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Comparative analysis of conventional and real time PCR for detection of haemoparasites in dogs

Lavinia Wahlang, Bindu Lakshmanan[1*], Naicy Thomas, Anu Bosewell, Jain Jose K, Sunanda C[2] and Aravindakshan, T V

[1]School of Applied Animal Production and Biotechnology, Department of Veterinary Parasitology,
[2]Department of Statistics, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala 680651, India

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Ehrlichiosis and babesiosis are the most pathogenic tick-borne diseases of dogs worldwide. The present study reports that the development of SYBR green based real time PCR (RT-PCR) protocols with novel primers targeting small subunit ribosomal RNA genes to detect natural infections of *Ehrlichia canis*, *Babesia vogeli* and *B. gibsoni* in dogs and its comparison with conventional PCR. Statistical analysis revealed that RT- PCR is more superior to conventional PCR assay to detect low level rickettsaemia ($p < 0.05$). The high prevalence of these pathogens in the study population also warrants immediate attention to the adoption of efficient and sustainable control strategies.

Keywords: *Babesia vogeli*, *Babesia gibsoni*, *Ehrlichia canis*, real-time PCR, conventional PCR

Introduction

The growing profit margin from dog breeding as well as the companionship preference of a large sector of society, has led to an escalation in dog ownership in India. Recent reports[1] have suggested that 17% of the households in India own a pet dog and the economic impact of tick-borne pathogens can no more be neglected in dog breeding units. This has made it utmost necessary to control these pathogens at a very early stage to reduce the spread of disease and to reduce the endemicity. Novel diagnostics based on nucleic acid amplification technologies have often been evaluated for their efficiency in the detection of pathogens during natural infection and are an inevitable adjunct to sustainable management strategies. The common conventional methods based on microscopy from disadvantages like reduced sensitivity and specificity, the involvement of expertise, labour intensiveness etc. In the last two decades, the introduction of molecular techniques has resulted in increased detection of emerging and re-emerging vector-borne pathogens in different parts of the world[2].

Canine ehrlichiosis and babesiosis are the most prevalent pathogenic tick-borne infectious diseases of dogs in India[3]. Canine ehrlichiosis is caused by three genogroups of ehrlichiae, among which, genogroup III includes *E. canis*, which is responsible for widespread disease in tropical and temperate areas of the world. The fatal nature of canine ehrlichiosis is unequivocal. Acute ehrlichiosis is characterized by fever, depression, dyspnoea, anorexia and lymphadenopathy. The subclinical phase is associated with persistence of *E. canis*, thrombocytopenia, variable leukaemia and anaemia. Bone marrow suppression with pancytopaenia, bone marrow hypoplasia and bleeding tendencies are marked during the chronic phase[4]. The geographical distribution of *E. canis* has expanded with the distribution of its potential vector, *Rhipicephalus sanguineus*[5].

Canine babesiosis is caused by four different large *rossi, B. vogeli* and *B. presenti*) and three different small *Babesia* species (*B. gibsoni, B. conradae* and *B. vulpes*)[6-7]. It has been assertively concluded that *B. canis vogeli* is the prevalent subspecies in the country[8-9]. This species is transmitted by *R. sanguineus*. *B. gibsoni* is mainly transmitted by *Haemaphysalis longicornis* and *R. sanguineus* ticks[10]. Clinically canine babesiosis is exhibited in a wide range of presentations from subclinical disease to serious illness characterized by fever, pallor, jaundice, splenomegaly, weakness and collapse associated with intra and extra vascular hemolysis.

*Author for correspondence:
Tel: 09447486722; Fax- 0487-237038891
bindul@kvasu.ac.in

Conventional PCR has been validated by several authors for detection of these species in the country[9,11-15]. However, because of its higher sensitivity and rapidity, real-time PCR is considered as an alternative to conventional molecular assays[16]. It is considered more advantageous since it yields results during the progression of the reaction itself. Though real-time PCR has been used to detect canine babesiosis and ehrlichiosis, there has been no attempt to utilize this assay for detection of these species in the country. Moreover only limited attempts have been made to evaluate the comparative efficiency of conventional and real time PCR to diagnose natural infections in dogs. In this context, a study was aimed to develop real time PCR protocols for the detection of *Babesia* and *Ehrlichia* spp. during natural infection in dogs and to compare it with conventional PCR.

Materials and Methods

Sample Collection

Blood samples of dogs (n=100) presented to different hospitals of Kerala, South India with symptoms suggestive of babesiosis/ehrlichiosis[4-5] formed the material for the study. Giemsa stained blood smears were also examined to initially screen the animals to serve as known positive controls.

Extraction of DNA

DNA was extracted from EDTA added whole blood samples using the phenol–chloroform technique[17] with modifications[18].

Conventional PCR Protocol

The PCR protocol was standardised separately for *B. vogeli, B. gibsoni* and *E. canis* using a gradient thermal cycling programme (MG Mini, Bio-Rad, USA). The primers for amplifying a 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 (www.ncbi.nlm.nih.gov). Species specific primers of *E. canis* were selected[19] to amplify an approximately 152 bp region of 16S rRNA gene. The primers were custom synthesised (Sigma Aldrich, USA) (Table 1). The PCR was performed in a 12.5 µl reaction volume containing 6.25 µl of 2 X PCR master mixes (TaKaRa Bio, Japan), 25 pmol each of forward and reverse primers and 2.0 µl of template DNA. A gradient thermal cycling program with an initial denaturation of 94°C for 5 min followed by 34 cycles of denaturation (94°C, 30s), annealing (57°C-62°C,15s) and extension (72°C, 20s) followed by final extension at 72°C for 5 min was adopted. Known positive control as revealed by heavy parasitaemia during blood smear examination was selected for initial standardisation of the protocol for each species. A known negative control and no template control were included in each run. The specificity of each set of primers was additionally cross checked with DNA templates of *Babesia* spp., *Ehrlichia* spp., *Hepatozoon* spp., *Mycoplasma haemocanis* and *Trypanosoma* spp. The amplicons obtained consequent to conventional PCR using the specific primers for the three different pathogens were electrophoresed in a 1.5% agarose gel.

Quantitative Real Time PCR (qPCR)

The real-time PCR protocol was standardised separately for *B. vogeli, B. gibsoni* and *E. canis* using a gradient thermal cycling programme in an Eco™ Real-Time PCR System (Illumina) employing the same set of primers (Table 1) and known positive and negative controls of each species. The qPCR was performed in a 12.5 µl reaction volume containing Maxima SYBR green qPCR master mix 6.25 µl, 10 pmol each of forward and reverse primers of each species and 2.0 µl of DNA. Cycling began with 2 min at 94°C, followed by 40 cycles of denaturation for 30s at 94°C, annealing for 15s at specific temperature and extension for 20s at 72°C. The annealing temperature was 60.1°C, 58°C and 60.5°C, for *B. vogeli*, *B. gibsoni* and *E. canis*, respectively.

Table 1 — Primers used for the study

S. no.	Target organism	Primer sequence	Amplicon size
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'	119 bp
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'	142 bp
3	*Ehrlichia canis*	EHCA F- 5′- AGA GCA TGA AGT CGG AAT CG - 3' EHCA R - 5′- CCA ACC TTA AAT GGC TGC TT – 3'	152 bp

A dissociation curve analysis was performed after each reaction for checking specificity of the amplification. The programme for melt curve analysis consisted of denaturation at 95ºC for 15s, annealing at 55ºC for 15s followed by denaturation at 95ºC for 15s. Data acquisition was performed during the final denaturation step. All the samples were tested in triplicates. In addition, the PCR products along with a 100 bp DNA ladder were resolved on a 3.5% agarose gel at 80 V/cm for 30 minutes and visualized in a gel documentation system (Gel doc EZ Imager, Bio-Rad, USA). The specificity of protocol for each species was checked with DNA templates of other pathogens as mentioned earlier.

Bidirectional sequencing of the purified amplicons was done with both forward and reverse primers and sequenced using Sangers dideoxy chain termination method (AgriGenome Labs Pvt Ltd, Cochin) and subjected to sequence analysis using BLASTn (www.blast.ncbi.nlm.nih.gov>blast).

Comparison of Conventional PCR and qPCR

The results of conventional PCR and qPCR from randomly selected twenty DNA samples each for the three pathogens were compared by McNemar test and kappa statistics (SPSS software version 24.1).

Results

Conventional Polymerase Chain Reaction

The microscopic examination had revealed that 8, 10 and 2 dogs were positive for *B. vogeli*, *B. gibsoni* and *E. canis*, respectively. Polymerase chain reaction targeting partial small subunit ribosomal RNA gene of the three pathogens yielded specific bright bands of approximately 119 bp, 142 bp and 152 bp sizes with known positive control DNA templates of *B. vogeli* (Fig. 1), *B. gibsoni* (Fig. 2) and *E. canis* (Fig. 3) at annealing temperature of 60.1ºC, 58°C and 60.5ºC, respectively. 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. The sequence corresponding to *B. vogeli* revealed 100% with *B. canis vogeli* isolates from different countries, including the three Indian isolates from Myanmar, Bhubaneswar and Ludhiana. Thus, it could be concluded that species of large *Babesia* obtained in this study were *B. vogeli*. This, along with the absence of cross amplification with known positive control DNA of other species, confirmed the specificity of the primers and the protocol. Out of the 20 canine

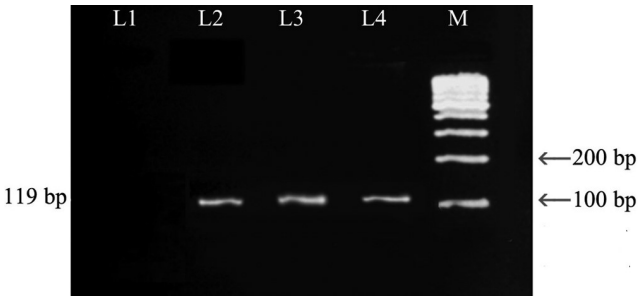

Fig. 1 — Conventional PCR for *B. vogeli*; M- DNA ladder; L2-L4 - positive samples of *B. vogeli*; L1- no template control (NTC).

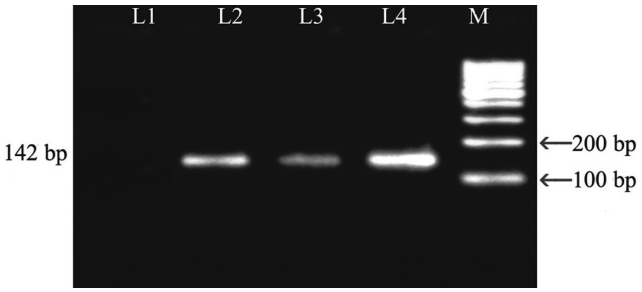

Fig. 2 — Conventional PCR for *B.gibsoni*; M- DNA ladder; L2-L4 - positive samples of *B. gibsoni* ; L1- NTC.

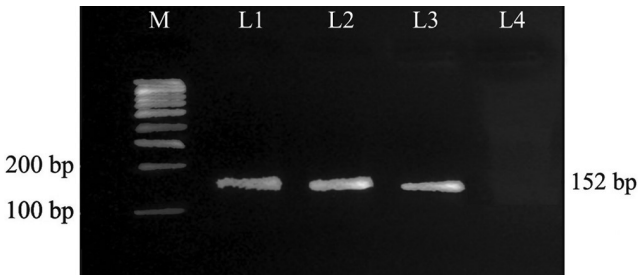

Fig. 3 — Conventional PCR for *E. canis*; M- DNA ladder; L1-L3 - positive samples of *E. canis* ; L4- NTC.

samples subjected to conventional PCR for each species, *B. vogeli*, *B. gibsoni* and *E. canis* infection were detected in 8 (40%), 10 (50%) and 7 (35%) samples, respectively. The nucleotide sequences were not submitted to the GenBank because the sequence length was lesser than 200 bp.

Real Time Quantitative PCR

Real time PCR targeting the 18S rRNA gene of *B. vogeli* was standardized by subjecting the respective known positive control DNA for amplification by a gradient protocol. Amplification plot (Figs. 4, 5 & 6) and melt curve were generated. Melt curve analysis for each species-specific protocol revealed the specificity of the protocol. There were no detectable peaks with no template control (NTC) and known negative control. Additionally, the identity of

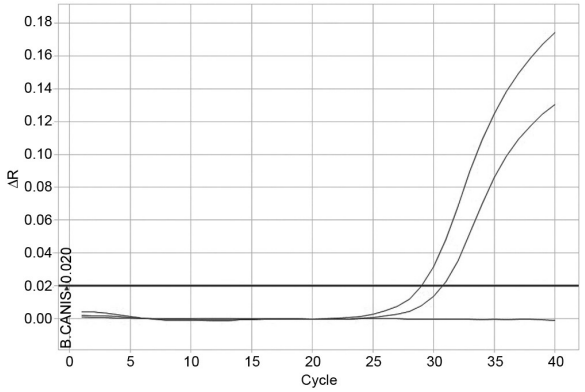

Fig. 4 — Amplification plot for *B. vogeli.*

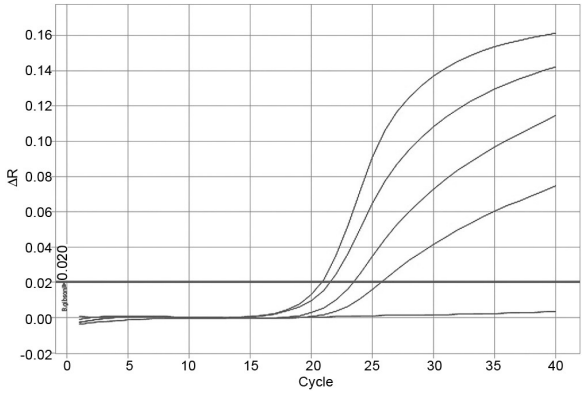

Fig. 5 — Amplification plot for *B. gibsoni.*

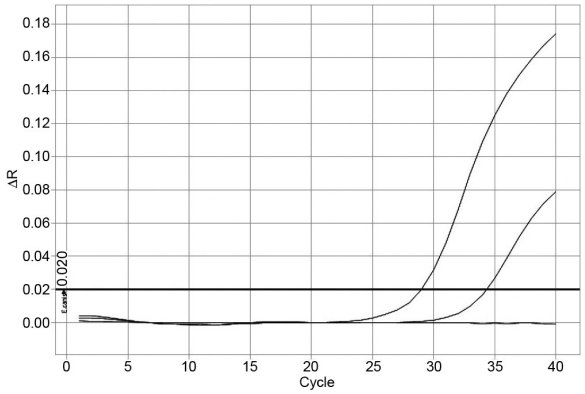

Fig. 6 — Amplification plot for *E. canis.*

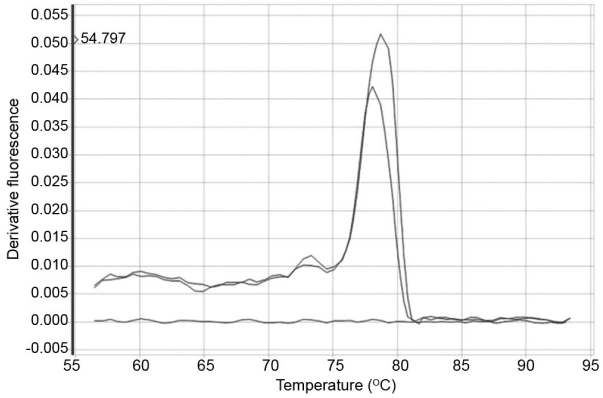

Fig 7 — Melt curve for *B. Vogeli.*

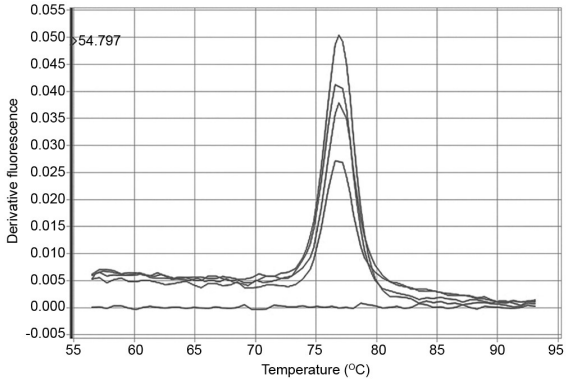

Fig 8 — Melt curve for *B. gibsoni.*

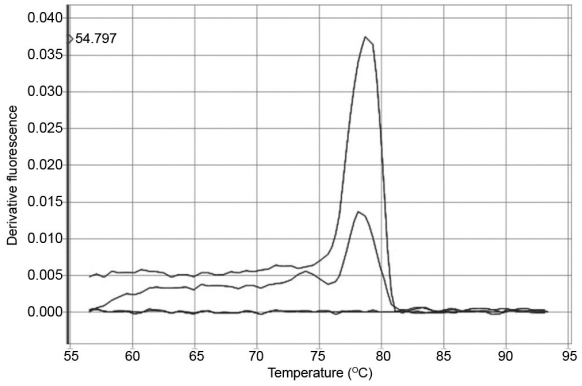

Fig 9 — Melt curve for *E. canis.*

amplicons was also confirmed by visualizing in 3.5% agarose gel. Out of the 20 canine blood DNA samples, *B. vogeli*, were detected in 11 cases (55%), while 14 samples (70%) each, revealed positive signals for *B. gibsoni* and *E. canis*.

Comparison of Conventional and Real Time PCR

The comparative evaluation of the two techniques for detection of pathogens revealed that real time PCR detected a higher percentage of positive cases when compared to conventional PCR based on visualization of ethidium bromide stained amplicons electrophoresed in agarose gel. Comparison of the two protocols revealed that both conventional as well as real time PCR had no significant difference in detecting natural infections of large (p value: 0.25, kappa value: 0.706) and small *Babesia* species (p value: 0.125, kappa value: 0.600). However, real time PCR had a statistically significant ($p < 0.05$) superiority in detecting *E. canis* infection (p value: 0.016, kappa value: 375).

Discussion

Despite the alarming prevalence of babesiosis and ehrlichiosis among dogs in South India along with the high rate of tick infestation, little attempts have been made to investigate the true prevalence of these pathogens in its natural hosts using sensitive detection protocols. The rapid evolution of molecular techniques has facilitated more sensitive and accurate detection of tick borne pathogens in a particular geographic environment. Though diagnosis and genetic diversity analysis of large and small *Babesia* spp. were attempted with several conventional PCR based assays,[8,12,14,20-22] and real-time protocols[23-30], comparative diagnostic efficacy of the two tests was rarely investigated. Hence, the present study for the development and evaluation of sensitive diagnostics for tick-borne pathogen species prevalent in India is attempted. The real-time protocol with newly designed primers detected a higher prevalence of natural infections of *B. vogeli* (55%) and *B. gibsoni* (70%) in canine hosts when compared with conventional PCR, even though, the difference in detection was not statistically significant. Nevertheless, when the operating procedures were compared, the real time PCR exhibited a decisive advantage. Moreover, it could also be speculated that the higher percentage of positivity observed with real time PCR offers definite superiority for detection of carrier status or subclinical infection with low parasitaemia, especially during epidemiological surveys involving both hosts and vectors.

Molecular detection of *E. canis* is extremely important owing to the low rickettsemia during natural infection which precludes its detection by conventional techniques. Different PCR based assays like nested PCR and real time PCR had been used for *E. canis* detection[11,31-42]. However, other methods like loop mediated isothermal amplification (LAMP) have been more sensitive than nested PCR for detection of *E. canis* in dogs[13]. The findings of our study support the enhanced sensitivity of the newly designed SYBR green based real time PCR assay than the conventional assay to detect rickettsemia during natural ehrlichiosis. Real time PCR could detect infection in 70% blood samples as against the 35% samples which turned out to be positive by conventional PCR. The statistically significant difference between between conventional and real time PCR for *E. canis* detection in blood samples were ascertained by McNemar test statistics ($p < 0.05$) and the low kappa value, revealing the lack of agreement between the two tests. This, along with indisputable technical advantages like speed, reduced chance of cross contamination and the option for delivery of results during the progression of reaction itself, bypassing the need for further analysis of the results in separate agarose gel electrophoresis as required in the case of conventional PCR, advocates the need for adoption of real time protocol for sensitive diagnosis of canine ehrlichiosis. Although, real time PCR was no more sensitive than conventional PCR[43], some of the validated studies showed that real time PCR was better for detection of both eukaryotic and prokaryotic pathogens. The enhanced potential of real time PCR to detect low level *Plasmodium* infections[44], visceral leishmaniasis[45] *E. canis*[46] and *Rickettsemia rickettsii*[47] are well documented.

The advantage of SYBR green real time protocol lies in its cost effectiveness when compared to fluorescence resonance energy transfer (FRET) and TaqMan probes, but it suffers from the major limitation of yielding a false positive signal with non-specific amplicons[48]. However, in the present study, the primers when tested with extracted DNA could yield only a single amplicon, specific with the desired size, when tested with agarose gel electrophoresis. Further, on melt curve analysis a single peak corresponding to a single amplicons was observed. The choice of gene targeted for amplification, fitness and robustness of primers and the degree of assay optimisation significantly affect the sensitivity and reliability of molecular assays[47]. Owing to the fact that, number of copies of the target DNA in the genome of the microorganism is an essential factor that affects sensitivity[43], the two test protocols in our study were designed to target gene of high copy number in each pathogen. Small subunit ribosomal RNA genes were targeted for amplification with the same set of species specific primers for conventional and real time PCR, thus assuring the uniformity for comparison of the two techniques for diagnosis of natural infection of each pathogen species. The template DNA for both the techniques was extracted using the same protocol to overcome the influence of efficiency of the extraction methods in assay sensitivity[49].

Moreover, the reaction and template volume for both the assays were kept the uniform, to circumvent the difference in analytical sensitivity due to the volume of template DNA input in the PCR[50]. However, the detection limits of agarose gel electrophoresis and ethidium bromide staining might

have contributed to the lower sensitivity of conventional PCR in this study. In order to avail the benefit of a simpler protocol for any diagnostic assay, we have not attempted more sensitive detection of amplicons through polyacrylamide gel electrophoresis followed by silver staining. The comparable performance of real time PCR and conventional PCR assays to detect canine babesiosis could be due to the relatively higher parasitaemia when compared to canine ehrlichiosis during natural infections in canine hosts.

The effective management of tick-borne haemoparasitic diseases requires rapid, reliable and highly sensitive diagnostic test, which can also serve to monitor the effectiveness of the therapeutic and prophylactic measures. Real time PCR had not been yet utilized to detect canine vector borne pathogens in South India. Despite the high cost of real time PCR, which may prevent the technique from being used in resource poor settings, its advantageous performance characteristics and rapid results warrant its use as a diagnostic adjunct for parasitic infections.

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