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# Molecular detection of canine Babesia in ticks of Bengaluru, South India

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**Abstract**
A molecular study was carried out for detection of canine *Babesia* spp. in tick vectors by PCR. In a total of 71 *R. sanguineus* subjected to genus specific PCR, 14 (19.71%) ticks showed amplification of 410 bp fragment specific for *Babesia* spp. Out of 14 *Babesia* spp. positive samples, 12 (16.90%) showed amplification of 671 bp fragment specific for *B. gibsoni* and 2 (2.81%) samples showed specific amplification of 590 bp specific for *B. c. vogeli.* None of the tick DNA samples from *R. haemaphysaloides* showed specific amplication for genus *Babesia.* Out of 110 dogs examined, 71 were infested with ticks, with an overall prevalence of 64.54 percent. Out of 764 ticks collected from infested dogs, 54.84 percent were found to be *R. sanguineus* and 45.15 percent of ticks were found to be *R. haemaphysaloides*.

**Keywords:** *Babesia* spp., dogs, ticks, pcr

## 1. Introduction
Among ectoparasites, ticks are the major problem as they suck blood, cause anaemia, irritation, annoyance to the dogs and act as vectors in transmission of many fatal diseases. In tropical and subtropical regions, tick-borne diseases *viz.,* bacterial, viral and most commonly the haemoprotozoan origin is a common feature due to their severe pathogenecity [1]. Amongst the various vector borne diseases, canine babesiosis is considered as one of the most widespread disease.

The disease is widely distributed in both domesticated dogs and wild canids. The disease is typically characterized by haemolytic anaemia, thrombocytopenia, fever and splenomegaly. Chronic infections are more common and infected dogs remain as carriers without any overt clinical signs [2]. It is life threatening and clinically significant disease caused by intraerythrocytic apicomplexan parasites of the genus *Babesia*. *B. canis* is a large form (3.0-5.0 µm), while *B. gibsoni* is a small pleomorphic organism (1.5-2.5 µm) and appears most commonly as ring form [3]. Three subspecies of *B. canis* have been described as *Babesia canis rossi, Babesia canis vogeli* and *Babesia canis canis* [4]. The disease is found in almost all parts of Asia, Europe, Africa, America and Australia and transmitted by *Dermacentor reticulatus* in Europe, *Rhipicephalus sanguineus* in tropical and subtropical regions and *Haemaphysalis leachi* in South Africa. Hence, a study was carried out for detection of canine babesiosis in tick vectors by Polymerase chain reaction.

## 2. Materials and Methods
### 2.1 Collection of ticks
Ticks were collected manually from different locations in and around Bengaluru namely, Veterinary College Hospital, Hebbal (284), Private Veterinary hospitals and clinics (176) and dog shelters and kennels (304) in a well ventilated plastic containers. The samples were labelled and brought to the laboratory. Ticks were stored in 70 percent alcohol until further use.

### 2.2 Identification of ticks
Adult male and nymphal ticks collected from each animal were stored in 70 percent alcohol and were identified under 10x and 40x objective magnification of the compound microscope based on the morphological characters [5, 6].

### 2.3 Extraction of genomic DNA from tick vectors
Tick samples stored in 70 percent ethanol were taken and blotted to remove ethanol. Then ticks were flash freezed in liquid N2 (-196°C) and pestle crushed to get fine powdered tissue sample for further extraction of genomic DNA. The genomic DNA was extracted from 25 mg of tick tissue (1-5 ticks) using the "DNeasy® Blood & Tissue Kit" (Qiagen, Germany) with slight modification. Proteinase K (20µl), 200 µl of blood sample and 200 µl of buffer AL was pipetted into a 1.5 ml tube and mixed by pulse-vortexing for 15 seconds. Homogeneous solution was incubated at 56°C for 10 min in waterbath and 200 µl of ethanol was added. The mixture was carefully applied to the QIAamp spin column and centrifuged at 6000 x g (8000 rpm) for 1 min. 500 µl of buffer AW1 was added centrifuged at 6000 x g (8000 rpm) for 1 min. 500 µl of buffer AW2 was added and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. QIAamp spin column was carefully opened and 200 µl buffer AE was added. Incubated at room temperature (15-25 °C) for 1 min and then centrifuged at 6000 x g (8000 rpm) for 1 min to obtain a final DNA yield of 200 µl and DNA was stored at -20°C until further use.

### 2.4 Polymerase chain reaction for detection of *Babesia* DNA in tick vectors
The DNA extracted from tick samples were subjected for *Babesia* genus specific PCR by using primers described by Gallego *et al.* (2008) [7]. The primers were synthesized and procured from M/s Bioserve biotechnologies private limited, Hyderabad. Forward primer PIRO-A F 5' AATACCCAATCCTGACACAGGG 3' and reverse primer PIRO-B R 5'TTAAATACGAATGCCCCCAAC 3' were used for the amplification of 410 bp fragment of genus specific PCR. The PCR cycling conditions for *Babesia* genus was 4 min incubation at 94°C for initial denaturation, followed by denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 1 min for 35 cycles with final extension at 72°C for 7 min.
The samples which were positive by genus specific PCR were subjected to species specific PCR using primers of *B. c. vogeli* [8] and *B. gibsoni* [9] by targeting 28S rRNA and 18S rRNA respectively. The forward primer BAB1 F 5' GTGAACCTTATCACTTAAAGG 3' and reverse primer BAB4 R 5' CAACTCCTCCACGCAATCG 3' were used for amplification of 590 bp fragment of *B. c. vogeli*. The thermal cycling conditions for this was 2 min incubation at 94°C for initial denaturation, 35 cycles of 30s at 94ºC, 56ºC for 30 sec, 72ºC for 1 min and a final extension at 72ºC for 7 min. The forward primer Gib599F 5' CTCGGCTACTTGCCTTGTC 3' and reverse primer Gib1270R 5' GCCGAAACTGAAATAACGGC 3' were used for amplification of 671 bp DNA fragment specific for *B. gibsoni* with the following cycling programme. 5 min incubation at 95°C to denature double strand DNA, 40 cycles of 30 sec at 95°C, 30 sec at 56°C and 1 min 30 sec at 72 °C with final extension at 72 °C for 5 min.
PCR amplification of 18S rRNA gene was performed using a gradient thermal cycler (Eppendorf, Germany) in a final reaction volume of 25 µl containing 12.5 µl of PCR master mix (Merck's), 2 µl of each forward and reverse primer (10 picomoles/µl), 5 µl of template DNA and 3.5 µl of nuclease free water. A known positive *Babesia* DNA obtained from Dr. Mohan Kumar, Department of Veterinary Medicine, Veterinary College, Hebbal, Bengaluru was used as positive control. Nuclease free water was used as a negative control. No template control (NTC) was maintained without DNA template in all PCR amplifications. The PCR products were analysed by using 1.5 percent agarose gel electrophoresis.

## 3. Results
### 3.1 Detection of *babesia* in tick vectors by PCR
During this study, *R. sanguineus* and *R. haemaphysaloides* tick DNA samples were analysed for the presence of *B. c. vogeli* and *B. gibsoni* DNA. Out of 71 *R. sanguineus* DNA subjected for genus specific PCR, 19.71 (14) percent were found positive for *Babesia* DNA (Fig. 1). *B. c. vogeli* was detected in 2.81 (2) percent of *R. sanguineus* whereas *B. gibsoni* was detected in 16.9 (12) percent of the ticks (Fig. 2 & 3).

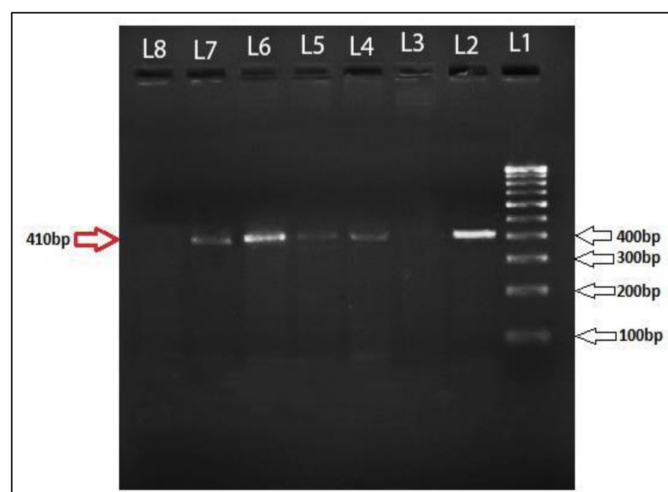


**Fig. 1** Gel showing amplification of 410 bp specific for *Babesia* spp. From *R. sanguineus* tick

L 1: 100 bp DNA Ladder
L 2: Positive Control
L 3: Negative Control
L 4, L 5, L 6 and L 7: tick DNA samples
L 8: No template control (NTC)

**Fig. 2** Amplification of 590 bp specific for *B. canis vogeli* from *R. sanguineus*

L 1: 100 bp DNA Ladder, L 2: Positive Control, L 3: Negative Control, L 4 and L 6: Tick DNA samples showing negative, L 5 and L 7: Tick DNA samples showing positive
L 8: No template control (NTC)

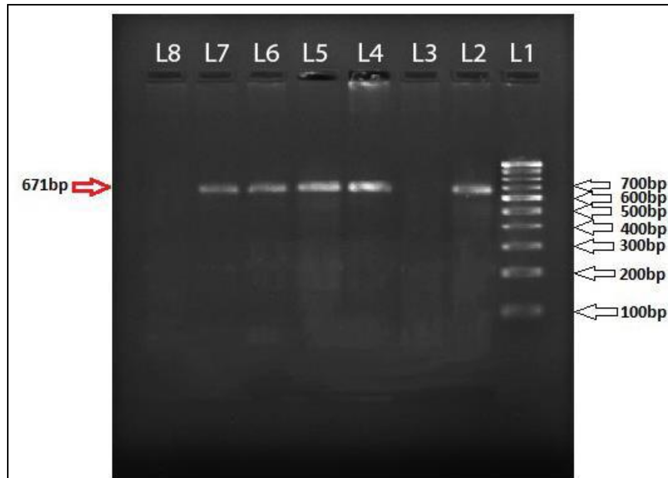


**Fig. 3** Amplification of 671 bp specific for *B. canis vogeli* from *R. sanguineus*

L 1: 100 bp DNA Ladder
L 2: Positive Control
L 3: Negative Control
L 4, L 5, L 6 and L 7: Tick DNA samples
L 8: No template control (NTC)

### 3.2 Prevalence and identification of ticks

During this study, a total of 764 ticks were collected from both clinically suspected and apparently healthy animals. In decreasing frequency, the most infested body sites were the head, back, interdigital spaces, lateral side of the body, neck, chest, ventral side of the body and the legs. All the ticks collected belonged to the genus *Rhipicephalus* and were identified based on the morphological characters. Out of 764 ticks collected from dogs, 54.84 (419/764) percent of ticks were found to be *R. sanguineus,* whereas 45.15 (345/764) percent of ticks were found to be *R. haemaphysaloides*.

## 4. Discussion

Babesiosis can be considered as one of the emerging / re-emerging tick borne haemoprotozoan disease of dogs in Bengaluru. In the present study, 19.71 percent of *R. sanguineus* tick DNA samples were positive for *Babesia* spp. by genus specific PCR. Out of 14, 12 (16.90%) genus specific DNA samples amplified DNA fragment specific for *B. gibsoni* and only 2 (2.81%) samples showed specific amplification for *B. c. vogeli*. These findings were in accordance with Rene *et al.* (2012) [10] who conducted molecular characterization of *B. vogeli* infection in dogs and *R. sanguineus* ticks in southern France and reported that 8 ticks (22.2%) were found to be infected with *B. vogeli* by using PCR. Similarly, Martellet *et al.* (2015) [11] detected DNA of *B. vogeli* and *B. canis* in 10.5 and 1.6 percent of *R. sanguineus* tick. In contrast to the present findings, three *D. reticulatus* tick samples showed specific amplification for *Babesia* DNA by seminested PCR based on the 18S rRNA gene with subsequent sequencing in a study conducted by Rar *et al.* (2005) [12] from southwestern Siberia, Russia. Oines *et al*. (2012) [13] detected *I. ricinus* tick harbouring *Babesia* spp. by real time PCR screening in Norway from a total of 1908 *I. ricinus* ticks, 17 (0.9%) indicated the presence of *Babesia* spp. Capligina *et al.* (2015) [14] conducted a prevalence and phylogenetic analysis of *Babesia* spp. in *Ixodes ricinus* and *Ixodes persulcatus* ticks in Latvia. The 18S rRNA gene fragment of *Babesia* spp. were detected in 1.4 percent of the *I. ricinus* ticks and in 1.9 percent of *I. persulcatus* ticks suggesting that both *I. ricinus* and *I. persulcatus* ticks play a role in the epidemiology of canine babesiosis. Schaarschmidt *et al.* (2013) [15] reported free living *D. reticulatus* ticks harbouring *B. canis* DNA in Switzerland by PCR targeting the 18S rRNA gene of *Babesia* spp. This variation is due to different tick species involved as vector in different geographical locations. Uilenberg, *et al.* (1989) [4] reported that *B. canis* and *B. gibsoni* are recognized to cause canine babesiosis worldwide and are transmitted by *D. reticulatus* in Europe, *R. sanguineus* in tropical and subtropical regions and *Haemaphysalis leachi* in South Africa.

During the present study, a total of 764 ticks were collected from both clinically suspected and apparently healthy animals. In decreasing frequency, the most infested body sites were the head, back, interdigital spaces, lateral side of the body, neck, chest, ventral side of the body and the legs. The present findings were in concurrent with Hadi *et al.* (2016) [16] who conducted a study on prevalence of ticks and tick-borne diseases in Indonesian dogs and reported 67.90 percent of prevalence. The infested body sites were back region (35.0%), the head, ears and neck (29.0%), the legs and the interdigital spaces (14.5%), the abdomen (10.9%) and the tail (10.9%).

The ticks encountered during this study were identified as *R. sanguineus* and *R. haemaphysaloides* based on morphological characters. Similarly, Zhang *et al.* (2017) [17] carried out an epidemiological survey of ticks and tick-borne pathogens in pet dogs in China and reported that *R. sanguineus* (68.2%), *Haemaphysalis longicornis* (18.4%) and *R. haemaphysaloides* (12.5%) as the major tick species in dogs. Bhadesiya *et al.* (2014) [18] and Amuta *et al.* (2008) [19] had reported 58.11 and 82.4 percent prevalence of *R. sanguineus* in dogs from Gujarat (India) and Nigeria, respectively. In the present study, the prevalence of *R. haemaphysaloides* (45.15%) which is commonly found on cattle, sheep and goat was observed in dogs may be due to contact of dogs with other animals in the domestic scenario. *R. sanguineus* was found to be predominant tick species infesting dogs in Bengaluru which is highly adoptable for the urban situations and is capable of multiplication in the environment in the same premises and hence it is so called urban dog tick.

In conclusion, PCR was found to be very sensitive, rapid and reliable method for detection of *Babesia* infection in both carrier animals and clinically suspected animals. The tick *R. sanguineus* was found to be the proven vector for babesiosis in dogs in Bengaluru. The environmental conditions present in the Bengaluru district are favourable for tick vector propagation. Normally, in Bengaluru district the temperature is around 25 to 34 °C with an average rainfall of 859 mm per annum. However, there has been a remarkable change and wide variation in the climatic conditions in the recent years. These alterations in the weather pattern have been favourable for development of ticks and also for the emergence of haemoprotozoan infections such as babesiosis to a large extent and also other predisposing factors that may increase animal's susceptibility to infection.

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