RESEARCH ARTICLE

Identification of Babesia Infection in Dogs with Tick Infestation in Chennai, Tamil Nadu: A Molecular Diagnostic Approach

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Abstract

Canine babesiosis, a tick-borne disease caused by protozoa of the genus *Babesia*, is a significant concern in tropical regions like India, where the warm, humid climate facilitates tick proliferation. This study was aimed to determine the prevalence of *Babesia* infection in dogs with tick infestation in Chennai, Tamil Nadu, using a molecular diagnostic approach. Blood and tick samples were collected from 158 dogs exhibiting clinical signs suggestive of haemoprotozoan infection. Following DNA extraction, PCR targeting the 18S rRNA gene of *Babesia* spp. was performed. Tick species identification was based on morphological characteristics and PCR amplification of the 12S rDNA gene of *Rhipicephalus sanguineus s.l*. Out of the 158 samples, 29 (18.35%) were positive for *Babesia* infection, with *Rhipicephalus sanguineus s.l.* identified as the predominant tick vector. These findings highlight the substantial prevalence of canine babesiosis in Chennai and emphasize the need for enhanced surveillance and preventive measures to control tick populations and safeguard canine health. Molecular diagnostic tools like PCR are essential for accurate and early diagnosis of this potentially fatal disease.

Key words: *Babesia*, Canine babesiosis, Chennai, India, PCR, *Rhipicephalus sanguineus*.

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Introduction

Haemoprotozoan infections are prevalent among domestic dogs in tropical regions like India, where the warm, humid climate promotes tick proliferation, leading to an increase in tick-borne diseases. The *Rhipicephalus sanguineus* complex, known as brown dog ticks, includes at least 12 species distributed globally, with *R. sanguineus sensu stricto* inhabiting temperate regions and the "tropical lineage" or *R. sanguineus sensu lato* prevalent in tropical areas (Slapeta *et al.,* 2021; Pascoe *et al.,* 2022). The geographical distribution of *Rhipicephalus sanguineus*, can make canines susceptible to a multitude of tick-borne haemopathogens, including *Babesia, Ehrlichia, Anaplasma,* and *Hepatozoon,* which pose a significant threat to canine health, with some additionally exhibiting zoonotic potential (Shaw *et al.,* 2001; Okubanjo *et al.,* 2013; Baneth, 2018). Among these, canine babesiosis is especially noteworthy due to its pathogenicity. This disease is caused by protozoa of the genus *Babesia*, transmitted primarily by the brown dog tick (*Rhipicephalus sanguineus*). In Asia, *Babesia vogeli* (large form) and *Babesia gibsoni* (small form) are the primary species infecting dogs. The disease presentation ranges from peracute, acute, chronic, or subclinical cases. The acute form is marked by recurring fever, progressive anaemia, lethargy, thrombocytopenia, haemoglobinuria and marked splenomegaly and hepatomegaly (Goo *et al.,* 2008). Chronic infections are more prevalent, with affected dogs often remaining asymptomatic carriers. The most pathogenic strains of *Babesia* can trigger severe complications, including disseminated intravascular coagulation (DIC), systemic inflammatory response syndrome (SIRS), and multi-organ dysfunction syndrome (MODS) (Baneth, 2018).

Diagnosing *Babesia* infections is challenging due to the diverse clinical signs. Preliminary diagnosis typically involves

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demonstrating intra-erythrocytic piroplasms in stained peripheral blood smears. While this conventional method is cost-effective, it lacks sensitivity for low-level parasitemia in chronic infections and is impractical for large-scale surveillance. Consequently, molecular diagnostics such as PCR, loop-mediated isothermal amplification (LAMP), are crucial for detecting chronic infections. Given the persistence of carrier stages and chronic infections, molecular diagnosis is crucial (Duarte *et al.,* 2008). Molecular techniques enable species identification and definitive diagnosis, especially when traditional methods yield negative results. Therefore, the primary objective of the current study was to employ PCR for screening dogs suspected of haemoprotozoan infections to achieve accurate diagnosis.

Materials and Methods

Collection of Blood Samples and Isolation of DNA

Over the course of a year, from March 2023 to May 2024, samples were collected from dogs presented at the Small Animal Clinics of the Teaching Veterinary Clinical Complex, Madras Veterinary College, Chennai (India). Blood samples were obtained from 158 dogs with a history of tick infestation and clinical signs including pyrexia, epistaxis, loss of appetite, swollen lymph nodes and pigmenturia. Blood samples were stored at 4°C till further processing. A total of 38 ticks collected from these dogs were stored at -20°C till further processing. Male and female ticks were separated based on their morphology using stereo zoom microscope (Fig. 1 & 2) according to the criteria described by Barker and Walker (2014). They were further subjected to molecular confirmation.

Genomic DNA was extracted from both whole blood and tick samples using commercial kits. For the blood samples, DNA was isolated from 100 µL of whole blood using the DNeasy Blood Mini Kit (Qiagen, Germany), following the manufacturer's protocol, and eluted in 35 µL of Buffer AE.

For the tick samples, five ticks were pooled as one set, washed in 70% ethanol, snap-frozen in liquid nitrogen, and ground into a fine powder. DNA extraction was then performed using the DNeasy Blood & Tissue Kit (Qiagen, Germany). The powder was lysed with Buffer ATL, Buffer AL and proteinase K, and incubated at 56°C for 4-5 h. The subsequent steps, including protein precipitation, DNA binding, and washing, were done according to the kit instructions. The DNA was eluted in 40 µL of Buffer AE. The extracted DNA was quantified and assessed for purity using a NanoDrop spectrophotometer (Thermo Fisher) and stored at –20°C until further use.

Nucleic Acid Amplification and PCR

DNA amplification and PCR were carried out in a Bio-Rad thermal cycler following the protocol described by Kledmanee *et al.* (2009) for *Babesia* spp. and Szabó *et al.* (2005) for *Rhipicephalus sanguineus sensu lato* (*s.l.*). Primers used for amplification of the 18S rRNA gene of *Babesia* spp. were Ba103F (5′ CCAATCCTGACACAGGGAGGTAGTGACA 3′) and Ba721R (5′ CCCAGAACCCAAAGACTTTGATTTCTCTCAAG 3′). Thermal cycling conditions used were initial denaturation at 95°C for 3 min, followed by 30 cycles each of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

The primers used for amplifying 400 bp fragment of the 3′ half of the mitochondrial 12S rDNA of *Rhipicephalus sanguineus s.l.* were forward, 5′-AAACTAGGATTAGATA CCCTATTATTTTAG-3′; and reverse, 5′-CTATGTAACGACTTATCTTAATAAAGAGTG-3′. The PCR conditions included an initial denaturation at 94°C for 3 min, followed by 35 cycles each of 45 sec at 94°C, 45 sec at 55°C for primer annealing, and 45 sec at 72°C for primer extension. A final extension step was performed at 72°C for 7 min.

All the PCR reactions included 50-80 ng of total DNA, 5 µL of Taq DNA Polymerase 2x Master Mix RED (Ampliqon), 10 pmol each of the forward and reverse primers, and nuclease-free water to adjust the final volume to 10 µL in 0.2 mL PCR tubes. To ensure assay specificity and detect potential contamination, no-template controls were included in each PCR run. Additionally, positive controls containing confirmed *Babesia* DNA (maintained at TRPVB) were included to verify the efficiency and accuracy of the PCR amplification.

Agarose Gel Electrophoresis

Following amplification by PCR, *Babesia* and *Rhipicephalus sanguineus s.l.* specific DNA fragments were separated on a 1.5% agarose gel with ethidium bromide for DNA staining. Electrophoresis was performed in 1X Tris acetate EDTA buffer at 100 V for 1 h. Separated DNA fragments alongside a DNA marker (MX-0916-02, 100bp DNA ladder, Medox®) were then visualized and documented under UV light using a Gel Documentation system (Bio-Rad, USA).

Results and Discussion

Canine babesiosis is a serious and emerging disease of global concern, posing a serious threat to canine health worldwide. Upon screening 158 samples suspected for canine haemoprotozoan infection presented to TVCC, MVC, 29 (18.35%) dogs tested positive for *Babesia* spp. on agarose gel electrophoresis, with bands observed at 619 bp (Fig. 3). Notably, most of these positive dogs harboured ticks on their bodies, which were subsequently identified as *Rhipicephalus sanguineus,* a known carrier of *Babesia* based on their distinct morphological features according to Barker and Walker (2014) (Fig. 1, 2). In Tamil Nadu, brown dog tick infestations are frequently reported as vectors for canine babesiosis, with recent literature confirming *R. sanguineus s.l.* as the predominant tick vector (Manoj *et al.,* 2020)

The *Rhipicephalus sanguineus* complex comprises at least 12 tick species that are commonly known as brown dog ticks

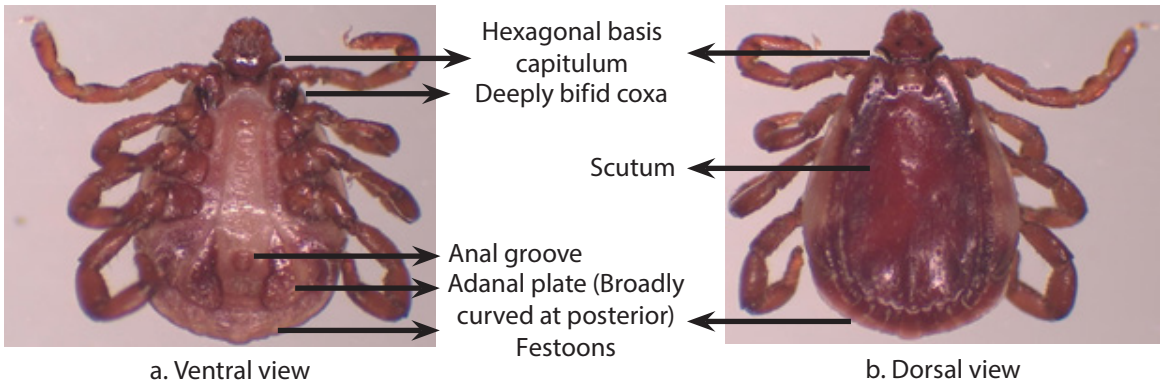

Fig. 1: *Rhipicephalus sanguineus*- Male

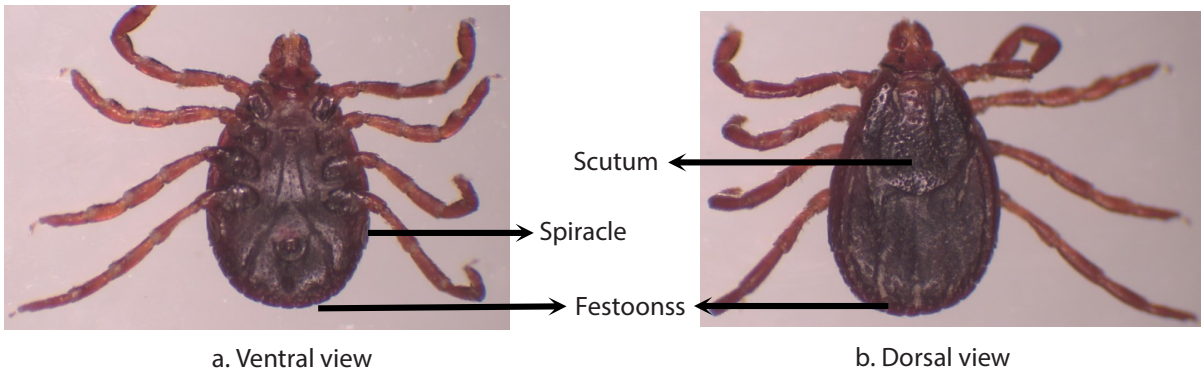

Fig. 2: *Rhipicephalus sanguineus*- Female

(Slapeta *et al.,* 2021; Pascoe *et al.,* 2022). So, the ticks were molecularly confirmed as *Rhipicephalus sanguineus s.l.* by targeting mitochondrial 12S rDNA, which produced a 400 bp product on 1.5% agarose gel electrophoresis (Fig. 4), consistent with previous reports of molecular confirmation for this tick complex (Szabó *et al.,* 2005; Almazán *et al.,* 2023).

Based on various studies, the prevalence of canine babesiosis in different regions of India has been reported as 14.06% in Gujarat (Bilwal and Mandali, 2016), 14.3% using multiplex PCR (Azhahianambi *et al.,* 2018), 9.09% by simple PCR and 15.45% by nested PCR in working dogs in Indian kennels (Mittal *et al.,* 2019), 28.3% through PCR in Andhra Pradesh (Kopparthi *et al.,* 2021), and 19.19% using conventional PCR around Bhubaneswar (Panda *et al.,* 2021).

India's diverse climates likely influence the varying prevalence of tick-borne diseases like canine babesiosis. The prevalence of canine babesiosis in India is exacerbated by the year-round presence of tick vectors and a large population of stray dogs. Recent studies indicate an increasing incidence of this disease among domestic dogs, posing a significant challenge due to inadequate diagnostic facilities, poor prognosis, and the lack of effective treatments (Mittal *et al.,* 2019). This suggests a significant number of dogs in India may be suffering from undiagnosed or unreported cases. Utilizing molecular diagnostic tools such as PCR could provide a clearer picture of the true prevalence of this disease.

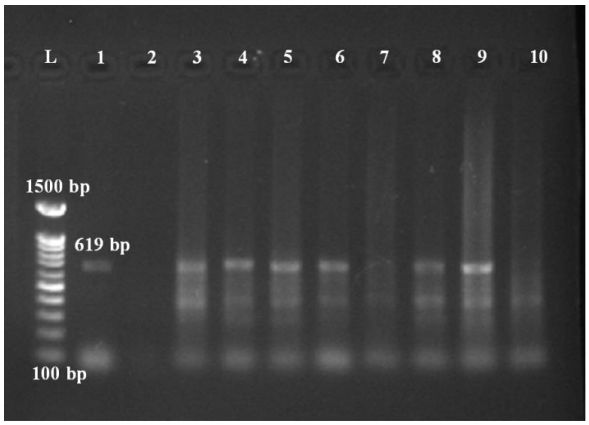

Fig. 3: PCR screening of *Babesia* spp. L- Ladder lane, Lane 1: Positive control, Lane 2: Negative control, Lane 3-6, 8, 9: Clinical positive samples, Lane 7, 10: Clinical negative samples

PCR stands out as the most sensitive and specific method for diagnosing *Babesia* infections. Unlike tests targeting anti-babesial antibodies, PCR directly targets the parasite's DNA, ensuring reliability in diagnosing peracute, acute, and chronic infections. The clinical presentation of babesiosis is highly variable, influenced by factors such as *Babesia* species, host immunity, age, and concurrent diseases. PCR can detect even low parasitemia in small blood samples, making it invaluable in clinical diagnosis, for identifying new parasite strains, and for differentiating between closely related and genetically

distinct Babesia species in epidemiological research. Nearly 1 in 5 dogs (29/158) tested positive for *Babesia* through PCR, indicating a prevalence rate of 18.35% in the Chennai region. The higher prevalence rate observed in the present study may be due to the hot and humid environmental conditions in Chennai region of Tamil Nadu, which favour the survival of tick vectors, making it highly enzootic for canine babesiosis.

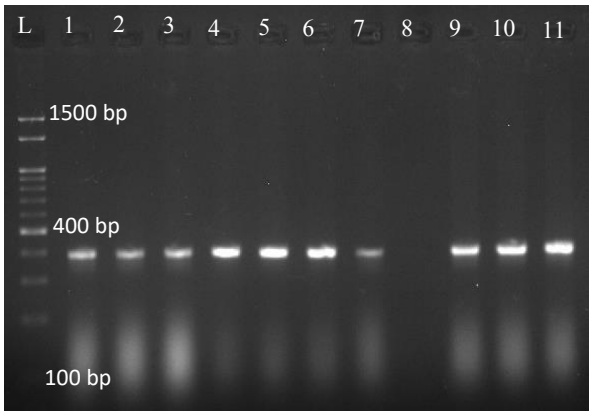

Fig. 4: Molecular identification of *Rhipicephalus sanguineus* s.l. L- Ladder lane, Lane 7- Positive control, Lane 8- Negative control, Lane 1-6: Female tick DNA, Lane 9-11: Male tick DNA

Conclusion

Canine babesiosis is a significant and growing concern in India, particularly in regions like Chennai, where environmental factors and the presence of stray dogs exacerbate its spread. This study confirms a substantial prevalence of 18.35% Canine babesiosis through PCR analysis, emphasizing the importance of sensitive diagnostic tools like PCR for accurate detection. The molecular identification of *Rhipicephalus sanguineus s.l.* as the primary tick vector, coupled with favourable climatic conditions for tick proliferation, contributes to the endemicity of canine babesiosis in Chennai. Ongoing surveillance, improved diagnostic and treatment strategies, are essential to mitigate the disease's impact and develop effective prevention and control measures.

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