Original Research Article

Hidden threat: Unmasking silent spreaders in dogs for better tick-borne disease management

ABSTRACT:

Aims: Canine vector-borne diseases (CVBDs) are a significant concern in India due to their widespread prevalence and impact on working dogs. This study aimed to screen National Disaster Response Force (NDRF) dogs in Arakkonam for canine hemoprotozoans using microscopy and PCR, while also assessing haematological and serum biochemical parameters as a part of regular health check-up.

Place and Duration of Study: The study was conducted in April 2024 at the 4th Battalion of the National Disaster Response Force (NDRF) in Arakkonam, Tamil Nadu, India.

Methodology: Blood samples from 39 dogs were examined using microscopy, complete blood count (CBC), serum biochemistry analysis, and PCR, including both hemoprotozoan and nested hemoprotozoan panels. The data related to hematological and serum biochemical parameters, as well as the molecular prevalence of hemoprotozoans, were statistically analyzed using Mean ± SD and Fisher's exact testin SPSS software.

Results: While microscopy did not detect piroplasms, PCR revealed *Babesia* spp. (28.2%), *Ehrlichiacanis* (2.56%), and *Anaplasma platys* (23.1%). Nested PCR further identified *Babesia gibsoni* (56.4%) being the most prevalent, followed by *Babesia vogeli* (10.3%). Coinfections were observed in 23% (9/39) of dogs. Older dogs (>1 year) had a significantly higher infection rate than younger dogs. Male dogs had a higher, though not statistically significant, infection rate than females. Labrador Retrievers showed higher infection rates, suggesting a possible breed-specific susceptibility.

Conclusion: Despite tick control efforts, NDRF dogs face significant risk due to environmental conditions and stray dog interactions. Subclinical infections pose a risk of transmission and warrant regular health screenings and preventive measures. The findings emphasize the need for comprehensive disease management strategies, including treatment of infected dogs, environmental tick control, and adherence to preventative protocols to potentially reduce transmission risks.

Keywords: Hemoprotozoa, Rickettsia, working dogs, subclinical infection, risk factors.

1. INTRODUCTION

Canine vector-borne diseases are widespread globally and highly prevalent in India, where diverse climatic zones provide favourable environments for a broad range of vectors and pathogens of medical and veterinary significance(1-3). The transmission and distribution of these vectors and pathogens are closely linked to regional variations in temperature, rainfall, and humidity.In India, the most common ticks on dogs are *Rhipicephalus sanguineus sensu lato,* followed by *R. haemaphysaloides*, *R.microplusHaemaphysalis longicornis* and *H.bispinosa*. Frequently encountered CVBDs (Canine Vector-Borne Diseases) in India include canine hepatozoonosis, canine monocytic ehrlichiosis, canine babesiosis, trypanosomosis and filariosis(1-4).

The global household dog population was 700 million to 1 billion and over 70% were free-roaming dogs(9-10). In India, approximately 10.2 million pet dogs coexist with 62 million

community dogs (WPR, 2024), a persistent issue exacerbated by inadequate birth control programs, abandonment, religious beliefs, and weak animal welfare laws(3,10). Community dogs outnumbering pet dogs, often acting as disease reservoirs, heighten disease transmission risk to pets through increased interactions in shared environments(3).

Canine Hepatozoonosis is an emerging tick-borne infection, while Anaplasmosis, with potentially zoonotic *Anaplasmaphagocytophilum* and *Anaplasma platys*, and *Ehrlichiacanis*, the predominant *Ehrlichia*species, are common tick-borne infections(2-3,5). Specifically, babesiosis caused by *Babesia gibsoni* and *Babesia vogeli* is widespread in India due to the abundance of their vector, *R. sanguineuss.l.*(1). These diseases pose significant challenges due to their clinical complexities, especially in terms of diagnosis and treatment. Many laboratories rely on conventional parasitological techniques, which have limited sensitivity and specificity, making precise identification of the specific species involved largely anecdotal(6-8). The pathogenicity of the disease varies with the specific species involved in infecting dogs. Therefore, species-level diagnosis in dogs ensures targeted treatment and more accurate prognosis of the disease.

Complete elimination of small *Babesia* spp. infections in dogs is often not achieved with treatment, leading to frequent relapses(11). *Babesia gibsoni* infection can range from subclinical to life-threatening multi-organ failure, with chronic presentations including intermittent fever, lethargy, and weight loss with the potential to persist within the body for years(12).

Moreover, co-infections can result in more complex disease conditions, often making them more resistant to treatment and leading to poorer prognoses(13). The global prevalence of ticks and their role in transmitting diseases to dogs, including zoonotic pathogens, underscores the critical need for routine screening of dogs at risk of tick infestations. Therefore, this study aimed to screen for canine haemoprotozoans as part of the regular health check-up for the National Disaster Response Force (NDRF) dogs serving the nation.

2. MATERIAL AND METHODS

2.1 Sample collection

In response to a request for regular health screening of National Disaster Response Force (NDRF) dogs serving the nation, we collected 1 ml blood samples from 39 dogs in EDTA tubes for hematological analysis and DNA extraction, as well as 1 ml in clot activator tubes for serum biochemical analysis. This group included 19 service dogs currently in training and 20 retired dogs. The samples were transported under ice-cold conditions to laboratory and processed within 4 hours of collection, and serum was separated immediately upon arrival.

2.2 Epidemiological data on Study population

Samples were obtained from the 04Bn NDRF unit in Arakkonam, an area where climatic conditions are conducive to the breeding of dog ticks. To investigate canine tick-borne haemoparasitic diseases within the unit, we conducted a standardized questionnaire, which comprehensively captured all relevant epidemiological data for the diseases under study.

i. Breed, gender and age of the dog
ii. Details of kennelling and the dog's interactions with other dogs
iii. Previous exposure/History of ingestion of ticks- Level of ticks manifestation
iv. Type of anti-tick measures used- Oral/Injection/Topical spot on/Anti-tick Collar
v. Has the dog been observed biting another dog or being bitten by one?
vi. Interstate travel history
vii. Previous history of Illness/Recurrence
viii. Previous treatment history: Blood Transfusion - If yes, donor details?

2.3 Microscopy

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In conjunction with blood sample collection, thin blood smears were prepared from all the 39 dogs. These smears were subjected to microscopic examination after staining with Leishman-Giemsa (LG) cocktail to detect the presence of blood parasites and identify any abnormalities in the blood picture.

2.4 Assessment of Blood/Serum Parameters

For the whole blood samples complete blood count *i.e.,* total haemoglobin concentration, erythrocyte count, packed cell volume (PCV), white blood cell count as well as differential leukocyte count and thrombocyte count were evaluated by automatic haemo-analyser (Auto HaemoAnalyser- 4 part- Exigo). Biochemical analysis for the serum samples *viz.,* Albumin, Bilirubin, Creatinine, Total protein (TP), Alanine amino transferase (ALT), Alkaline phosphatase (ALP), was performed using standard kits in an automated clinical chemistry analyser (Auto Biochemistry Analyser - A15).

2.5 DNA extraction

For all the 39 EDTA blood samples (stored at 4°C), DNA was isolated from 200 µl aliquots using the DNeasy® Blood Mini Kit (Qiagen, Germany) as per manufacturer's instructions. The DNA, eluted in 35 µl of nuclease-free water, was checked for concentration and quality using a NanoDrop spectrophotometer (Thermo Fisher) and preserved at −20°C until subsequent use.

2.6 Nucleic acid amplification

The investigation employed two distinct sets of PCR reactions. The first set included a broad haemoprotozoan and rickettsial panel including *Babesia* spp., *Ehrlichiacanis*, *Trypanosoma evansi*, and *Hepatozooncanis*. The second panel, a nested PCR, focused on the specific identification of *Babesia* species, including *Babesia gibsoni*, *Babesia canis*, and *Babesia vogeli*, along with *Anaplasma platys.* The details regarding the oligonucleotide primers employed in the study and the PCR cycling parameters for each organism are provided in Table 1 & 2.

PCR amplification was conducted in 10 µl reactions, each containing approximately 80 ng of dog blood DNA, 5 µl of Taq DNA Polymerase 2x Master Mix RED, 2 mM MgCl2 (Ampliqon, Denmark), and 10 pmol each of both forward and reverse primer combinations. The reaction mixture was adjusted to final volume with nuclease-free water. For the nested PCR, following the established primary PCR protocol, the resulting PCR amplicons were diluted at a ratio of 1:10 and employed as the template in a subsequent secondary PCR reaction.

All PCR assays included positive controls, with species-specific DNA, as well as non-template controls to detect contamination and non-specific amplifications, ensuring the validity of the experiment. The positive controls, confirmed by PCR, were maintained at the TRPVB lab facility, which regularly provide diagnostic services for canine haemoprotozoans. Subsequently, PCR amplifications were carried out in a Biorad T100 thermocycler. The amplified products were resolved by electrophoresis on a 1.5% agarose gel containing ethidium bromide in 1X Tris acetate EDTA buffer at 100 V for 45 min. After electrophoresis, they were visualized under UV light and documented using a Gel Documentation system (Bio-Rad, USA).

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Table 1 Singular PCR primer details

Primers	Sequence	Product size	Cycling conditions	Reference
Babesia Spps.	Partial 18S rRNA gene Ba103F- 5'CCAATCCTGACACAGGGAGGTAGTGACA 3' Ba721R- 5'CCCCAGAACCCAAAGACTTTGATTTCTCTCAAG 3'	619 bp	95°C/3min (94°C/45s, 60°C/45s, 72°C/90s) 30 cycles, 72°C/10 min	20
E. canis	Partial VirB9 gene Ehr1401F - 5'CCATAAGCATAGCTGATAACCCTGTTACAA 3' Ehr1780R - 5'TGGATAATAAAACCGTACTATGTATGCTAG 3'	377bp	95°C/3min (94°C/45s, 60°C/45s, 72°C/90s) 30 cycles, 72°C/10 min	20
T.evansi	Partial VSG gene (Rotat 1.2) TevF -TGCAGACGACCTGACGCTACT TevR -CTCCTAGAAGCTTCGGTGTCCT	227bp	95°C/3min (94°C/45s, 60°C/45s, 72°C/90s) 30 cycles,72°C/10 min	28
H. canis	Partial 18S rRNA HepF: 5'- ATACATGAGCAAAATCTCAAC-3' HepR: 5'- CTTATTATTCCATGCTGCAG-3'	666bp	95°C/3min (94°C/45s, 60°C/45s, 72°C/90s) 30 cycles,72°C/10 min	29

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Table 2 Nested PCR primer details

Primers	Sequence	Product size	Cycling conditions	Reference
B.gibsoni	1st round: 455-479F: GTCTTGTAATTGGAATGATGGTGAC 793-772R: ATGCCCCCAACCGTTCCTATTA 2nd round: BgibAsia-F: ACTCGGCTACTTGCCTTGTC 793-772R: ATGCCCCCAACCGTTCCTATTA	340 bp 185bp	95°C/5min (95°C/45s, 56°C/45s, 72°C/45s) 30 cycles, 72°C/5 min	30
B.canis	1st round: 455-479F: GTCTTGTAATTGGAATGATGGTGAC 793-772R: ATGCCCCCAACCGTTCCTATTA 2nd round: BCC-F: TGCGTTGACGGTTTGACC 793-772R: ATGCCCCCAACCGTTCCTATTA	340 bp 198bp	95°C/5min (95°C/45s, 56°C/45s, 72°C/45s) 30 cycles, 72°C/5 min	30
B.vogeli	1st round: 455-479F: GTCTTGTAATTGGAATGATGGTGAC 793-772R: ATGCCCCCAACCGTTCCTATTA 2nd round: BCV-F: GTTCGAGTTTGCCATTCGTT 793-772R: ATGCCCCCAACCGTTCCTATTA	340 bp 198bp	95°C/5min (95°C/45s, 56°C/45s, 72°C/45s) 30 cycles, 72°C/5 min	30
A.platys	Partial 16S rRNA gene 1st round: 8-F - AGTTTGATCATGGCTCAG 1448-R- CCATGGCGTGACGGGCAGTGT PLATYS-F -GATTTTTGTCGTAGCTTGCTATG EHR16S-R -TAGCACTCATCGTTTACAGC	1445 bp 678 bp	95°C/5min (95°C/45s, 56°C/45s, 72°C/45s) 30 cycles, 72°C/5 min	31

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2.7 Statistical analyses

The Mean±SD for all blood and serum parameters were calculated using standard statistical functions in Microsoft Excel. Fisher's exact test was employed to analyze the differences in diagnostic results obtained through conventional PCR and nested PCR, using SPSS software (version 20.0; IBM Corp., Armonk, USA). A p-value below 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

Canine haemoparasites hold global significance due to their impact on veterinary health, zoonotic potential, widespread distribution, vector-borne transmission, effects on working dogs, and economic implications. They have significant impact on working dogs, which face higher risk of vector exposure and are more prone to stress during training activities that can compromise their immune systems. For the current study, samples were collected as a part of routine health screenings from the 04Bn NDRF unit in Arakkonam, where the hot and humid climate, creates ideal conditions for vector breeding and poses a high risk of tick exposure for the dogs. Training of the NDRF dogs often takes place on open grounds accessible to stray dogs, which are known tick carriers.

3.1 Microscopic examination of blood smears

All Leishman-Giemsa (LG) cocktail-stained blood smears from 39 dogs were examined under 1000X of binocular light microscope (Lawrence & Mayo, India). Microscopic examination did not reveal any evidence of pathogenic stages in the blood smears, but only a few abnormalities like polychromasia and spherocytosis were detected.Detecting haemoprotozoans in chronic or subclinical infections is challenging, as it relies on the microscopist's experience and requires a sufficient level of parasitemia in the blood, which is often lacking in these cases. This can lead to a high probability of false-negative results. Additionally, *Babesia* parasites can easily be mistaken for artifacts, increasing the risk of false-positive findings(12-13).

3.2 Blood Parameters

Upon physical examination at the time of blood collection, all dogs appeared clinically healthy. Analysis of the blood samples revealed no significant abnormalities in hematological and serum biochemical parameters (Table 3). However, blood profiles of five dogs that tested positive for *B. gibsoni* exhibited a reduced platelet count, indicating thrombocytopenia as the primary hematological abnormality associated with blood parasite infection(13-15). An increased lymphocyte count, also known as lymphocytosis indicates some chronic inflammatory conditions. Elevated bilirubin levels, a condition known as hyperbilirubinemia, indicate liver insufficiency or dysfunction (15-16).

Table 3:Hematological and serum biochemical parameters of dogs

Parameters	Units	Mean±SE	Reference range
HEMATOLOGICAL PARAMETERS			
Hb	g dL^{-1}	12.7±0.23	11.9-18.9
RBC	$\times10^6 \mu L^{-1}$	5.96±0.1	4.95-7.87
PCV	%	38±0.69	35-57
WBC	$\times10^3 \mu L^{-1}$	10.16±4.39	5-14.1
Neutrophils	%	59±1.59	58-85
Lymphocytes	%	34.67±1.51	8-21
Monocytes	%	5.47±0.14	2-10
Platelets	$\times10^3 \mu L^{-1}$	168±1.1	211-621
SERUM BIOCHEMICAL PARAMETERS			
Creatinine	mg dL^{-1}	1.06±0.04	0.5-1.7
Total protein	g dL^{-1}	6.8±0.11	5.4-7.5
Albumin	g dL^{-1}	2.79±0.05	2.3-3.1
Bilirubin	mg dL^{-1}	0.4±0.02	0-0.3
ALT	IU L^{-1}	90±10	10-109
ALP	IU L^{-1}	106±6.6	1-114

3.3 PCR screening for Canine haemoprotozoans

PCR analysis of canine DNA samples detected tick-borne infections in a significant portion of the study population. The parasite-specific PCR successfully amplified specific gene fragments: a 619 bp partial *18S rRNA* gene of *Babesia*spp (Fig 1), a 377 bp partial *VirB9* gene of *Ehrlichiacanis*, and a 227 bp partial *VSG* gene of *Trypanosoma evansi,* 666 bp of partial *18S rRNA* gene of *Hepatozoancanis*(Fig 2). Among the 39 dog DNA samples subjected to PCR, *Babesia* spp. was the most prevalent parasite, identified in 28.2% (11/39) of dogs, followed by *E. canis* in 1 (2.56%), while none of the samples showed amplification of the *T. evansi* and *H. canis* genes.

Subsequent nested PCR was conducted for the specific identification of *Babesia* species, resulting in the amplification of a 185 bp fragment for *Babesia gibsoni* (Fig 3) and a 198 bp fragment for *Babesia vogeli*(Fig 4). Additionally, *Anaplasma platys* was detected through amplification of a partial *16S rRNA* gene fragment yielding a 1445 bp product during initial round, followed by a second round amplifying a 678 bp fragment (Fig 5). Nested PCR differentiated *Babesia* species, with *Babesia gibsoni* (56.4%, 22/39 dogs) being the most prevalent, followed by *Babesia vogeli* (10.3%, 4/39 dogs). Additionally, *Anaplasma platys* infection was confirmed in 23.1% (9/39 dogs) through amplification of a partial *16S rRNA* gene.

The study found a total infection rate of 69.23% (27/39) for canine haemoprotozoan and rickettsial pathogens screened through PCR. The prevalence rates of canine haemoprotozoans was reported earlier as 15.45%, 1.25%, and 28.3%, by various studies, respectively(17-19). These variations in infection rates are attributed to the differing environmental conditions that affect tick populations, which in turn influence the risk of canine tick-borne diseases.

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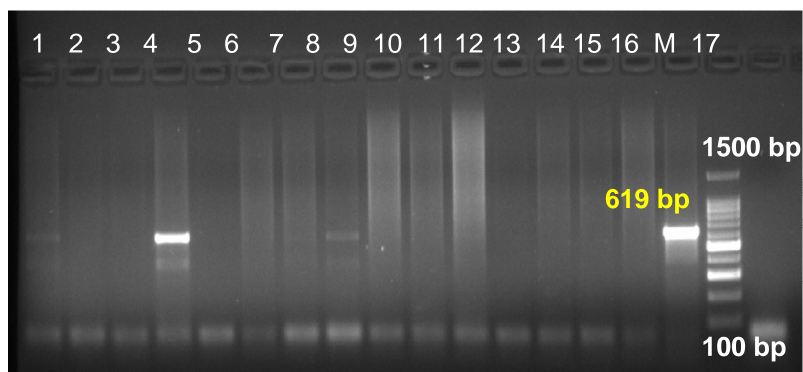

Fig 1 PCR for screening of *Babesia* spp.
M: Ladder lane
1-15: Samples screened for *Babesia* spp.
16: Positive control
17: Negative control

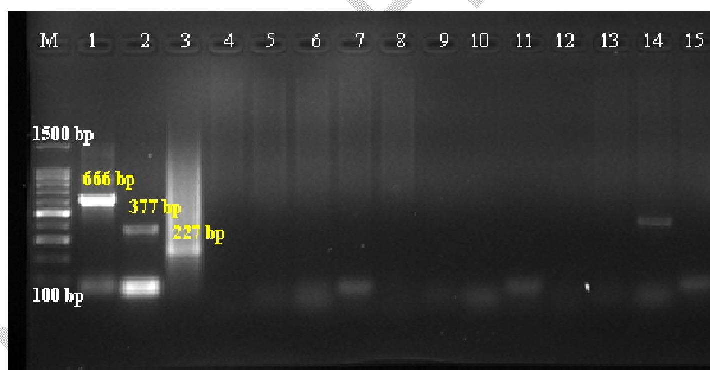

Fig 2 PCR for screening of *E.canis*.
M: Ladder lane
1: Positive control for *H.canis*
2: Positive control for *E.canis*
3: Positive control for *T.evansi*
4-15: Samples screened for *E.canis*

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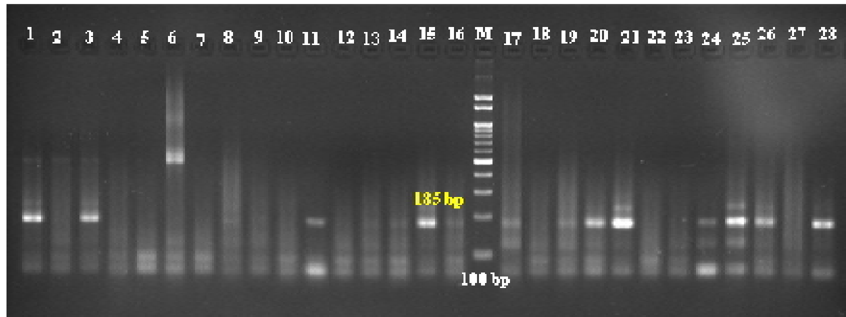

Fig 3 Nested PCR for screening of *Babesia gibsoni*
M: Ladder lane
1-26: Samples screened for*B gibsoni*
27: Negative control
28: Positive control

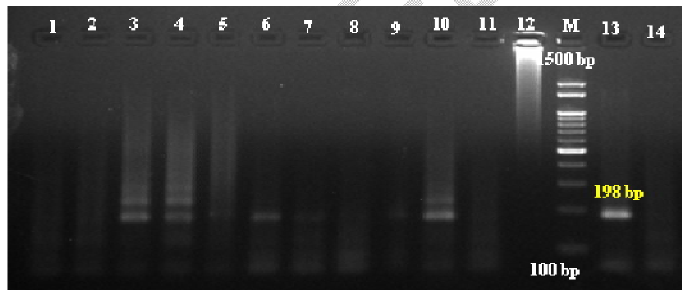

Fig 4 Nested PCR for screening of *Babesia vogeli*
M: Ladder lane
1-12: Samples screened for*B.vogeli*
13: Positive controlfor*B.vogeli*
14: Negative controlfor*B.vogeli*

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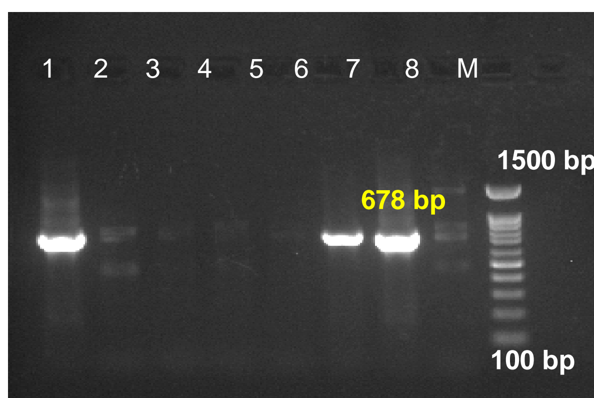

Fig 5 Nested PCR for screening of *Anaplasma platys*
M: Ladder lane
1-6: Samples screened for*A. platys*
7: Positive controlfor*A. platys*
8: Negative controlfor*A. platys*

Animals can be exposed to multiple pathogens transmitted by ticks. A single tick species may carry several pathogens, and individual ticks themselves can be co-infected with different pathogens. In endemic areas, dogs can experience co-infections with *Ehrlichia*, *Babesia*, and *Hepatozoon*, which may contribute to variations in clinical presentation, pathogenicity, and response to treatment(20). Intriguingly, the study also revealed concerning co-infections of 23% (9/39), such as *B. gibsoni*&*B. vogeli* (2/39), *B. gibsoni*&*E. canis* (1/39), *B. gibsoni*&*A.platys* (5/39) and *B. vogeli*&*A. platys* (1/39). A study found a 12.67% co-infection rate of major tick-borne pathogens in dogs in South India(8). This underscores the importance of employing comprehensive assays in epidemiological studies to precisely discern pathogen patterns and develop targeted treatment strategies (8,22-23).

3.4 Epidemiological data

3.4.1 Age, Gender and Breed

In the study population of 39 dogs, 24 were older than one year, and 15 were younger than one year. Notably, older dogs had a significantly higher infection rate (20/27, 74.07%) compared to younger dogs (7/27, 25.93%) (P = .05), suggesting a potential link between age and susceptibility to tick-borne haemoparasites.

Gender distribution within the sample revealed, 23 dogs were male, and 16 were female. Male dogs had a higher infection rate (15/27, 55.56%) compared to females (12/27, 44.44%), but this difference was not statistically significant (P> .05). These were in accordance with the earlier reports stating female dogs less prone for *B. gibsoni* infection than male animals. Increased immune reactivity in females due to estrogens provides effective resistance, making them less susceptible to infections(19,24).

Notably, Labrador Retrievers were the predominant breed (n=33), followed by Golden Retrievers (n=3) and Belgian Malinois (n=3). Interestingly, all Golden Retrievers and Belgian Malinois tested positive for at least one parasite, while only a certain group of Labrador Retrievers (21/33, 63.6%) were infected. These findings warrant further investigation into breed-specific susceptibility factors, suggesting a heightened susceptibility of purebred dogs to diseases.

3.4.2 Kennelling and tick history

The dogs were individually kenneled at the 04Bn NDRF unit in Arakkonam, an area with climatic conditions conducive to the breeding of dog ticks. Although the dogs were

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individually kenneled, they participated in mass training activities on open grounds accessible to stray dogs, which act as carriers of ticks. The dogs had a history of previous tick exposure.

3.4.3 Previous history of illness, treatment & blood transfusion

One dog in the group received treatment for babesiosis with a blood transfusion a week before sampling due to complications. This dog showed improvement after treatment. However, because of the close contact and interaction among the dogs during training, the investigators recommended regular health screenings for all dogs. All other dogs appeared healthy during blood collection, except one with an ear hematoma.

The study further substantiates that *B. gibsoni* infection is not associated with age, as even apparently healthy dogs irrespective of age, without manifesting clinical signs, tested positive in both conventional PCR and nested PCR, aligning with earlier findings of subclinical and chronic *B. gibsoni* infections in adult dogs(25).

This study demonstrates that *B. gibsoni* is more prevalent than *B. vogeli* as a major pathogen among working dogs, raising a serious concern over increase in the prevalence of canine babesiosis, especially considering that stray dogs, which were not included in the study, make up a large portion of the domestic canine population in India. Stray dogs often act as disease reservoirs, further heightens the risk of disease transmission through increased interactions in shared environments (3).

Babesia gibsoni infections can range from subclinical to severe chronic presentations. Subclinical infections of canine haemoparasites significantly impact both individual dogs and the broader community. Affected dogs, often appearing healthy despite harbouring the parasite, can act as chronic carriers and pose a zoonotic risk. Premunition or concomitant immunity often keeps these infections asymptomatic. Asymptomatic chronic carriers can develop high antibody levels and may be at risk for chronic conditions involving liver and kidneys, such as hepatitis or glomerulonephritis(13,17,27-28). This asymptomatic form, particularly in cases of *Babesia* infection, presents a diagnostic challenge, as these dogs can yield false negative PCR results(12,26). This study underscores the importance of surveillance and intervention strategies to manage haemoparasite infections within the canine population.

4. CONCLUSION

Upon screening 39 NDRF dogs, as a part of their regular health check-up, the study found a total infection rate of 69.23% (27/39) for canine haemoprotozoan infections detected through PCR, among which *Babesia gibsoni* was the predominant species indicating the subclinical infection of the dogs, posing potential risk of transmission. Despite diligent care, NDRF dogs face significant risk of tick infestation and tick-borne diseases due to the environmental conditions and stray dog interactions.

Effective management of canine tick-borne diseases requires a comprehensive strategy beyond diagnostics. This includes targeted treatment of infected dogs with prophylactic antibabesial therapy and supportive measures to bolster the dog's immune system. Additionally, environmental control measures to reduce tick populations, along with consistent surveillance and intervention strategies are crucial to manage vector borne diseases within the canine population.

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