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MOLECULAR DETECTION AND PHYLOGENETIC ANALYSIS OF *BABESIA VOGELI* INFECTIONS IN DOGS OF THRISSUR, KERALA, INDIA

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ABSTRACT

Babesiosis in dogs is a severe, life-threatening blood parasite disease that is found all over the world. The molecular detection and phylogenetic analysis of *Babesia vogeli (B. vogeli*) was investigated in dogs from Thrissur, Kerala by using polymerase chain reaction (PCR) assay. A total of 200 dogs suspected of tick-borne haemoprotozoan infections were screened by both light microscopy and PCR targeting the *18S rRNA* gene of *B. vogeli*. The comparative evaluation of the two techniques for detection of pathogens revealed that PCR detected a higher percentage of positive cases (27 per cent) when compared to light microscopy (18.50 per cent). Phylogenetic analysis showed that the sequence obtained in this study had close relations to sequences from India, France, the USA, Germany, Thailand, Taiwan, Brazil, China, and Tunisia.

Keywords: *Babesia vogeli*, Light microscopy, Polymerase chain reaction (PCR), 18S rRNA

Tick-borne diseases (TBDs) in domestic pets has received more public attention recently due to the emergence of new endemic areas, improved diagnostic sensitivity, and increased awareness among pet owners and veterinarians alike (Leschnik *et al.,* 2008). In India, there reports of high prevalence of ticks and tick-borne diseases in Gujarat and South India, and these diseases are reportedly gradually spreading to other parts of the country (Negi *et al.,* 2021). Canine babesiosis and ehrlichiosis were considered as the most prevalent

vector-borne haemoparasites among dogs in India, and Ajith *et al.* (2024) found a high prevalence of haemoparasitism (28.35 per cent) in dogs from Thrissur, Kerala, which included infections such as *Babesia gibsoni* (40.89 per cent), *Babesia vogeli* (16.67 per cent) and *Ehrlichia canis* (15.17 per cent).

Babesia vogeli was the large babesia species reported in Kerala (Augustine *et al.,* 2017: Jose *et al.,* 2018). Though the symptoms of canine babesiosis are pathognomonic, fever, anorexia, epistaxis, petechiae, splenomegaly along with haemoglobinuria, anaemia and thrombocytopaenia are common (Coralic *et al.,* 2018). Traditionally, laboratory diagnosis has relied on the light microscopic detection of intraerythrocytic piroplasms. However, this technique is laborious, time-consuming and has the limitation that demonstration of intraerythrocytic piroplasms depends on the stage of disease (Costa-Junior *et al.,* 2012) and is hence not confirmatory. On the other hand, molecular diagnosis shows high sensitivity and specificity, making it useful and adequate to detect low parasitaemia levels even during the subclinical or chronic stages of the disease (Ionita *et al.,* 2023).

In India, the occurrence of canine babesiosis has increased exponentially over the past two decades, transitioning from occasional cases to endemic regions, especially in Kerala. However, there exists a paucity of data concerning the genetic profiling of *B. vogeli* in the study area. Hence, the current investigation was undertaken with a view to enhance our understanding of the molecular detection and phylogenetic analysis *B. vogeli* infections in dogs of Thrissur, Kerala.

MATERIALS AND METHODS

Study area

The study was carried out in Thrissur, located in the central part of Kerala. The geographical coordinates of Thrissur are approximately 10.5276° N latitude and 76.2144° E longitude. The climate of Thrissur, Kerala, in general, plays a significant role in the prevalence and transmission of tick-borne infections in dogs. The warm, humid, and relatively stable climate of Thrissur, Kerala provides a conducive environment for the survival and transmission of ticks that carry babesia parasites.

Study plan

This molecular study was conducted from January 2024 to June 2024 at the University Veterinary Hospitals, Kokkalai and Mannuthy. Dogs with clinical signs suggestive of haemoparasitism like fever, anaemia, thrombocytopaenia, weakness, etc. were included in the present study.

Peripheral blood smears were collected from the suspected dogs on the day of presentation for initial screening and were examined using Field's staining technique. For molecular studies, the EDTA anticoagulated blood samples were obtained from the dogs and stored at 4°C for DNA extraction.

DNA extraction, molecular and phylogenetic analysis

Genomic DNA was isolated from 200 µL of the EDTA buffered whole blood using ORIonX genomic DNA kit, Kerala (Cat. No. ODP304), according to the manufacturer's instructions. The isolated DNA was eluted in 200 µL of Tris-EDTA buffer and stored at -20°C until further analysis. The molecular screening for *B. vogeli* was conducted as per Duarte *et al.* (2008) with the forward primer BAB1 (5'-GTG AAC CTT ATC ACT TAA AGG-3') and reverse primer BAB4 (5'-CAA CTC CTC CAC GCA ATC G-3'). The amplification was conducted in an automated thermal cycler (BIORAD C1000 Touch™, Poland) under the following cycling conditions: an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. The PCR products underwent analysis using 1.5 per cent agarose gel electrophoresis, visualized by the molecular imager (BIO-RAD ChemiDoc™ XRS+, USA) and the test results were documented. Products obtained after polymerase chain reaction were sequenced in both directions using available sequencing facility at GeneSpec, Private Ltd., Kerala. The obtained nucleotide sequences were assembled and aligned using the BioEdit software, and their homology with other published sequences in GenBank was analysed using the NCBI BLAST tool before being submitted to the GenBank database. Phylogenetic analysis was carried out with MEGA-X software, which generated a maximum likelihood tree based on the Kimura-2 parameter model with 1000 bootstrap replicates.

Statistical analyses

The results of light microscopy and conventional PCR were compared by receiver operating characteristic (ROC) and kappa statistics was employed to find the agreement between the tests using SPSS software version 24.0. According to Thrusfield *et al.* (2018), kappa statistics is an approach to assess the agreement between different tests, without assuming that one test is the best. It is interpreted as follows: ≤0.2 as poor agreement, 0.21 to 0.40 as fair agreement, 0.41 to 0.60 as moderate agreement, 0.61 to 0.80 as good agreement and >0.80 represents a very good agreement.

RESULTS AND DISCUSSION

Babesiosis, a serious and potentially life-threatening tick-borne disease in dogs caused by *Babesia gibsoni* (small Babesia) and *Babesia vogeli* (large Babesia), varies in severity based on the babesia species and the host's immune response (Mittal *et al.,* 2019; Guo *et al.,* 2020). In the study conducted by Abd-Rani *et al.* (2011), both *B. gibsoni* and *B. vogeli* were co-endemic with its pathogenicity varying in different regions of India.

The current study involved 200 dogs of different breeds and age groups, presented at the outpatient wards of the university veterinary hospitals in Kokkalai and Mannuthy from January 2024 to June 2024, with a range of clinical conditions. The age of dogs varied from 43 days to 16 years and included both male and female animals belonging to different breeds including non-descript dogs. Examination of the Field's-stained peripheral blood smears from suspected dogs revealed typical pear-shaped pyriform bodies (Fig. 1) in the red blood cell of infected dogs in 18.50 per cent (37/200) of cases presented. According to Obeta *et al.* (2020), this protozoan haemoparasite usually occurs in pairs or single pear-shaped or as multiple merozoites divided by binary fission within the erythrocytes.

Isolated DNA samples from all 200 dogs were tested for the presence of *Babesia vogeli* species-specific primers that target the *18S rRNA* gene. Electropherogram revealed that, 54 samples (27.00 per cent) were positive for *B. vogeli* with a product size of 590 bp (Fig. 2). The infection with *B. vogeli* was confirmed through sequencing a segment of the *18S rRNA* gene from positive cases. Sequencing of the amplified product revealed a nucleotide identity of 100 per cent with *B. vogeli* sequence from a domestic dog of Delhi (India) available in GenBank under accession number MN165667.

Comparing the present sequence to those published in GenBank revealed identity rates ranging from 98 to 100 percent. The obtained sequence was deposited in GenBank under the accession number PP976490 and was aligned with partial *18S rRNA* sequences of *B. vogeli* and other *Babesia* spp. available in GenBank, using the ClustalW tool integrated within MEGA-X software (Kumar *et al.,* 2018). The dendrogram (Fig. 3) was constructed using maximum likelihood method with Kimura-2 parameter model and bootstrap replicated 1000 times in MEGA-X. It included isolates of *B. vogeli* from dogs from India, Brazil, China, Colombia, Thailand, Taiwan, USA and Vietnam.

The present isolate (Dog, Kokkalai, India) was found to be closely related to Delhi isolate (MN165667) with 100 per cent query cover and 100 per cent identity to *B. vogeli*. The Kokkalai isolate of this study was closely related to the Kerala isolates (Mannuthy - MN190278, Thrissur - KY494646, Kozhikode - OK626770, Trivandrum – MN399204), from the other Indian states (Tamil Nadu - MN165666, Gujarat - MZ646048, Uttar Pradesh - MN165664, Haryana - OR566934, Punjab- MN190701) and also to the isolates from Thailand (ON005139), Taiwan (EF180054), France (KF953983), Brazil (JX535812), China (MK881125), USA (EU084675), Austria (GQ395377), Germany (AF394534) and Tunisia (KT445941). The present isolate (PP976490) showed 98.70-100 per cent similarity with the above isolates from different countries. These findings were in concordance with the previous reports of Caccio *et al.* (2002), Duarte *et al.* (2011), Hirata *et al.* (2022), Selim *et al.* (2022) and Mahmoud *et al.* (2024). The *B. vogeli* Kokkalai isolate formed distinct lineages and was part of a well-supported subclade with parasites from the same host, which was in congruence with the findings of Mahmoud *et al.* (2024). Gaining insights into the evolutionary relationships among *B. vogeli* isolates was crucial for performing a comprehensive diversity analysis, which will aid in enhancing the prevention and control of this tick-borne pathogen's spread.

The evaluation of PCR results versus light microscopy for detecting *B. vogeli* was conducted using ROC and kappa statistics. The comparative evaluation of the two techniques for detection of pathogens

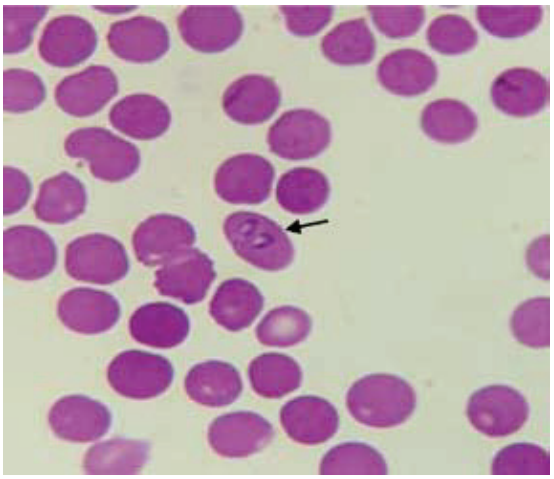

Fig. 1. Intraerythrocytic pair of pear/tear drop shaped merozoites of *Babesia vogeli* (black arrow) within a red blood cell (Field's staining, ×1000).

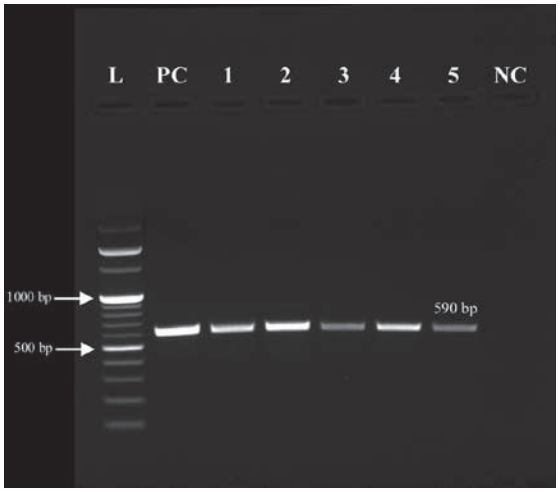

Fig. 2. Electropherogram of 18S rRNA gene of *B. vogeli.* Lane L: 100 bp ladder, Lane NC: negative control, Lane PC: positive control, Lane 1,2,3,4,5: positive samples.

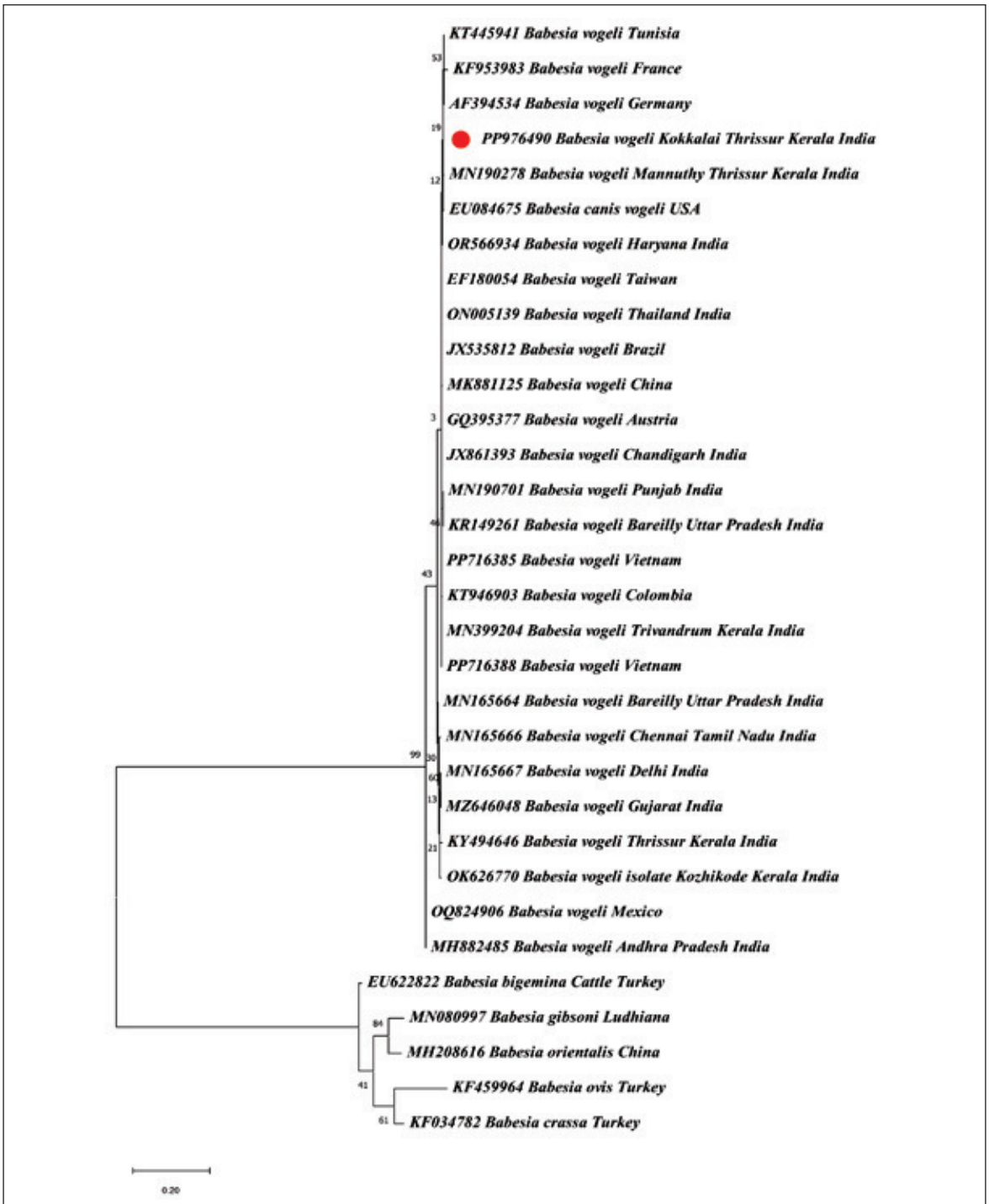

Fig. 3. Maximum-likelihood tree based on the alignment of partial *18S rRNA* sequences (590 bp) of *B. vogeli* and other sequences of *Babesia* spp. Multiple sequence alignments were generated with MEGA-X. Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting the nodes. The sequence of *B. vogeli* obtained in the present study is represented by a circle coloured in red. The *18S rRNA* partial sequence of *Babesia* spp. of other hosts were taken as outgroup. The GenBank accession number, organism and the country of origin are indicated.

revealed that PCR based on visualization of ethidium bromide-stained amplicons electrophoresed in agarose gel detected a higher percentage of positive cases (27 per cent) when compared to light microscopy (18.50 per cent). Conventionally, light microscopic examination of thin blood smears was the most common method resorted for diagnosing canine babesia infections (Abd-Rani *et al.,* 2011). The comparison of PCR and microscopic findings reveals that babesia infections are difficult to detect by microscopy, because of the low number of parasites in peripheral blood (Lakshmanan *et al.,* 2007; Alvarez *et al.,* 2019). With the progress of molecular biology techniques like PCR, the infection could be detected with greater sensitivity and specificity than the conventional methods (Selim *et al.,* 2022). Kappa statistics was used to analyse the consistency between two tests which revealed score of 0.761 (SE, 0.054, $p<0.001$ level) indicative of good agreement between the tests (Thrusfield *et al.,* 2018). The true positive rate (sensitivity) was plotted against the false positive rate (100 - specificity) to create a receiver operating characteristic (ROC) curve. The ROC curve for PCR was calculated, yielding an area under the curve (AUCROC) of 0.948. This value was significantly higher than the true area of 0.5 (SE, 0.015, $p<0.001$), indicating that it is an excellent test for diagnosing *B. vogeli* infection.

According to Ionita *et al.* (2023), *B. vogeli* vectored by *Rhipicephalus sanguineus* (*R. sanguineus*) is regarded as the least pathogenic large species of babesia causing asymptomatic or subclinical infections in adult dogs and severe disease in young dogs. Among the 200 dogs screened, 88 per cent had previous exposure to ticks and 29.54 per cent of them were infected with the haemoprotozoan. At the time of presentation to the hospital, 57 per cent of dogs were having moderate (94/200) level of tick infestation. Morphological identification of ticks collected from sampled dogs revealed that all the dogs were infested with the brown dog tick, *R. sanguineus* with deeply bifid first coxa and hexagonal basis capitulum. Jose *et al.* (2018), Wahlang *et al.* (2019), Manoj *et al.* (2020) and Hirata *et al.* (2022) have reported the occurrence of *B. vogeli* in this tick species. Each active developmental stage of *R. sanguineus* feeds on a different host and every active tick stage feeds on a different dog and therefore termed as a three-host monotropic tick (Dantas-Torres, 2010). In addition to this, Zygner *et al.* (2023) reviewed that, the transovarial transmission of *B. vogeli* in this tick may promote its survival by getting transmitted to dogs by larvae, nymphs, and adult

ticks. Penzhorn (2011) pointed out that, in subclinical infections, dogs were not been treated with babesicidal drugs, thus acted as a contributing risk factor for the haemoprotozoan infection. Most of the pet parents were using fipronil and cypermethrin (68.50 per cent) as anti-tick measures. Still, 32.11 per cent of the dogs treated with fipronil and cypermethrin were found to be affected with babesiosis. This result pointed towards the development of resistance to ticks towards the conventional acaricide treatments. Prevention of babesiosis primarily involves using topical and environmental acaricidal treatments designed to minimize contact with vector ticks and limit the transmission of the pathogen to dogs. Typically, topical ectoparasiticides has to be employed to either repel ticks and prevent their attachment or to eliminate ticks within 24 to 48 hours after application (Otranto *et al.,* 2010; Solano-Gallego and Baneth, 2011).

SUMMARY

The present study revealed that among the 200 dogs examined, PCR based on *18S rRNA* gene of *B. vogeli* detected a higher percentage of positive cases (27 per cent) when compared to light microscopy (18.50 per cent). Traditionally, the identification of canine babesiosis has predominantly depended on the examination of stained microscopic blood smears. However, with advancements in nucleic acid amplification techniques like PCR, the diagnostic sensitivity has significantly increased in recent years. Phylogenetic analysis reaffirmed that the *B. vogeli* identified in our study were closely related to those previously reported in dogs from India and other *B. vogeli* isolates around the world. The hot, humid tropic climate of Thrissur, Kerala provides a favourable and conducive climate for the survival and propagation of ticks throughout the year. Consequently, priority should be given to implement tick control strategies in order to eliminate babesiosis among dogs.

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