

## Research Article

# First Molecular Detection of *Babesia canis vogeli* in Dogs and *Rhipicephalus sanguineus* from Mexico

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• Canine babesiosis; PCR-RFLP; nPCR; *Babesia canisvogeli*; DNA sequencing

**Abstract**

At the global level, three distinct subspecies for *B. canis* have been identified: *B. caniscanis* in Europe; *B. canisrossi* in Africa, while in America the presence of *B. canis vogeli* has been demonstrated. However, *B. canisrossi* has recently been reported in the USA and *B. canis vogeli* in South Africa and Europe. In order to optimize two methods for the molecular detection of the subspecies of canine babesiosis in Mexico from blood samples and *Rhipicephalus sanguineus* ticks, 30 blood samples and 18 tick specimens were collected in dogs with clinical manifestations compatible with canine babesiosis and a history of exposure to ticks. The analysis of Restriction Fragment Length Polymorphisms in PCR-amplified and digested DNA with restriction enzyme *TaqI* and *HinfI* (PCR-RFLP) allowed detection of the corresponding pattern for the *B. canis vogeli* subspecies (203 bp, 171 bp and 26 bp) in two blood samples and in one of the specimens of ticks subject to the PCR-RFLP analysis. With the nested PCR assay, the fragment of interest (192 bp) was detected in three blood samples and in three of the ticks that were analyzed for the *B. canis vogeli* subspecies. The presence of *B. canis* in Mexico had already been demonstrated microscopically, however, so far this is the first report on the molecular detection of the subspecies *B. canis vogeli* in our country and confirmed by DNA sequencing.

**ABBREVIATIONS**

PCR: Polymerase Chain Reaction; nPCR: nested Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; DNA: Deoxyribonucleic Acid

**INTRODUCTION**

Canine babesiosis is a tick-borne cosmopolitan disease affecting domestic dogs, and its distribution is related to vector presence being more prevalent in regions with tropical and subtropical climates. The disease is caused by *B. canis* and *B. gibsoni* Intraerythrocytic parasites [1]. The species of *B. canis* has been re-classified into three subspecies based on the vector that transmits the pathogen and its distribution: *B. caniscanis*, whose vector for transmission is the *Dermacentor reticulatus* tick; *B. canisrossi* transmitted by *Haemaphysalis leachi*; and *B. canisvogeli* transmitted by *Rhipicephalus sanguineus* in tropical and subtropical areas. These tick species are distributed in Europe, South Africa and America, respectively [2], although the presence of *B. canis rossii* in the USA and *B. canis vogeli* in South Africa and Europe has recently been reported [3-5]. The direct diagnosis can be made through the microscopic observation of the parasites in blood smears stained with Giemsa dye, and the indirect type using commercial serological packages. The main signs of the disease are characterized by fever, lethargy, vomiting, hemoglobinuria and anemia [1,6], whereas in more severe cases

clinical manifestations may also include anorexia, Weight loss, jaundice, hematuria, depression, ascites, lymphadenopathy, splenomegaly, central nervous system abnormalities and renal complications [7].

In Mexico, the molecular diagnosis for canine babesiosis has not been implemented, therefore, there are no reports that demonstrate and identify what subspecies of the *B. canis* protozoan is present in the blood of infected domestic dogs or in the tick vector. The polymerase chain reaction (PCR) has been used for amplification of the 18S rRNA gene, which has facilitated the detection and identification of *B. canis rossii*, and *B. canis vogeli* in samples of naturally infected dogs in regions of Italy [4], these subspecies have also been described in Northern Portugal, suggesting the presence and distribution of the vector tick [5] whereas in Brazil *B. canis vogeli* is the only subspecies that has been identified [8,9]. The analysis of polymorphisms in length of amplified and digested restriction enzyme fragments (PCR-RFLP), is an alternative for the discrimination among the subspecies of *B. canis*. In France and Italy, PCR amplification of the 18S rRNA gene fragment (400 bp) allowed the differentiation of the *B. canis canis*, *B. canis vogeli* and *B. canisrossii* subspecies using the *TaqI* and *HinfI* enzymes, obtaining fragments of 203bp, 171 bp and 26 bp for *B. canis vogeli* and 227 subsets of *B. canis* [BCC] bp and 174 bp for *B. canis rossii*. In the case of *B. canis canis*, and since the amplified sequence does not have restriction sites

for both enzymes [10,11], the fragment is not digested with any of these enzymes, in this way it is possible to discriminate the subspecies of *B. canis canis*. Currently in Mexico there is little information about infection of *Rhipicephalus sanguineus* ticks with the protozoan parasite *Babesia canis*, however, in other countries such as Australia, France and Tunisia, they have been able to implement the molecular characterization of the parasite using the PCR assay [12-14]. The objective of this work was to optimize two methods for the molecular detection of the subspecies of the etiological agent of canine babesiosis in Mexico from blood samples and *Rhipicephalus sanguineus* ticks collected from dogs.

## MATERIALS AND METHODS

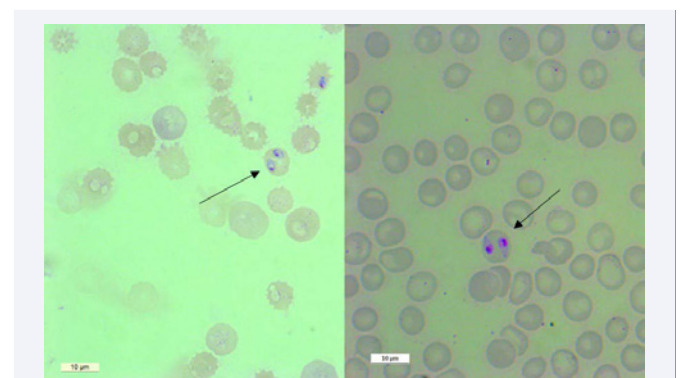
A total of 30 blood samples were collected in dogs by puncture of the cephalic vein in tubes with anticoagulant (EDTA). The dogs were referred to a particular veterinary clinic in the city of Cuautla, Morelos, Mexico with clinical manifestations, including fever, anemia, lethargy or epistaxis, consistent and suspicious of canine babesiosis and history of previous exposure or presence of ticks. An aliquot was taken and spread on a slide to be fixed with methanol and stained with Giemsa dye, each slide was observed by microscopy for at least 20 minutes. The cellular package containing red and white blood cells was separated from the plasma by centrifugation at 4,000 rpm and stored at -20°C until use. At the time of the physical inspection of the dogs, 18 specimens of ticks were collected, which were identified by taxonomic codes as *R. sanguineus* [15], only one of them could be checked by light microscopy from a dog infected with *Babesia canis* for which a parasitemia of about 80% was estimated.

Extraction of the genetic material from the cellular package was done with a commercial kit (ZR Genomic DNA II Kit; ZYMO RESEARCH). For the DNA extraction of the ticks analyzed in this study, the mortar crushing method was used prior to the use of a commercially available kit (ZR Tissue and Insect DNA Miniprep; ZYMO RESEARCH) according to the manufacturer's recommendations. The PCR test was performed with the enzyme Go Taq Green Master Mix 2X (PROMEGA), with a final volume of 25µl in a thermocycler (BIO-RAD Icyler). The detection of *Babesia* sp. was performed by amplifying the variable portion of the 18S rRNA gene using the pair of generic primers named PIRO-A sense (5'-AATACCCAATCCTGACACAGGG-3') and antisense PIRO-B (5'-TTAAATACGAATGCCCCACC-3'), under the following protocol: 94°C for 5 min in the initial denaturation, (94°C, 1 min, 55°C 1 min, 72°C 1min) for 35 cycles and 72°C for 15 min for the final extension [10], The semi-nested PCR assay included a first step with external primers 455-479 sense (5'-GTCTTGTAATTGGAATGGTGAC-3') and antisense 793-772 (5'ATGCCCCCAACCGTTCCTATTA-3) under the amplification protocol (95°C 5 min, 95°C 45 sec, 58°C 45 sec, 72°C 45 sec, 72°C 5 min with 50 repeating cycles, subsequently for the semi-nested PCR specific primers were included for each one of the subsets of *B. canis canis* [BCC] sense (5'-TGCGTTGACGGTTTGACC-3'), *B. canis rossii* (BCR) sense (5'-GCTTGGCGGTTTGTTC-3'), and *B. canis vogeli* (BCV) sense (5'GTTCGAGTTTGCCATTCGTT-3'), and antisense primer 793-772 (5'ATGCCCCCAACCGTTCCTATTA-3') using the same prior amplification protocol with 30 repeat cycles [1].

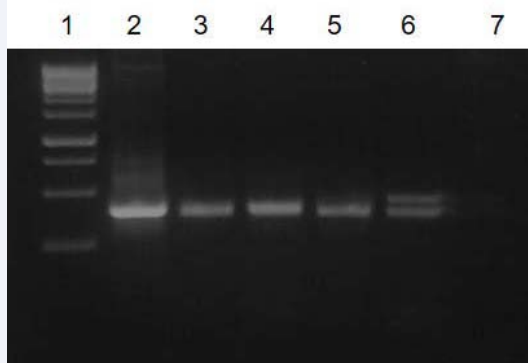
The PCR products amplified with the generic primers were digested with the restriction enzymes *TaqI* and *HinI* for the differentiation of the species of *Babesia* sp. For the analysis of PCR and PCR-RFLP products, 3% agarose gels were electrophoresed for 45 min at 100 V and visualized in a transilluminator with ultraviolet light (UV). The obtained amplicons were cloned into a T vector and the transformation was carried out with chemically competent *E. coli* cells using a commercial kit (pGEM-T Easy Vector System II; PROMEGA). Two representative white (recombinant) colonies were selected, which were scored in boxes with LB medium and ampicillin. Purification of the plasmid DNA was performed with a commercial kit (PureYield Plasmid Miniprep System Protocol; PROMEGA), for later use as a template for sequencing by the fluorinated dideoxynucleotide termination method in an automated sequencer (Applied Biosystems). The DNA sequences obtained were analyzed by searching for sequence identity in the databases using the blastn application (<http://blast.ncbi.nlm.nih.gov/Blast/blastn>).

## RESULTS AND DISCUSSION

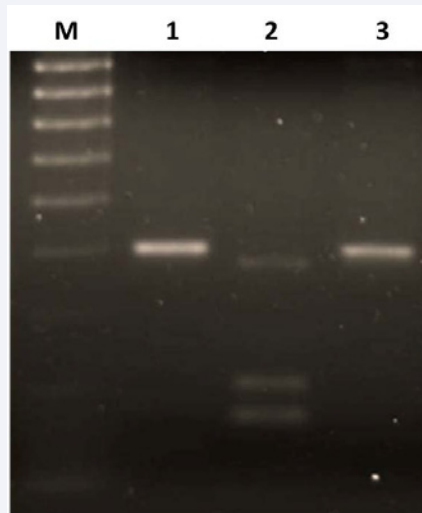
Two positive samples were identified by microscopic analysis, with predominance of trophozoite stages in one sample and trophozoites and merozoites consistent with *Babesia* spp. in the second sample. However, the parasitemias were very low to be quantified (< 0.01%), (Figure 1). DNA amplification by means of the PCR test with the generic primers for the *B. canis* species allowed the identification of two positive blood samples from the total of samples analyzed, whereas from the tick samples analyzed in this study it was demonstrated consistently the presence of a 400 bp fragment in three samples (Figure 2). The results of the amplified products that were digested with the restriction enzymes could demonstrate partial digestion with the enzyme *TaqI* in two blood samples and only one of the tick samples subject to the reaction, which corresponded to the expected size of the fragments with bands of approximately 203 bp, 171 bp and 26 bp (Figure 3) coincident with the RFLP pattern corresponding to *B. canis vogeli* [10]. The analysis of results with the semi-nested PCR assay allowed identifying three positive samples from blood and three from ticks consistent with the amplification of a 192 bp fragment (Figure 4) when the BCV subspecies specific primer was used in the semi-nested PCR test [1]. Two amplified



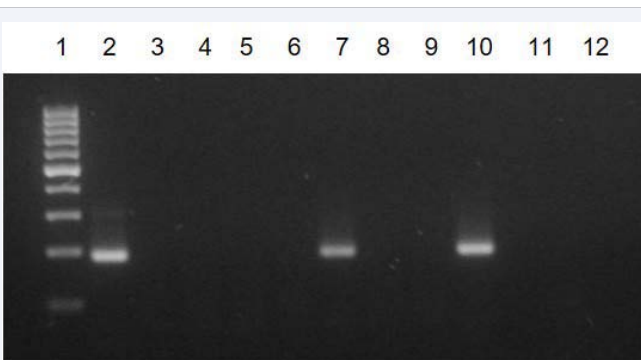
**Figure 1** Intraerythrocytic parasites in blood smears stained with Giemsa from two clinically infected dogs, later confirmed as *Babesia canis vogeli*.



**Figure 2** Representative image of PCR products obtained with the generic PIROA-PIROB primers (400 bp) in specimens of *Rhipicephalus sanguineus* ticks. Lanes 1): 1 Kb ladder DNA marker; 2): positive control plasmid DNA containing 18S ssRNA gene of *B. canis*; 3): positive control of *B. canis* DNA; 4-6): Specimens of *R. sanguineus* ticks; 7): negative DNA control.



**Figure 3** Representative PCR-RFLP result. Amplicon From dog 1 digested with restriction enzyme *TaqI* (lane 2) and *HinfI* (lane 3). Lane 1 represents the undigested product; M) 100 bp molecular marker.



**Figure 4** PCR seminested detection of *Babesia canis vogeli* DNA (192 bp) in blood samples from dogs. Lanes 1): 100 pb ladder DNA marker; lane 2): positive DNA control; lanes 3-10): blood samples from dogs; lanes 11-12): negative DNA controls.

fragments obtained by PCR using the primers PIRO-A and PIRO-B, containing the 400 bp variable portion of the 18S rRNA gene were cloned and sequenced. The result of blast analysis of the cloned fragments showed an identity of 99% in nucleotide sequences (403 out of 405 residues), existing between the sequences of the two Mexican cases of *B. canis vogeli* (Genbank accession numbers MG430179 and MG430180, respectively) and those corresponding to isolates from Venezuela (*Babesia canis vogeli* from Venezuela 18S rRNA gene, gb DQ297390.1), Brazil, Japan and the United States.

The classic form for the detection of *Babesia canis* is by observing the intraerithrocytic forms in blood smears stained with Giemsa dye, which is considered as the gold test in acute cases of canine babesiosis and it is a relatively simple technique to perform. Molecular tests are highly reliable tools for the genotyping of parasites that cause canine babesiosis in acute and subacute clinical cases. They are a valid and necessary resource because it allows to classify the different subspecies of *B. canis* at the regional and global levels to determine the origin or a possible route of transmission of *B. canis* from the tick to the canine host, as it has been done in other latitudes [4,5,10,11]. However, nested PCR proved to be the test with the highest analytical sensitivity and specificity to differentiate *B. canis* species in both blood and tick DNA samples [12-14].

According to the work done on the distribution of the brown dog tick (*Rhipicephalus sanguineus*) in the state of Morelos, Mexico, the results of the present study on *R. sanguineus* ticks found in dogs coincide with previously reported in the cities of Cuernavaca [16], and Morelos State in Mexico [17]. The detection of infection in the positive ticks of this study suggests that *B. canis vogeli* is most likely transmitted by this tick species, according to what has been previously reported in the literature [1,2,10]. In Mexico, the presence of *B. canis* in Mexico had already been demonstrated microscopically [18], however, so far this is the first report on the molecular detection of the subspecies *B. canis vogeli* in our country and confirmed by DNA sequencing.

## CONCLUSION

The semi-nested PCR technique used in this study has a higher analytical sensitivity and a better specificity for the differentiation between the subspecies of *B. canis*. The subspecies detected for the first time in Mexico, *B. canisvogeli*, associated with canine babesiosis in this study, was confirmed by DNA sequencing.

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