia* species is not the aim of the present paper, the classical nomenclature (*B. canis*, *Babesia vogeli* and *Babesia rossi*) will be used as suggested by Schetters et al. (1997), Zahler et al. (1998), Carret et al. (1999) and Depoix et al. (2002).

In Brazil, canine Babesiosis is commonly distributed as reported in a previous study when anti-*B. canis* (sensu lato)-antibodies were detected in 66.9% of dogs examined at a veterinary hospital in Minas Gerais state (Ribeiro et al., 1990). *Rhipicephalus sanguineus* Latreille, 1806 ticks have been considered the vector of canine Babesiosis. Because *R. sanguineus* is a three-host tick, very well adapted to urban areas, this would support the fact that the disease assumes an endemic character.

Although the importance of canine Babesiosis has increased over the last years in the state of Minas Gerais (Bastos et al., 2004), few epidemiological studies have been carried out in the country and little is known about its epidemiological aspects in Brazil. Characterization of *Babesia* isolates on a molecular basis is completely lacking. Therefore, the aim of the present study was to characterize Brazilian isolates of canine *Babesia* species with molecular biological methods in order to fit them into a taxonomic system, together with *Babesia* isolates from other geographic regions.

2. Materials and methods

Five canine samples were obtained in four separate geographic locations in Brazil: four dogs from the state of Minas Gerais (two in Belo Horizonte, one in Lavras and one in Uberlândia) and finally one dog from the state of São Paulo. Peripheral blood smears were made from dogs naturally infested with *R. sanguineus* showing clinical manifestations suspicious of Babesiosis. Giemsa-stained smears were examined under the light microscope for direct detection of intra-erythrocytic stages. Blood samples from infected dogs were collected in EDTA tubes and the DNA was extracted using a commercial kit (Wizard Genomic DNA Purification, Promega, Madison, USA).

For comparison, blood from a dog naturally infected with a large *Babesia* in the United States of America was included as well as an isolate of *B. vogeli* originated from Egypt (Egypt 1), which had served as a reference isolate for the description of the species (Uilenberg et al., 1989). From these two samples DNA was extracted from 200-μl aliquots of EDTA blood using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR amplifications of the 18S rDNA were carried out according to Zahler et al. (2000a), with the PCR primers RIB-19 (5′-CGG GAT CCA ACC TGG TTG ATC CTG C-3′) and RIB-20 (5′-CCG AAT TCC TTG TTA CGA CTT CTC-3′), which are located at the 5′ and 3′ end of the small subunit rRNA gene, respectively. Using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), PCR products of a size of 1.6 kb were purified and sequenced with the primers RIB-19, RIB-20, RIB-21 (5′-TTT CCC CGT GTT GAG TC-3′) and RIB-24 (5′-GAC GGT AGG GTA TTG GC-3′), (Zahler et al., 2000a). The sequences are available in GenBank

under the accession numbers AY371194 (Lavras), AY371195 (Belo Horizonte), AY371196 (Uberlândia), AY371197 (Egypt 1), and AY371198 (USA). Sequence alignments were performed using CLUSTAL W of the DNAMAN software package (Lynnon Bio Soft, Quebec, Canada). For comparison, *B. canis* sequences from France (GenBank accession number AY072925), Spain (AY150061), Egypt 2 (AJ009796), Japan (AB083374), Australia (AY102163), and South Africa (AF548006) were included in the analyses.

3. Results and discussion

Typical large merozoites and trophozoites (size > 2.5 μm) were detected in blood smears from all infected dogs. The DNA sequences obtained from the PCR products of Babesia isolated from dogs of Belo Horizonte (sequence length of 1542 bp), Lavras (1608 bp), Uberlândia (1599 bp) and São Paulo (660 bp) demonstrated a 100% identity to each other. In comparison with the 18S rDNA sequence of other *Babesia* species, these sequences were completely identical to a *B. vogeli* sequence from Japan (GenBank accession number AB083374). Compared with the *Babesia* isolate from Egypt, which had served as a reference isolate for the description of the taxon *vogeli* (Uilenberg et al., 1989), only two substitutions were found (Table 1). These results indicate a very close genetic relation and the affiliation of the canine *Babesia* from Brazil to the species *B. vogeli*. When the sequences were compared with the sequence of the canine *Babesia* from USA and with other *B. vogeli* sequences deposited in GenBank, 13 polymorphic positions and pair wise identities of at least 99.4% were found (Table 1). Interestingly, in *B. microti*, very similar identities of atleast 99.2% were found between the 18S rDNA of parasites from Japan, Germany and USA (Zahler et al., 2000c).

Three subspecies of *B. canis* with differing vector specificities have been proposed: *B. canis* canis, transmitted by *Dermacentor reticulatus*, *B. canis vogeli*, transmitted by *R. sanguineus* and *B. canis rossi*, transmitted by *Hemaphysalis leachi* (Uilenberg et al., 1989). Although these subspecies are morphologically indistinguishable, they can be differentiated by molecular biological methods (Zahler et al., 1998; Carret et al., 1999). Parasites from Europe have been classified as *B. canis canis* (Zahler et al., 1998; Carret et al., 1999; Cacciò et al., 2002; Criado-Fornelio et al., 2003). *B. canis rossi* was detected in South Africa (Zahler et al., 1998; Carret et al., 1999) while *B. canis vogeli* was found in Europe (Zahler et al., 1998; Carret et al., 1999; Cacciò et al., 2002; Criado-Fornelio et al., 2003), Northern and Southern Africa (Zahler et al., 1998; Carret et al., 1999; Matjila et al., 2004), Asia (Inokuma et al., 2003), Australia (Jefferies et al., 2003) and Northern America (Birkenheuer et al., 2003). In the past, these subspecies have even been assigned separate species status (Zahler et al., 1998), a view that is adopted here. However, classification of canine *Babesia* species occurring in South America was still unknown.

On the basis of presumed vector specificity, pathogenicity and morphological aspects, it was believed that *Babesia vogeli* was the agent involved

Table 1
Comparison of *Babesia vogeli* 18S rDNA sequences from Brazil with other *B. vogeli* sequences base numbering of polymorphic positions is according to the genotype from Lavras (Brazil) with a deletion included at position 432

Genotype (GenBank accession number)	Polymorphic position												
	432	459	556	570	603	640	746	759	811	829	878	929	1387
Brazil	–	A	A	T	T	T	G	G	A	A	C	T	T
Japan (AB083374)	·	·	·	·	·	·	·	·	·	·	·	·	·
Australia (AY102163)	·	·	·	·	·	·	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
USA (AY371198)	·	C	·	·	·	·	·	·	·	·	·	·	C
France (AY072925)	·	C	·	·	·	·	·	·	·	·	·	·	·
Egypt 1 (AY371197)	·	·	·	C	C	·	·	·	·	·	·	·	·
Spain (AY150061)	·	C	·	·	·	A	·	·	G	G	A	A	n.d.
Egypt 2 (AJ009796)	·	·	·	·	·	·	·	T	n.d.	n.d.	n.d.	n.d.	n.d.
RSA (AF548006)	G	·	G	·	·	·	·	·	·	·	·	·	n.d.

(–), Deletion; (·), same base as in genotype Brazil; and n.d., not determined.

in cases of canine Babesiosis in Brazil; however, characterisation of *Babesia* isolates on a molecular basis were completely lacking. The present study is the first report in which this species of *Babesia* has been confirmed and characterised by molecular methods in dogs naturally infected in the states of Minas Gerais and São Paulo and even in South America. The high genetic homology demonstrated here between *B. vogeli* parasites originating from Brazil, Japan, and Egypt, and thus even from different continents, supports the validity of the concept that these protozoans with the same proven or presumed vector specificity belong to a taxonomic entity.

Previous studies using serological methods in Minas Gerais have demonstrated high prevalence of infection in dogs (Ribeiro et al., 1990; Spiewak, 1992). A retrospective study, from March 1998 to September 2001, carried out at the Veterinary Hospital of the Federal University of Minas Gerais (UFMG), Belo Horizonte, showed that from a total of 145 dogs with suspicious cases of hemoparasites, 61 (42%) dogs were infected with *Babesia spp* by direct examination of blood smears (Bastos et al., 2004).

Canine Babesiosis is prevalent in other regions of Brazil, as demonstrated in a recent microscopic examination of 250 dogs from Rio de Janeiro state, where a prevalence of 5.2% was reported (O'Dwyer et al., 2001). Considering that *Babesia* parasites in blood smears are usually visualized only during the acute phase and that percentage of infected cells is commonly low, an absence of parasites in blood smears does not exclude the possibility of infection.

The brown dog tick *R. sanguineus* is highly prevalent in urban areas where canine Babesiosis is endemic. The climatic conditions in tropical regions, such as those in the state of Minas Gerais, favour the development of this tick species, allowing up to four generations per year (Ligia M. Borges, personal communication, 2002). The large population of dogs available guarantees the development of all stages of *R. sanguineus* ticks. However, in rural areas, where *R. sanguineus* is less common, further epidemiological studies are necessary for a better understanding of transmission of canine Babesiosis. The potential occurrence of different species or subspecies transmitted by other tick species remains to be clarified.

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