RESEARCH ARTICLE

Vector-Borne Dynamics of Canine *Babesia* Species: A Study on Trans-Stadial and Trans-Ovarian Transmission

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Abstract

Canine babesiosis is an emerging haemoprotozoan disease affecting domestic and wild canids, globally. Among the primary vectors, *Rhipicephalus sanguineus* and *Haemaphysalis longicornis* play crucial roles in the disease transmission dynamics. The present study was aimed to investigate the potential role of different life stages of *R. sanguineus* in the transmission of canine babesiosis. Genomic DNA was extracted from various life stages of *R. sanguineus* ticks. 18S rRNA based molecular analysis revealed that 7.0% (23/330) of the samples were positive for *Babesia* species. Semi-nested PCR analysis showed no amplification for any *Babesia* species in DNA extracted from male ticks. A single DNA sample from a pool of eggs laid by an infected female tick tested positive for *Babesia gibsoni*, although further molecular and sequencing analyses are required for confirmation. This vector-based molecular epidemiological study indicated that *R. sanguineus* is more commonly associated with the transmission of *Babesia vogeli* (74.01%) than *Babesia gibsoni* (25.95%).

Key words: *Babesia gibsoni*, Canine babesiosis, Semi-nested PCR, *Rhipicephalus sanguineus*, Trans-ovarian transmission.

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Introduction

Canine babesiosis is one of the more common tick-borne, intra-erythrocytic haemoprotozoan disease caused by apicomplexan protozoan disease distributed globally (Penzhorn *et al.,* 2017). *Babesia canis*, *B. vogeli*, *B. rossi* and *B. gibsoni* are the more common canine *Babesia* species distributed more often in tropical and subtropical countries. Among which, *B. gibsoni* is the smaller form (1.0-2.5 µm), whereas other three species classified as larger forms (2.5-5.0 µm) based on morphometric analysis (Mittal *et al.,* 2019). However, *B. gibsoni* and *B. vogeli* are considered as the major etiological agents for canine Babesiosis in India (Kundu *et al.,* 2012; Roopesh *et al.,* 2018). This infection is primarily caused by blood feeding infected Ixodid ticks. However, the infection can be transmitted through infected dog bite (Konishi *et al.,* 2008; Yeagley *et al.,* 2009), blood transfusion (Stegeman *et al.,* 2003), trans-placental transmission (Fukumoto *et al.,* 2005).

Rhipicephalus sanguineus and *Haemaphysalis longicornis* are more common hard ticks (Ixodidae) transmitting canine Babesiosis in India (Shaw *et al.,* 2001). Sporadic outbreaks of canine babesiosis have been reported in Tamil Nadu (Lakshmanan and John 2007; Sundar *et al.,* 2004; Senthilkumar *et al.,* 2009). The clinical manifestation of *B. vogeli* infection can vary from moderate sub-clinical (Irwin, 2009; Solano-Gallego and Baneth, 2011) to fatal (Abalaka *et al.,* 2018; Ajoke *et al.,* 2019). The infected adult dogs can act as foci of infection (carrier) to other animals (Beck *et al.,* 2009; Hamel *et al.,* 2009). However, *Rhipicephalus sanguineus* plays significant role in transmitting *B. vogeli* (Nava *et al.,* 2015).The pathogenicity of canine babesiosis can vary from targeting erythrocyte to multiple visceral organs such as hepatic failure, immune-mediated haemolytic anaemia (IMHA), cerebral babesiosis, acute renal failure (ARF), acute respiratory distress syndrome (ARDS), based on extrinsic factors (species of *Babesia,* vector potentiality) and intrinsic factors (host factors such as age, immune response and breed) (Obeta *et al.,* 2020). To date, only a limited number of studies have been carried out to investigate the transmission dynamic pattern of canine babesiosis by *Rhipicephalus sanguineus* (Chao *et al.,* 2016). Hence, the present study was undertaken to study the disease transmission dynamics of various canine *Babesia* species in commonly found brown dog tick vector, *R. sanguineus*.

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Materials and Methods

The study was conducted for a period of 6 months (January to June 2023). Various developmental stages of *Rhipicephalus sanguineus* were collected from 330 owned dogs suspected

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for babesiosis presented at Teaching Veterinary Clinical Complex (TVCC), Madras Veterinary College, Chennai, Tamil Nadu (India). Chennai is located on the Coromandel Coast, at the northern end of the Indian state of Tamil Nadu. The city stretches along this coastline, boasting a long expanse of sandy beaches, and extends inland with an irregular shape that covers approximately 426 square kilometers. The current estimated population of Chennai is around 7.1 million, with at least 1.81 lakh stray dogs in the city.

Sample Size Determination

Brown dog ticks of various life stages were collected from 330 dogs of clinically suspected babesiosis cases by using simple random sampling method and the sample size were determined using Thrusfield formula (1995).

$$n = Z^2 * P*(1- P)/ d^2$$

Where, n- required sample size; P- expected prevalence from previous study; d- margin of error (5%); Z= 1.96 (at 95% confidence interval). The P-value was considered as 31% from the previous epidemiological study on canine haemoprotozoan infections in Chennai (Senthil and Chakravarthi, 2023).

Sample collection

Around 639 male ticks, 425 engorged female ticks and 378 semi-engorged nymphs, 524 larval stages of *R. sanguineus* were collected from dogs (n=330) clinically suspected for canine babesiosis or tick infestation. Ticks were collected directly from the infested dogs by manual picking. All these developmental stages of ticks were stored in 70% alcohol, except engorged female ticks. The engorged female ticks were incubated at 28ºC and 85% relative humidity in a dark place for oviposition and further development. All the developmental stages from the engorged female ticks were subjected to molecular assay.

Extraction of DNA from Various Stages of Tick

The morphological identification of ticks was done through microscopic examination (Dantas-Torres *et al.,* 2013). The ticks collected from various individuals were pooled together as male ticks, female ticks, larva, nymph and eggs under stereomicroscope. The genomic DNA of various developmental stages of *R. sanguineus* was extracted as described by Roopesh *et al.* (2018). Briefly, the ticks were snap frozen in liquid nitrogen (-196°C) and then crushed into fine powder using sterile mortar and pestle. The genomic DNA was extracted from the powdered tick tissue using the DNeasy® Blood and Tissue Kit (Qiagen, Germany) as per the manufacturer's guidelines and the DNA samples were stored at -20°C. The concentration of DNA elute was determined at 260 nm in Nanospectrophotometer (Nano drop, Thermoscientific, USA) and purity at 260:280 nm ratio. Samples which yielded a ratio between 1.8 and 1.9 were selected for analysis.

Molecular Identification

Molecular screening of tick genomic DNA was done using *Babesia* genus specific primer (Table 1) (Birkenheuer *et al.,* 2013) with the same thermal cycling conditions and composition of reaction mixture. The resultant PCR products using genus specific-primers were subjected to semi-nested PCR for species identification (Augustine *et al.,* 2017). Briefly, in the semi-nested PCR, 50 μL reaction volume containing 1.25 U of AmpliqonTaq /reaction, 25 pmol of each primer, 200 μM concentrations of each deoxynucleoside triphosphate, 1.5 mM MgCl and a 1 μL concentration of PCR buffer II. The first reaction of semi-nested PCR with outer primer pair was performed in a thermal cycler at the following conditions: initial denaturation at 95°C for 5 min, followed by 50 amplification cycles (95°C for 45 s, 58°C for 45 s and 72°C for 45 s) and a final extension step at 72°C for 5 min. In the second reaction, same thermal cyclic conditions were followed using 0.5 μL of amplicon from the first reaction as template DNA and the outer reverse primer with species specific forward primers in separate tubes for 34 cycles.

The PCR products were subjected to gel electrophoresis, with 2% agarose gel and the product sizes were determined based on standard DNA ladder using Bio-Rad Gel Documentation system XR+ with Image Lab software version 3.0, USA (Bio-Rad, USA).

Table 1: Details for genus specific primers and semi-nested PCR primers

S.No.	Primer	Primer Sequence(5'-3')	Target organism
1	5-22F	GTTGATCCTGCCAGTAGT	18S rRNA forward primer
2	1661R	AACCTTGTTACGACTTCTC	18S rRNA reverse primer
3	455-479F	GTCTTGTAATTGGAATGATGGTGAC	Outer forward primer
4	793-772R	ATGCCCCCAACCGTTCCTATTA	Outer reverse primer
5	BgibAsia-F	ACTCGGCTACTTGCCTTGTC	*B. gibsoni* specific forward primer
6	BCV-F	GTTCGAGTTTGCCATTCGTT	*B. vogeli* specific forward primer
7	BCC-F	TGCGTTGACGGTTTGACC	*B. canis* specific forward primer
8	BCR-F	GCTTGGCGGTTTGTTGC	*B. rossi* specific forward primer

Statistical Analysis

The occurrence of species of canine Babesiosis in ticks and the significant difference in its frequency of various life-stages of *R. sanguineus* were analysed by Chi-square test using SPSS version 18.0.

Results and Discussion

A total 639 male ticks, 425 engorged female ticks and 378 semi-engorged nymph, 524 larval stages, 272 batches of eggs of *R. sanguineus* were collected from dogs of 3 months to 2 years old (n=330). Among all the samples, 158 samples showed amplification at ~ 1.7 kb using 18S rRNA gene targeted genus specific primers in 1.5 % agarose gel electrophoresis (Fig. 1) indicating tick infected with *Babesia* species. This research finding was in accordance with the previous molecular epidemiological study on canine babesiosis (Birkenheuer *et al.,* 2013).

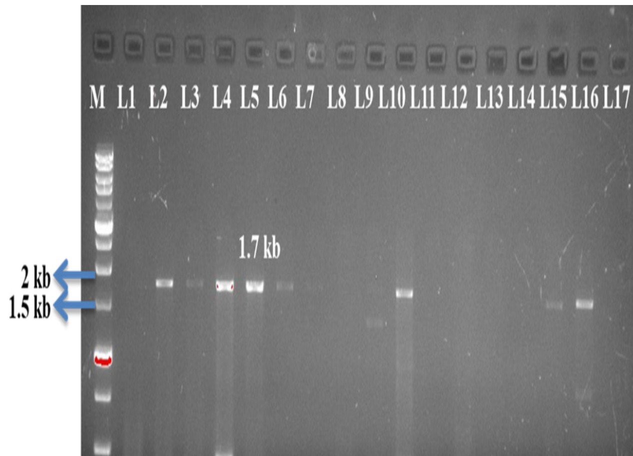

Fig. 1: 1% Agarose gel electrophoresis showing amplification at 1.7 kb using 18S rRNA based primers of *Babesia* genus. Mà 1 kb ladder; L1,L7-L9, L11-14à Negative samples; L2-L6,L10,L15à Positive samples.L15- DNA extracted from egg of *Rhipicephalus sanguineus*.

The PCR analysis using genus specific primer showed the prevalence rate of canine *Babesia* in female ticks, nymph, male ticks, larva and eggs of *R. sanguineus* as 17.65% (75/425), 5.56% (21/378), 0 % (0/639), 11.64 % (61/524), and 0.4% (1/272), respectively. However, no amplification was observed with 18S rRNA-based primers in the DNA extracted from male *R. sanguineus,* indicating potentially a lower vector competence and implies a negligible role of male ticks in the transmission dynamics of *Babesia* species. On the contrary, Chao *et al.* (2016) recorded nymphal stages (2.42%) of *R. sanguineus* were more commonly infected with the canine babesiosis than female (1.97%) and male (0.98%) ticks in northern Taiwan using 18S ssrRNA based nested PCR assay.

The primary reaction using BgibAsia-F revealed amplification at 340 bp (Fig. 2) with the overall occurrence of *B. gibsoni* in various life-stages of *R. sanguineus* as 1.83%. This finding was consistent with the observations of Birkenheuer *et al.* (2013), who stated successful amplification of *B. canis*, *B. rossi*, *B. vogeli* and *B. gibsoni* (Asian genotype) in the primary reaction of semi-nested PCR at 340 bp.

Secondary reaction of semi-nested PCR using species specific forward primers with common outer reverse primer yielded resultant products at 192 bp and 185 bp for *B. c. vogeli* (Fig. 3) and *B. c. gibsoni* (Fig. 4), respectively, along with the primary reaction product at 340 bp as mentioned by Birkenheuer *et al.* (2013).

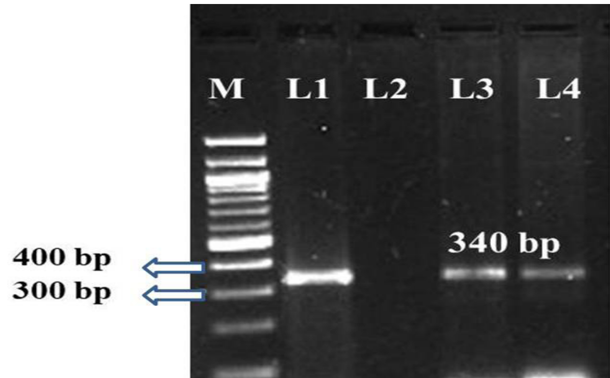

Fig. 2: 1.5% Agarose gel electrophoresis showing amplification at 340 bp for the primary reaction products of semi-nested PCR. M- 100 bp DNA ladder, L1- Positive control, L2- Negative control, L3 and L4- Positive *Babesia* samples.

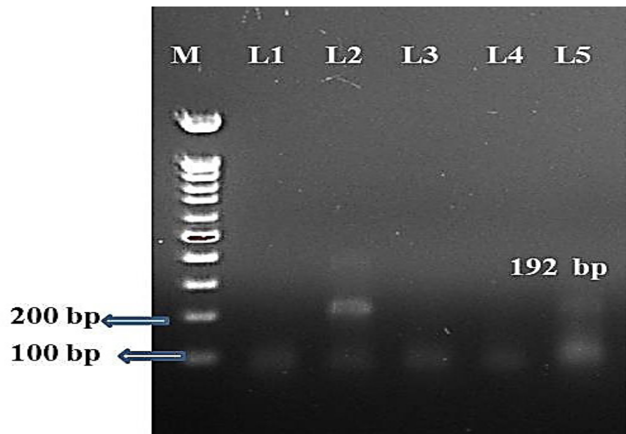

Fig. 3: 2% Agarose gel electrophoresis revealed amplification of 18S rRNA gene of *Babesia vogeli* at 192 bp. L2- Positive sample, L1 & L3- Negative samples, L4- Negative control, L5- Positive control

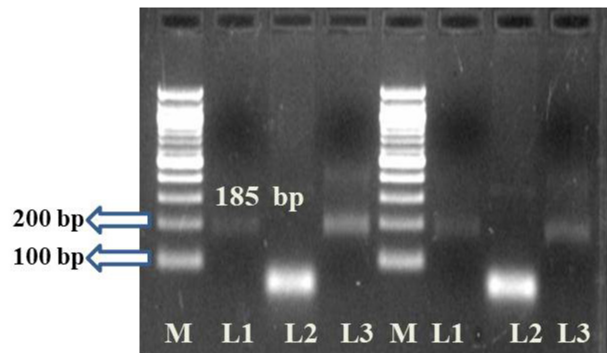

Fig. 4: 2% Agarose gel electrophoresis revealed amplification of 18S rRNA gene of *Babesia gibsoni* at 185 bp. L1- Positive control, L2- Negative control, L3- Positive samples.

Table 2: Developmental stages of *R.sanguineus* infected with *Babesia vogeli by* PCR assay

Female ticks		Male ticks		Eggs (Batches)		Larva		Nymph	
No. screened	No. (%) Positive	No. screened	No. (%) Positive	No. screened	No. (%) Positive	No. screened	No. (%) Positive	No. screened	No. (%) Positive
425	53(12.47)	639	0	272	0	524	48 (9.16)	378	16 (4.23)

Chi-square value (X^2) =112.36, Degree of freedom (df)= 4, p-value= 2.28E$^{-23,}$ Highly significant ($p<0.01$)
Mean Square Contingency (F2) =0.0502, Coefficient of Mean-square Square Contingency=0.2186

Table 3: Developmental stages of *R.sanguineus* infected with *Babesia gibsoni by* PCR assay

Female ticks		Male ticks		Eggs (Batches)		Larva		Nymph	
No. screened	No. (%) Positive	No. screened	No. (%) Positive	No. screened	No. (%) Positive	No. screened	No. (%) Positive	No. screened	No. (%) Positive
425	22(5.18)	639	0	272	1(0.37)	524	13 (2.48)	378	5 (1.32)

Chi-square value (X^2) =43.37, Degree of freedom (df)= 4, p-value= 8.66E$^{-09,}$ Highly significant ($p<0.01$)
Mean Square Contingency (F2) =0.0194, Coefficient of Mean-square Square Contingency=0.1379

The disease transmission potential of different developmental stages of *R. sanguineus* was statistically evaluated for *B. vogeli* (Table 2) and *B. gibsoni* (Table 3) using semi-nested PCR analysis.

These findings were in accordance with Augustine *et al.* (2017), who observed the amplification of 18SrRNA gene at 185 bp for *B. gibsoni*, while, the amplicon of secondary semi-nested PCR observed at 192 bp for *B.vogeli* using the respective species specific forward primer. On the contrary, 18S rRNA based molecular based epidemiological study on canine babesiosis in Rajasthan showed only 24% of suspected canine blood samples were positive for *B. canis*. In addition, no other canine *Babesia* species were identified in that study (Choudhary *et al.,* 2025). Additionally, DNA samples extracted from various developmental stages of *R. sanguineus* showed no amplification with the species-specific forward primers (BCC-F and BCR-F), indicating the absence of *B. canis* and *B. rossi* in the study population. This study reveals the identification of *B. vogeli* and *B. gibsoni* in female, nymph and larval stages of ticks that suggests the epizootological relevance of trans-stadial transmission of *R. sanguineus*. Similarly, an epidemiological study on canine babesiosis conducted in Peninsular Malaysia revealed the occurrence of both *B. vogeli* and *B. gibsoni* in *R. sanguineus* (adult and nymph) as 1.43% using 18S rRNA based molecular assay (Prakash *et al.,* 2018). Likewise, Do *et al.* (2021) reported that the infection of *R. sanguineus* with *B. vogeli* was the second most common tick-borne haemoprotozoan infection (9.4%) in Thailand, based on 18S rRNA molecular assay, with an overall haemoprotozoan infection prevalence of 38.8% in ticks.

In the present study, DNA extracted from a pool of eggs laid by infected female ticks showed amplification at 185 bp using species-specific forward primers for *B. gibsoni* in semi-nested PCR assay, indicating the mere possibility of transovarian transmission of *B. gibsoni* by *R. sanguineus*. However, this needs further confirmation through comprehensive molecular investigations. Transovarial transmission of *B. gibsoni* has been recorded in *Haemaphysalis hystricis* in Taiwan (Jongejan *et al.,* 2018). But there are no reports on transovarian transmission of *Babesia* species by *R.sanguineus*. Therefore, subsequent extensive studies with larger sample sizes, accompanied with sequencing analyses are required to confirm the transovarial transmission pattern of *Babesia* species by *R. sanguineus.*

Conclusion

Canine babesiosis is a significant emerging haemoprotozoan disease affecting both domestic and wild canids worldwide. The present study aimed to investigate the potential for trans-stadial and transovarian transmission of four major *Babesia* species using molecular assays. The findings demonstrated evidence supporting the possibility of trans-ovarian transmission of *Babesia gibsoni* in infected *R. sanguineus* ticks. However, further investigation integrating advanced molecular diagnostics and vector biology techniques are warranted to better elucidate the mechanisms underlying the transmission dynamics of canine babesiosis from tick vectors.

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