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SYBR green-based real-time PCR detection of canine *Babesia* spp. in ixodid ticks infesting dogs in Kerala, South India

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Abstract: Ticks, being hematophagous ectoparasites, are notorious vectors that transmit an array of pathogenic viruses, bacteria, and protozoa while simultaneously exhibiting a zoonotic potential by transmitting pathogens that affect the health of owners in contact. The distribution of ixodid ticks of the genera *Rhipicephalus* and *Haemaphysalis* spp. in tropical climate of the state contributes to many serious tick-borne parasitic and rickettsial infections in domestic and wild canines. In south India, canine babesiosis is one of the most prevalent vector-borne parasitic diseases. Detection of parasites in tick vectors has significant epidemiological implications. The present study was designed to identify the presence of the most common vector-borne parasites of dogs in ixodid ticks using sensitive detection protocols. SYBR green-based real-time protocols detected *Babesia vogeli* in *Rhipicephalus sanguineus sensu lato*, while *B. gibsoni* was detected in *R. sanguineus* (s.l.) and *Haemaphysalis bispinosa*. The present study points out the need to reinvestigate the vectorial capacity of ticks in different geographical regions.

Key words: *Babesia vogeli*, *Babesia gibsoni*, conventional PCR, real-time PCR

Ticks are notorious vectors of pathogenic viruses, bacteria and protozoa of veterinary, medical and zoonotic importance. Tick-borne infectious diseases of domestic and wild mammals pose significant diagnostic and therapeutic challenges for veterinary practitioners around the world [1]. Canine babesiosis is one among the most prevalent and pathogenic tick-borne infections among canine population of India [2,3]. Hence sensitive and specific detection of these pathogens had always been a focus of research to deliver timely control measures. Conventional PCR has been validated and used for the detection of *Babesia* spp. in canines [3, 4, 5] and in ticks [6] in India. Though *B. vogeli* is transmitted by *Rhipicephalus sanguineus* (s.l.), and *B. gibsoni* by *Haemaphysalis longicornis* and *R. sanguineus*(s.l.) ticks [7], the reports of *B. vogeli* in *Ixodes ricinus* and *Dermacentor reticulatus* [8] and that of *B. gibsoni* in *H. bispinosa* [6], warrants further investigation using sensitive detection protocols. Adoption of novel molecular techniques has resulted in increased detection of emerging and re-emerging vector-borne pathogens in different parts of the world in the last two decades [9]. Real-time PCR is considered as an alternative to conventional molecular assays owing to its higher sensitivity and rapidity [10]. Though real-time PCR has been used to detect canine babesiosis [11, 12], there has been no attempts in the country to utilise this tool for sensitive detection of pathogens in ticks. In this context, a study was proposed to standardise real-time PCR protocols to explore the presence of the most common canine *Babesia* spp in ixodid ticks and to compare the results with conventional PCR detection.

Ticks (n=132) were collected in sterile vials from client owned dogs (n=100) presented to the Veterinary Hospitals as well as from private kennels in Kerala State. They were identified under a stereozoom microscope using standard keys [13]. Three ticks of the same species recovered from the same animal were sampled as a tick pool.

DNA was extracted from tick pools by salting out method [14]. Real-time PCR protocol targeting the small subunit ribosomal RNA gene was standardized separately for *B. vogeli* and *B. gibsoni* using a gradient thermal cycling program in an Eco™ Real-Time PCR System (Illumina).

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The primers for amplifying partial 18S rRNA gene of *B. vogeli* and *B. gibsoni* were designed using the Primer 3 software (www.bioinfo.ut.ee>primer3-0.4.0) based on corresponding gene sequences available in the Genbank (KX-766397- *B. canis* ;KX-766396- *B. gibsoni*). The primer sequences (Table 1) were custom synthesized (Sigma Aldrich, USA). Known positive control DNA of *B. canis* and *B. gibsoni* available in the Department of Veterinary Parasitology were used for initial standardization of the protocol for each pathogen species. Known negative control and no template control (NTC) were included in each run. In addition, the known positive controls of *B. canis*, *B. gibsoni*, *Trypanosoma evansi*, and *Hepatozoon canis* were also used as specificity controls to check any cross amplification of primers. The qPCR was performed in a 12.5 µL reaction volume containing 6.25 µL of Maxima SYBR green qPCR master mix (Thermoscientific, USA), 10 pmol each of forward and reverse primers of each species and 2.0 µL of DNA. The thermal cycling protocol included an initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation (94 °C for 30 s), annealing (60.1 °C and 58 °C for *B. vogeli* and *B. gibsoni*, respectively for 15 s) and extension (72 °C for 20 s). A dissociation curve analysis was performed after each reaction for checking specificity of the amplification. The program for melt curve analysis consisted of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s followed by denaturation at 95 °C for 15 s. Data acquisition was performed during the final denaturation step. In addition, the PCR products along with a 100 bp DNA ladder were resolved on a 3.5% agarose gel and visualized in a gel documentation system (Biorad, USA). Bidirectional sequencing using Sangers dideoxy chain termination method (AgriGenome Labs Pvt Ltd, Cochin) and sequence analysis using BLASTn (www.blast.ncbi.nlm.nih.gov>blast) were performed to confirm the specificity of the amplicons.

The conventional PCR was performed separately for *B. vogeli* and *B. gibsoni* using a gradient thermal cycling program (MG Mini, Biorad, USA) with the same set of primers and controls. The amplification was done in a 12.5 µL reaction volume containing 6.25 µL of 2X PCR master mix (TaKaRa Bio, Japan), 25 pmol each of forward and reverse primers and 2.0 µL of template DNA. A thermal cycling program was performed with an initial denaturation of 94 °C for 5 min followed by 34 cycles of denaturation (94 °C, 30 s), annealing (57–62 °C, 15 s) and extension (72 °C, 20 s) and a final extension at 72 °C for 5 min. The amplicons were visualized after agarose gel electrophoresis as mentioned earlier.

The tick DNA samples were analyzed by real-time as well as by conventional PCR protocols separately for detection of *B. vogeli* (n = 44) and *B. gibsoni* (n = 43) with specific primer sets and the results were compared by McNemar's test and kappa statistics using SPSS software version 24.1.

Out of the 132 ticks collected, 117 were identified as *R. sanguineus* (s.l.) and the rest were *H. bispinosa*. Adult males, females, and nymphs were used for the study. Real-time PCR targeting partial small subunit ribosomal RNA gene with *B. vogeli* specific primer sets yielded positive signals in 5 out of the 44 tick pools analyzed (11.35%). All the 5 positive tick pools were *R. sanguineus* (s.l.) which included two male pools. Out of the 43 tick DNA samples analyzed for *B. gibsoni*, 22 samples (51.16%) were positive. Majority of *B. gibsoni*-positive tick pools (n = 20) belonged to *R. sanguineus* (s.l.), and the rest (n = 2) were *H. bispinosa* females. The evidence of *B. gibsoni* could be detected in adult female (n = 14), male (n = 5) and nymph (n = 1) *R. sanguineus* (s.l.) ticks pools. The amplification plots (Figures 1 and 2) and melt curves were analyzed using the software (Eco™ Real-Time PCR System, Illumina). Melt curve analysis for each species-specific protocol revealed the specificity of the protocol. There were no detectable peaks with NTC, specificity controls and known negative control. The amplicons were also visualized in 3.5% agarose gel for additional confirmation during standardization.

Conventional PCR yielded specific bright bands of approximately 119 bp and 142 bp with known positive DNA templates of *B. vogeli* and *B. gibsoni* at annealing temperatures of 60.1 °C and 58 °C, respectively. All the tick DNA samples subjected to real-time PCR were also tested with conventional PCR for detecting the three pathogens under study. In comparison to the results of real-time PCR, lesser number of *R. sanguineus* (s.l.) ticks pools were tested positive for *B. vogeli* (n = 2) and *B. gibsoni* (n = 17). The proportion of *B. gibsoni* (n = 2) positive *H. bispinosa*

Table 1. Primers used in the study.

Sl no.	Target organism	Primer sequence
1	*Babesia vogeli*	RT. B.CA F- 5'- AGC AAT TGG AGG GCA AGT CT -3' RT. B.CA R – 5'- TGG CAA ACT CGA ACA CGC TA -3'
2	*Babesia gibsoni*	RT.B.GB F – 5' - GCC TTTTTGGCGGCGTTT AT – 3' RT.B.GB R – 5' – CTG CCT CGG TAG GGC CAA TAC – 3'

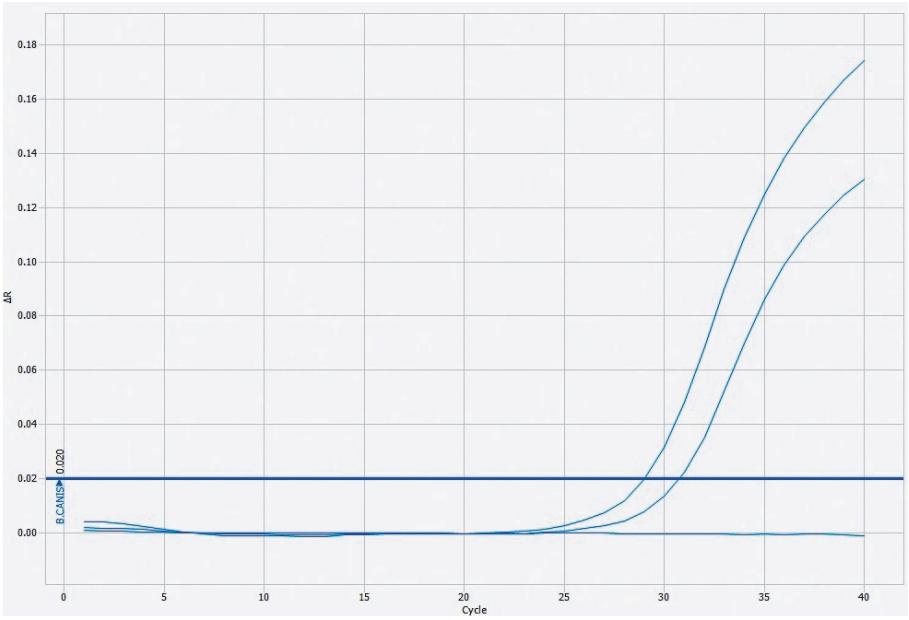

Figure 1. Amplification plot for *B. vogeli*.

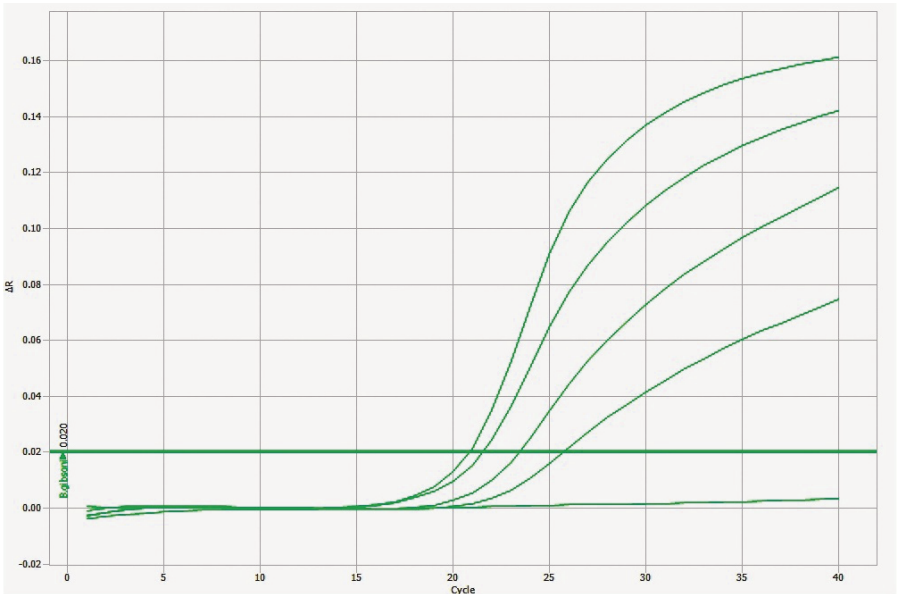

Figure 2. Amplification plot for *B. gibsoni*.

was also low when compared to that with real-time PCR protocols.

The bidirectional nucleotide sequences of the amplicons after alignment revealed 99%–100% similarity with corresponding gene sequences of the respective organisms in Genbank during BLASTn. These were submitted to Genbank and assigned with accession numbers (MK-734058.1- *B. vogeli*; MK 734060.1 - *B. gibsoni*).

The comparative evaluation of the two techniques for detection of the *Babesia* spp. parasites revealed that real-time PCR detected a higher percentage of infection when compared to conventional PCR. Mc Nemar's test performed for the comparison of results of conventional PCR and real-time PCR for detection of ticks infected with pathogens revealed that despite the higher percentage positivity obtained by real-time PCR, it was not statistically different from conventional PCR in detecting *Babesia* spp. The kappa statistics also supported the observation (Table 2). However, it is noteworthy that real-time PCR yielded positive signals for *Babesia* spp. rapidly. The cycle threshold

Table 2. Results of McNemar's test and kappa statistics.

	Real-time PCR Negative	Positive	P-value	Kappa value
Conventional PCR				
B. vogeli				
Negative	39	3	0.250[ns]	0.542
Positive	0	2		
B. gibsoni				
Negative	21	5	0.63[ns]	0.769
Positive	0	17		

ns – Non significant (P > 0.05)

values for all the samples were below 25. Conventional PCR failed to yield positive PCR signals when the number of cycles was below 34.

Real-time PCR protocol was successfully utilized for the detection of *B. vogeli* and *B. gibsoni* in ixodid ticks of dogs in India. Though *B. gibsoni* has been reported in *R. sanguineus* (s.l.) and *H. bispinosa* in India [6], it is noteworthy that *B. vogeli* could be detected in *Rhipicephalus* spp. only as observed in several studies conducted within and outside the country [6,15] by amplification of the 18S rRNA gene utilizing PCR. Real-time PCR protocols have been used to detect [16] and quantify [17] *Babesia* spp. in tick tissues. Except in a few reports [6,18], the evidence of *B. gibsoni* in *R. sanguineus* (s.l.) is rare and hence warrants further investigation into the vectorial capacity of brown dog ticks in India. Nevertheless, the protocol offered results in less than 25 cycles, thus proving to be a rapid diagnostic aid for infectious diseases. Although SYBR green real-time PCR protocol is cost-effective when compared to FRET and Taq man probes, it might yield a false-positive signal with nonspecific amplicons [19]. Visualization of the specific amplicon of desired size after agarose gel electrophoresis confirmed the specificity. Furthermore, on melt curve analysis a single peak corresponding to a single amplicon was observed. The sensitivity of molecular assay is also significantly influenced by the choice of gene targeted, robustness of primers, and the extent of protocol optimization [20]. In the present study, small subunit ribosomal RNA gene was targeted for amplification with the same set of species-specific primers for conventional and real-time PCR to ensure uniformity of comparison of the two techniques. The lower percent positivity with conventional PCR could be due to the lower detection limits of ethidium bromide stained agarose gels. Moreover, the template DNA extraction protocol as well as the reaction and template volume also were kept uniform for both the techniques, to circumvent the difference in analytical sensitivity [21,22]. The effective management of tick-borne hemoparasitic diseases demands sensitive tools to map the vector potentiality of the different tick species in an endemic region. Despite its high cost, real-time PCR possesses the definite advantage of not only yielding rapid results, but also of circumventing post-PCR handling of amplicons [23].

The present communication places on record the successful detection of the most prevalent babesial parasites in dog ticks in India utilizing SYBR green-based real-time PCR protocols with newly designed primers. The relatively simple protocol and the quick delivery of the results make it a preferred diagnostic during epidemiological surveys. In the era of climate change, the vectorial status of arthropods needs to be re-evaluated to provide better insight into transmission dynamics. The results of the present study are thus pertinent and needs to be carried out in a wider geographical area targeting different pathogens.

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