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Short communication

Use of a Real Time PCR for detecting subspecies of *Babesia canis*

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ABSTRACT

This paper reports the development and use of a Real Time PCR for detection of *Babesia canis canis*, *B. canis rossi*, and *B. canis vogeli* in endemic areas of Brazil. The sequences of the internal transcribed spacer (ITS) of several organisms were aligned and five primers and four probes were designed for amplification of a fragment (around 125 bp) which differentiates subspecies of *B. canis*. Blood samples collected from dogs living in farms in three distinct rural regions within the State of Minas Gerais (Lavras, Belo Horizonte and Nanuque) were tested. Blood samples had been collected during a dry season (Lavras, $n = 100$; Belo Horizonte, $n = 50$; Nanuque, $n = 102$); the dogs were re-sampled in the subsequent rainy season (Lavras, $n = 71$; Belo Horizonte, $n = 29$; Nanuque, $n = 66$). From each sample, DNA was extracted and Giemsa stained smears were microscopically examined for direct detection of *Babesia* parasites. *B. canis vogeli* was the only subspecies found, with an overall prevalence of 9.9% during the dry season and 10.8% during the rainy season. Dogs living in Nanuque and Belo Horizonte showed significantly higher prevalence rates than those living in Lavras (13.7%, 12.0% and 5.0%, respectively). The Real Time PCR developed proved to be appropriate to detect *B. canis* subspecies in endemic areas.

1. Introduction

Babesia species are tick-transmitted apicomplexa parasites that infect a wide range of vertebrate hosts and cause severe diseases in wild and domestic animals (Kuttler, 1988). *Babesia canis* and *Babesia gibsoni* are recognized as the two species that cause canine babesiosis, a clinically significant hemolytic disease of dogs (Yamane et al., 1993; Lobetti, 1998).

Three subspecies of *B. canis* have been proposed (Uilenberg et al., 1989): *B. canis rossi*, transmitted by the tick *Haemaphysalis leachi* in South Africa and causing a usually fatal infection in domestic dogs even after treatment; *B. canis canis*, transmitted by *Dermacentor reticulatus* in Europe and showing a more variable pathogenicity; and *B. canis vogeli*, transmitted by *Rhipicephalus sanguineus* in tropical and subtropical countries, and leading to a moderate, often clinically unapparent infection (Uilenberg et al., 1989; Hauschild et al., 1995; Zahler et al., 1998; Cacciò et al., 2002).

A molecular study carried out with Brazilian samples from infected dogs living in urban areas has shown that *B. canis vogeli* was the etiological agent involved in all cases (Passos et al., 2005) and only recently few cases of *B. gibsoni* infections have been molecularly characterized in dogs from a region in Southern Brazil (Trapp et al.,

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2006). Although the importance of canine babesiosis has increased over the last years in urban areas of the State of Minas Gerais (Bastos et al., 2004), only recently the prevalence rates in rural areas of Minas Gerais have been determined (Maia et al., 2007; Costa-Júnior et al., 2009).

Usually, the diagnosis of *Babesia* infections is made upon size and morphological appearance of intra-erythrocytic forms in peripheral blood smears. However, parasitemias are usually very low or not detectable particularly in animals undergoing a chronic phase of infection. The Polymerase Chain Reaction (PCR) and the nested PCR provide a practical means to detect and differentiate infections with various *Babesia* spp. and constitute sensitive tools for assessing treatment outcomes (Birkenheuer et al., 2003; Duarte et al., 2008). Detection of infection by Real Time PCR can replace conventional and nested PCR, as well as sequencing methods in the diagnosis and follow-up of many diseases, providing the ability to perform very sensitive, accurate and reproducible measurements of specific DNA present in a sample (Bell and Ranford-Cartwright, 2002; Matsuu et al., 2005; Oyamada et al., 2005).

In the present study, a Real Time PCR was developed and used to detect babesia infections in dogs living in rural areas of Brazil, and to determine the subspecies of *B. canis* occurring in these areas.

2. Materials and methods

2.1. Primers and probes designing

Consensus sequences were performed using CLUSTAL W with successive alignment of internal transcribed spacer (ITS) of a large number of sequences of *B. canis vogeli*, *B. canis canis*, *B. canis rossi*, *B. gibsoni*, *Babesia microti*, *Rhipicephalus* (*Boophilus*) *microplus*, *R. sanguineus*, *Amblyomma variegatum*, *Ixodes scapularis*, *Mus musculus*, *Homo sapiens* and *Oryctolactus cuniculus* available in Genbank, and specific primers and probes for *B. canis vogeli*, *B. canis canis*, *B. canis rossi* (Table 1) were designed using the DNAMAN software package (Lynnon Bio Soft, Quebec, Canada).

2.2. PCRs conditions

For detection of *B. canis vogeli*, *B. canis canis* and *B. canis rossi*, a Real Time PCR was performed with the primers (Table 1) for amplifying a fragment (around 125 bp) at the 3′end of the ITS 2 of the rDNA. Detection of specific products was based on hybridization with a fluorescent probe of the molecular beacon type (Table 1). Amplifications were done in a total volume of 25 μl containing Tris–HCl, KCl, $(NH_4)_2SO_4$, 8.0 mM of $MgCl_2$, and 1.25 U Hot-Star Taq Polymerase (Qiagen, Hilden), as well as 200 μM of each dNTP, 1 μM of each primer, 0.2 μM of each probe and 250 ng of DNA. For each PCR test, positive controls of genomic babesial DNA from each subspecies and negative controls containing DNA from uninfected dogs were included in every run. Amplifications of target genes were done using an iCycler (Biorad, Munich) with an initial Taq Polymerase activation step of 13 min at 95 °C, followed by 50 cycles at 95 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 30 s. Fluorescence was measured at each annealing step. Reactions were evaluated using the software version 3.1 (iCycler iQ Real Time PCR detection system, Biorad) and were regarded as positive if the amount of fluorescence exceeded a threshold value (basal emission plus the 10 fold of its standard deviation) and followed a curve of a sigmoid shape.

2.3. Samples

Blood samples were collected from dogs living in farms in three regions within the State of Minas Gerais, Brazil: Lavras (latitude – S 21°20′; longitude – W 45°00′), Belo Horizonte (latitude – S 19°55′; longitude – W 43°56′) and Nanuque (latitude – S 17°49′; longitude – W 40°20′). In these areas two seasons are well defined during the year: a dry season (from April to September) and a rainy season (from October to March). The climatic data, as referred in http://www.agritempo.gov.br/agroclima/pesquisaWeb?uf=MG, were obtained throughout the experimental period for each region.

Blood samples were collected during a dry season of 2004 from 252 dogs living in the following locations: Lavras ($n = 100$), Belo Horizonte ($n = 50$) and Nanuque ($n = 102$). In the subsequent rainy season, a total of 166 dogs were re-sampled, as followed: Lavras ($n = 71$), Belo Horizonte ($n = 29$) and Nanuque ($n = 66$).

From each sample, DNA was extracted using the Wizard Genomic DNA Purification (Promega, Madison, USA) and Giemsa stained smears were microscopically examined for direct detection of *Babesia* parasites. DNA concentration was determined using the spectrophotometer NanoDrop ND-1000 (NanoDrop, Wilmington, USA) and DNA samples were diluted using ultrapure water to reach a concentration of 50 ng/μl.

2.4. Statistical analysis

The Chi-square test was used to evaluate associations between prevalence and incidence among municipalities and seasons. The Kappa test was performed to compare detection in blood smears and by the Real Time PCR.

3. Results

3.1. Prevalence and incidence of Babesia canis infections

Although the Real Time PCR developed in this study had been designed to detect all subspecies of *B. canis* (Fig. 1), only *B. canis vogeli* was found in all three regions analyzed. Prevalence rates by region and season are shown in Table 2. Direct examination of blood smears detected few positive animals (0.8% during dry season, and 0.0% during the rainy season), while the Real Time PCR detected 9.9% of positive animals during the dry season and 10.8% during the rainy season. All positives animals in blood smears were positive for Real Time PCR. The index Kappa showed poor agreement between the tests (0.08).

A comparison among the prevalence rates observed in each region showed that Nanuque (13.7%) and Belo Horizonte (12.0%) had significant higher prevalence rates than Lavras (5.0%) ($p < 0.05$).

Table 1
Oligonucleotide primers and probes developed for detection of *Babesia canis* subspecies.

Primers/probes	Sequence 5′–3′	Subspecies
BC-1F	TGT GTA CTT GTT ACC GAA TCT TC	*B. canis canis*
BC-2F	TGC TTA AAT TCA GCG GAT AG	*B. canis* spp.
BC-3F	TGT GTA TGT GAT ACC GAA TCT TC	*B. canis vogeli*
BC-4R	GCT TAA ATT CGG CGG ATA G	*B. canis rossi*
BC-6R	CTT AAA TTC GGC GGA TCC	*Babesia* spp.
BC-S1	6-FAM CGCGATGGCGTTGCCCGAGACTGTGATCGCG Dabcyl	*B. canis canis*
BV-S2	HEX CGCGATGTGAGCCGTCTGACTCCGTGTATCGCG Dabcyl	*B. canis vogeli*
BR-S3	BHQ-3 CGCGAGACGCTGTATGGTTGTGGATGATCGCG Cy5	*B. canis rossi*
BR-S4	BHQ-3 CGCGATGAGCTTGCAACCACAACCATATAAATCGCG Cy5	*B. canis rossi*

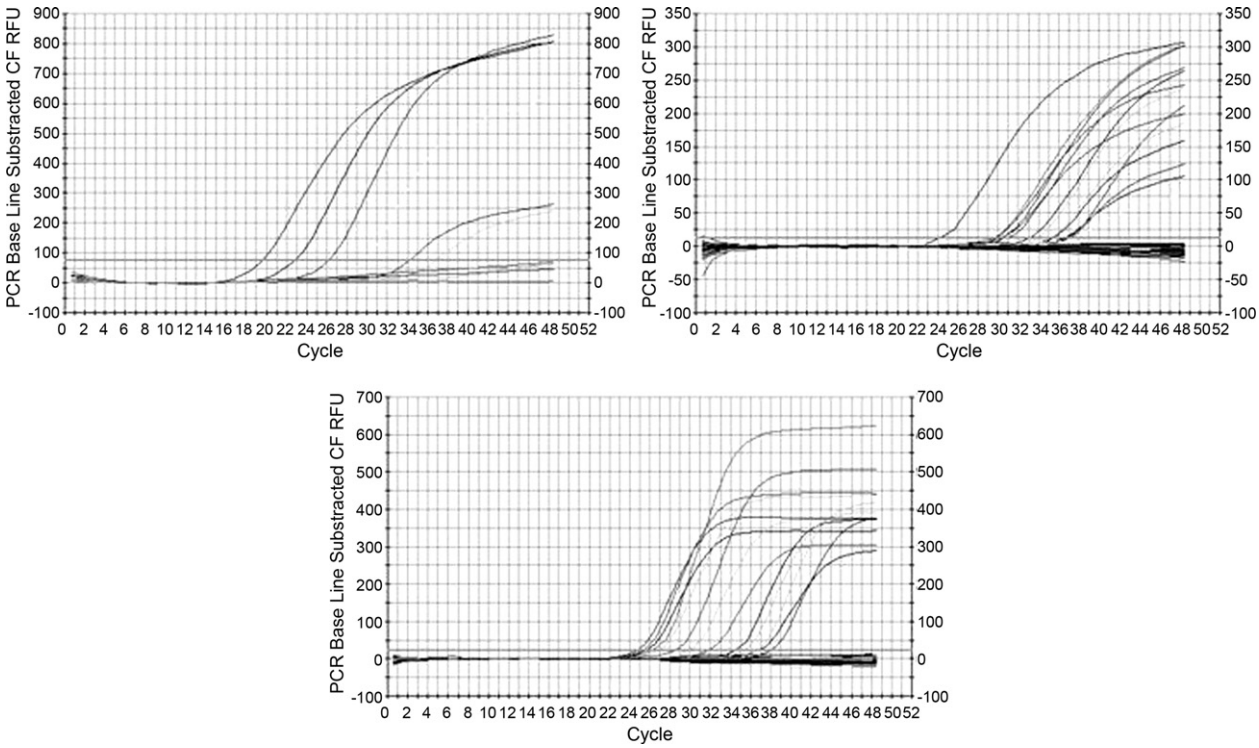

Fig. 1. Representative Real Time PCR amplification curve to *Babesia canis canis* (A), *Babesia canis rossi* (B), and *Babesia canis vogeli* (C).

Re-sampling the same dogs during the subsequent rainy season allowed calculating the incidence rates of *Babesia* infections in the three regions. The Real Time PCR detected only one new case of infection among 68 negative dogs in Lavras and only one new case among 25 negative dogs in Belo Horizonte, resulting incidence rates of 1.5% and 4.0%, respectively. However, a much higher incidence rate (24.1%) was observed in Nanuque, where 14 new cases were identified among 58 negative animals.

4. Discussion

Three subspecies of *B. canis* have been proposed (Uilenberg et al., 1989) and detected in many countries on the world (Martinod et al., 1986; Uilenberg et al., 1989; Matjila et al., 2004). However the differentiation between these subspecies is impossible by direct examination of blood smears. In the present study we developed a Real Time PCR for specific detection of *B. canis canis*, *B. canis*

Table 2
Prevalence rates (%) of *Babesia canis vogeli* infections detected by microscopic examination of blood smears and by the Real Time PCR from dogs living in rural areas of Minas Gerais State, Brazil.

	Microscopy (positive/*n*)		Real Time PCR (positive/*n*)	
	Dry season	Rainy season	Dry season	Rainy season
Belo Horizonte	2.0 (1/50)[a]	0.0 (0/29)[a]	12.0 (6/50)[b]	3.4 (1/29)[a]
Lavras	0.0 (0/100)[a]	0.0 (0/71)[a]	5.0 (5/100)[a]	1.4 (1/71)[a]
Nanuque	0.9 (1/102)[a]	0.0 (0/66)[a]	13.7 (14/102)[b]	24.2 (16/66)[b]
Total	0.8 (2/252)	0.0 (0/166)	9.9 (25/252)	10.8 (18/166)

Different letters indicate significant differences ($p < 0.05$).

rossi and *B. canis vogeli*. In the three studied rural areas in Brazil the only subspecies present was *B. canis vogeli*, as previously reported for urban areas (Passos et al., 2005).

The standard method for quantification of parasites is the microscopic examination of blood smears. Although this is an inexpensive diagnostic test, it has a low sensitivity in detecting the parasites when an animal has low parasitemia (Böse et al., 1995). This was confirmed in the present study by the low prevalence found in blood smears from all three regions, indicating the inappropriateness of this technique for epidemiological studies.

Real Time PCR is a method that can be used to monitor amplicon formation throughout the PCR reaction providing the ability to perform very sensitive, accurate and reproducible measurements of specific DNA present in a sample (Bell and Ranford-Cartwright, 2002; Matsuu et al., 2005; Oyamada et al., 2005). In the present study, we development and validated a highly sensitive qualitative Real Time PCR that amplifies a 125 bp fragment at the 3′end of ITS2 of the rDNA of *B. canis* subspecies. The overall prevalence rate of *B. canis vogeli* by the Real Time PCR (9.9%) was higher than that found by direct examination of blood smears (0.8%), confirming the higher sensitivity of the method.

The highest prevalence was found in Nanuque (13.7%) and Belo Horizonte (12.0%) regions where temperatures were higher. On the other hand, the lowest prevalence was found in Lavras where temperatures were lower than the other regions (data not shown). The results presented here indicate that canine babesiosis is endemic in rural areas in the State of Minas Gerais and the only subspecies present is *B. canis vogeli*. Besides, infection transmission is influenced by climatic conditions, mainly the temperature.

The developed Real Time PCR was proved to be appropriate to detect *B. canis* subspecies in endemic areas.

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