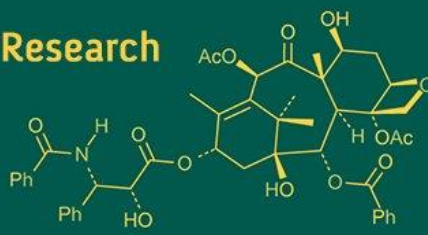


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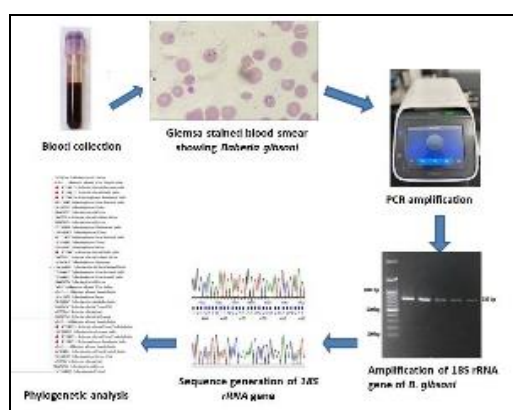
Molecular detection, phylogenetic analysis, and genetic diversity of *Babesia gibsoni* isolates from dogs in India based on the 18S rRNA gene

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Abstract

Babesia gibsoni is an important tick borne haemoprotozoan parasite causing canine babesiosis worldwide. Molecular characterization of circulating isolates is essential for understanding the epidemiology and genetic diversity of the parasite. This study investigated the molecular prevalence and phylogenetic relationships of *B. gibsoni* in dogs from India using partial 18S rRNA gene sequences. A total of 180 blood samples from clinically suspected canine babesiosis cases in Uttar Pradesh, Delhi, Haryana, and Tamil Nadu were examined by microscopy (Giemsa-stained blood smear) and PCR assay targeting the 18S rRNA gene. Representative PCR-positive amplicons were sequenced, and phylogenetic analysis was performed using the Maximum Likelihood method with representative global *B. gibsoni* sequences retrieved from GenBank. Microscopy detected *B. gibsoni* in 42 (23.3%) samples, whereas PCR identified 58 (32.2%) positives, confirming its greater diagnostic sensitivity. Seven partial 18S rRNA gene sequences (787 bp) were generated and submitted in GenBank (accession numbers PV018514-PV018520). Phylogenetic analysis showed that the Indian isolates clustered with previously reported isolates from India and other Asian countries, including China, Taiwan, Korea, Japan, Nepal, Myanmar, and Sri Lanka, forming a genetically conserved lineage distinct from the *Babesia canis* outgroup. Pairwise genetic distance analysis revealed a low overall mean distance (~0.004), indicating high conservation of the 18S rRNA gene and minimal genetic diversity among geographically diverse isolates. These findings demonstrate the utility of PCR for sensitive diagnosis and indicate that *B. gibsoni* isolates circulating in India belong to a highly conserved Asian lineage, providing valuable insights into the molecular epidemiology of canine babesiosis.



Graphical abstract: Molecular detection, phylogenetic analysis, and genetic diversity of *Babesia gibsoni* isolates from dogs in India based on the 18S rRNA gene

Keywords: *Babesia gibsoni*, 18SrRNA, India, genetic diversity, phylogenetic analysis

Introduction

Canine babesiosis is a globally significant tick-borne haemoprotozoan disease caused by intraerythrocytic protozoa belonging to the genus *Babesia* resulting in subclinical infections to severe multisystemic illness, thereby posing a major challenge to canine health worldwide.

Based on the morphology of intraerythrocytic piroplasms, canine *Babesia* species are broadly classified into two groups: large forms (3-5 µm), represented primarily by *B. canis* complex, and small forms (1-2.5 µm), which include *B. gibsoni*, *B. conradae*, and *B. vulpes* (formerly referred to as *B. microti*, *B. annae*, or *Theileria annae*) (Karasova *et al.*, 2022) [14].

Among the small *Babesia* spp., *B. gibsoni* is considered one of the most clinically important pathogens infecting domestic dogs. It is a small pleomorphic intraerythrocytic protozoan that predominantly appears as ring-shaped organisms, although oval and elongated forms spanning the erythrocyte may occasionally be observed (Conrad *et al.*, 1991; Casapulla *et al.*, 1998) [6, 4]. Based on genetic diversity, *B. gibsoni* has been classified into three major genotypes: the Asian genotype, the Californian strain, and the Spanish isolate (Karasova *et al.*, 2022) [14]. The parasite has a worldwide distribution and has been reported from all continents except Antarctica (Conrad *et al.*, 1991; Casapulla *et al.*, 1998) [6, 4].

In India, *Rhipicephalus sanguineus* is the principal vector of *Babesia gibsoni*, with confirmed transstadial transmission (Chao *et al.*, 2017) [5]. The parasite can also be transmitted through blood transfusion, dog bites, contaminated needles, and transplacental infection (Fukumoto *et al.*, 2005; Jefferies *et al.*, 2007) [8, 13]. Clinical manifestations range from anorexia, lethargy, weakness, pale mucous membranes, fever, hypoalbuminemia, and hyperbilirubinemia to severe complications including haemolytic anaemia, thrombocytopenia, renal and hepatic dysfunction, pancreatitis, neurological and cardiac disorders, systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), and septic shock (Irwin, 2009; Solano-Gallego and Baneth, 2011; Solano-Gallego *et al.*, 2016) [11, 15, 16]. While clinical manifestations often vary depending on the infecting strain of *B. gibsoni*, molecular surveillance as well as characterization of circulating *B. gibsoni* isolates is crucial for understanding the genetic diversity, evolutionary relationships, geographical distribution, and epidemiology of the parasite. Reliable molecular diagnostic assays facilitate the detection and characterization of *B. gibsoni*, enable surveillance of emerging genetic variants, and contribute to effective epidemiological monitoring and disease control. In this regard, the *18S rRNA* gene is considered valuable molecular marker for genotype differentiation and elucidating the epidemiological distribution of *B. gibsoni* isolates. (He *et al.*, 2017; Jain *et al.*, 2017) [10, 12]. The correlation between these molecular markers and clinical severity remains critical, as distinct genotypes may influence the pathogenesis and disease progression observed in both domestic and wild canids (Guo *et al.*, 2020) [9]. Although some investigations have highlighted clinical signs such as depression, anorexia, and lymphadenopathy, the potential for zoonotic transmission remains poorly substantiated in current literature (Alobaidii *et al.*, 2024; Badawi & Yousif, 2020) [1, 2]. Consequently, rigorous molecular characterization targeting highly conserved *18S rRNA* sequences gene will be useful to elucidate the phylogenetic relationship of *B. gibsoni* isolates as suggested by (Birkenheuer *et al.*, 2003) [3].

The present study aimed to characterize *B. gibsoni* isolates by analyzing their genetic diversity and phylogenetic relationships with globally reported isolates, thereby

providing molecular insights into the epidemiology of the parasite.

Materials and Methods

Sample collection, microscopy and DNA extraction

A total of 180 whole blood samples were aseptically collected from dogs clinically suspected of babesiosis presented to veterinary clinics and hospitals in Uttar Pradesh, Delhi, Haryana, and Tamil Nadu, India. Thin blood smears were prepared from each sample, stained with Giemsa, and examined microscopically under the oil immersion lens (1000X) for the presence of intraerythrocytic *B. gibsoni* piroplasms. Genomic DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was quantified and stored at -20 °C until further analysis.

PCR amplification of the *18S rRNA* gene

Molecular detection of *B. gibsoni* was performed by PCR targeting the *18S rRNA* gene using the primer pair: forward 5'-CCTTTTGGCGGCGTTTATTA-3' and reverse 5'-CAATCCCTAGTCGGCATGGTTTA-3'.

PCR amplification was carried out in a final reaction volume of 25 µL containing 2X PCR Master Mix, 10 pmol of each primer, template DNA, and nuclease-free water.

The thermal cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. Amplified products were resolved by electrophoresis on 1.5% agarose gel stained with RedSafe™ nucleic acid stain (Intron Biotechnology, South Korea) and visualized using a Gel Documentation System (Syngene, USA).

DNA sequencing and molecular characterization

Representative PCR-positive amplicons were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and subjected to bidirectional Sanger sequencing. The obtained chromatograms were manually inspected, edited, and assembled to generate consensus sequences. Newly generated sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database, and accession numbers were assigned. Sequence similarity was determined using the Basic Local Alignment Search Tool (BLAST), and homologous reference sequences were retrieved from the GenBank database for comparative sequence analysis.

Phylogenetic analysis

Phylogenetic analysis was performed using seven newly generated partial *18S rRNA* gene sequences (787 bp) together with 32 representative *B. gibsoni* sequences retrieved from the NCBI GenBank database (Supplementary table 1). Multiple sequence alignment was carried out prior to phylogenetic reconstruction. The evolutionary relationships among the isolates were inferred using the Maximum Likelihood (ML) method and Tamura model implemented in MEGA version 11. The robustness of the inferred phylogeny was evaluated by bootstrap analysis using 1,000 replicates.

Results

Detection of *Babesia gibsoni*

Of the 180 canine blood samples examined, 42 (23.3%) were positive for *Babesia gibsoni* by Giemsa-stained blood smear examination, whereas PCR targeting the 18S rRNA gene detected 58 (32.2%) positive samples. PCR identified 16 additional infections that were negative by microscopy, indicating its superior sensitivity for detecting low parasitaemia.

Sequence analysis

Seven representative PCR amplicons were successfully sequenced, generating partial 18S rRNA gene sequences of 787 bp. The sequences were submitted to the NCBI GenBank database and assigned accession numbers PV018514-PV018520.

Phylogenetic analysis

Phylogenetic analysis of the 18S rRNA gene revealed that all *B. gibsoni* isolates formed a well-supported monophyletic group with bootstrap value 76 that was clearly separated from the outgroup, *Babesia canis* (KT844899, Poland) (Fig. 2). Within the *B. gibsoni* clade, isolates obtained from different geographical regions showed a high degree of genetic similarity, with relatively short branch lengths throughout the tree. The Indian isolates were distributed among sequences originating from several Asian countries, including China, Korea, Japan, Taiwan, Nepal, Myanmar, and Sri Lanka, rather than forming a completely independent lineage. The newly generated Indian sequences (PV018514-PV018520) clustered within the principal *B. gibsoni* lineage. Most of these isolates grouped closely with previously reported Indian isolates from Uttar Pradesh, Delhi, Haryana (Hisar), Tamil Nadu, West Bengal, Kerala, Assam, Maharashtra, and Odisha, demonstrating close genetic relatedness among Indian populations. Several international isolates, including those from Taiwan, China, Korea, Japan, Nepal, Myanmar, and Sri Lanka, were interspersed among Indian isolates, further supporting the presence of a genetically homogeneous Asian population. In contrast, isolates from Brazil, Malawi (Africa), Italy, and Texas (USA) occupied nearby branches within the same major lineage without showing extensive divergence, reflecting the conserved nature of the 18S rRNA gene.

The pairwise genetic distance matrix demonstrated very low nucleotide divergence among *B. gibsoni* isolates. The overall mean genetic distance was approximately 0.004, indicating high conservation of the 18S rRNA gene across geographically diverse isolates.

Discussion

The present study provides molecular evidence of *B. gibsoni* infection in dogs across India, employing a PCR based diagnostic approach that demonstrated significantly higher sensitivity than conventional microscopic examination. While 42 samples were identified as positive via Giemsa stained blood smears, PCR targeting the 18S rRNA gene successfully detected 58 positive samples. These results corroborate findings from previous studies (Birkenheuer *et al.*, 2003; Fukumoto *et al.*, 2001; Jain *et al.*, 2017; Liu *et al.*, 2019) [3, 7, 12, 17], which consistently show that PCR assays overcome the inherent limitations of microscopic examination, specifically, the difficulty of detecting low level parasitemia common in subclinical or chronic infections.

Molecular characterization of the 18S rRNA gene further elucidated the genetic structure of the *B. gibsoni* isolates identified in this study. The phylogenetic analysis revealed that all Indian isolates clustered within the established Asian *B. gibsoni* lineage, which is characterized by a high degree of genetic homogeneity and conservation. The calculated low overall mean genetic distance of 0.004 is consistent with reports of minimal nucleotide divergence in the 18S rRNA gene among *B. gibsoni* populations across geographically diverse regions in Asia (Guo *et al.*, 2020; He *et al.*, 2017; Jain *et al.*, 2017; Yin *et al.*, 2023; Zahler *et al.*, 2000) [9, 10, 12, 17, 18].

Our findings demonstrate that Indian isolates group closely with those reported from other Asian countries, including China, Korea, Japan, Nepal, Myanmar, and Sri Lanka. This clustering pattern, often referred to as the Asian genotype (Birkenheuer *et al.*, 2003; Jain *et al.*, 2017) [3, 12], supports the existence of a genetically homogeneous population, likely facilitated by the widespread circulation of related strains. In contrast, while sequences from other regions (e.g., Brazil, Italy, Texas) may show minor divergence, they remain within the same major lineage, reinforcing the high conservation of the 18S rRNA gene. Although this gene is highly effective for species identification, the observed lack of extensive differentiation suggests that additional, more polymorphic markers may be required for finer-resolution strain differentiation and epidemiological tracing of *B. gibsoni* isolates in future studies.

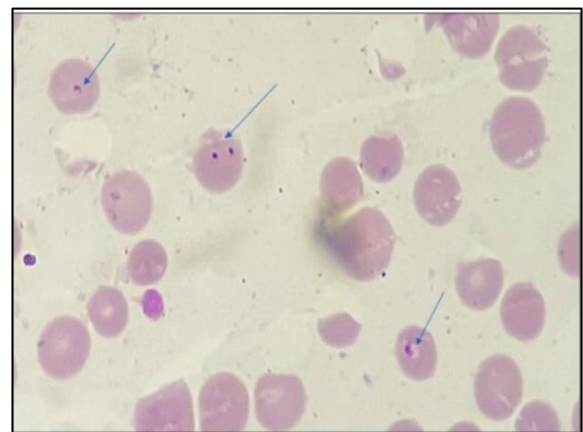


Fig 1: Microscopic view of *Babesia gibsoni* in a Giemsa stained peripheral blood smear showing small intraerythrocytic piroplasms (1000X)

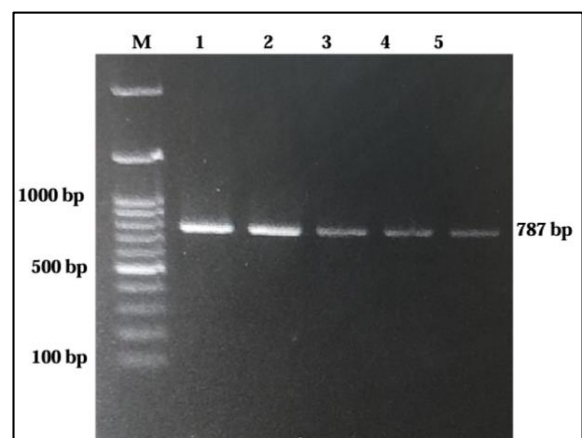


Fig 2: Agarose gel electrophoresis showing PCR amplification of the 18S rRNA gene of *Babesia gibsoni*. M: 100 bp DNA ladder; Lanes 1-5: Amplicons of 787 bp

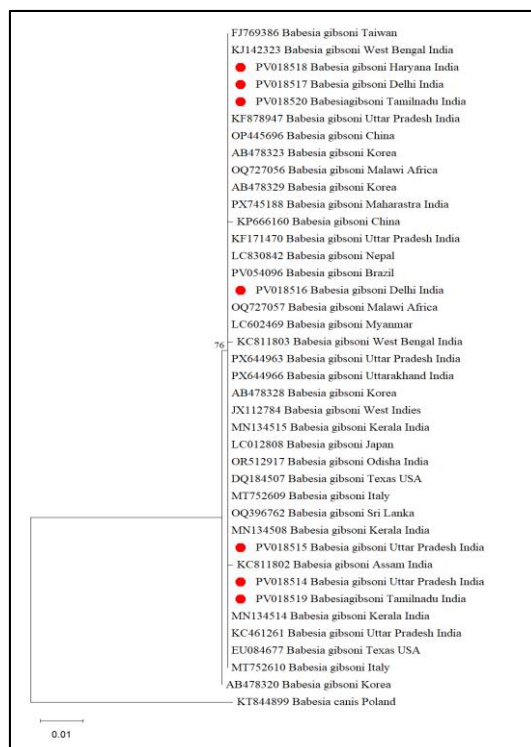


Fig 3: Maximum Likelihood phylogenetic tree inferred from partial 18S rRNA gene sequences (787 bp) of *Babesia gibsoni*. The tree includes seven sequences generated in this study (PV018514-PV018520) together with representative isolates from India and other countries retrieved from GenBank

Supplementary Table 1: List of accession numbers of 18S rRNA gene of *Babesia gibsoni* used for phylogenetic analysis

S. No	Accession number	Parasite	Name of country/state	Host	Isolation year	Sequence length (bp)
2	OP445696	<i>Babesia gibsoni</i>	China	Dog (<i>Canis lupus familiaris</i>)	2022	1,673
3	KC461261	<i>Babesia gibsoni</i>	Uttar Pradesh, India	Dog (<i>Canis lupus familiaris</i>)	2013	1,673
4	KF171470	<i>Babesia gibsoni</i>	Uttar Pradesh, India	Dog (<i>Canis lupus familiaris</i>)	2014	1,665
5	KJ142323	<i>Babesia gibsoni</i>	West Bengal, India	Dog (<i>Canis lupus familiaris</i>)	2014	1,673
6	PV018514	<i>Babesia gibsoni</i>	Uttar Pradesh, India	Dog (<i>Canis lupus familiaris</i>)	2024	787
7	LC602469	<i>Babesia gibsoni</i>	Myanmar	Dog (<i>Canis lupus familiaris</i>)	2014	1,426
8	LC012808	<i>Babesia gibsoni</i>	Japan	Dog (<i>Canis lupus familiaris</i>)	2015	1,622
9	MN134514	<i>Babesia gibsoni</i>	Kerala India	Dog (<i>Canis lupus familiaris</i>)	2019	1,524
10	JX112784	<i>Babesia gibsoni</i>	West Indies	Dog (<i>Canis lupus familiaris</i>)	2013	882
11	MN134515	<i>Babesia gibsoni</i>	Kerala India	Dog (<i>Canis lupus familiaris</i>)	2019	1,525
12	PX644963	<i>Babesia gibsoni</i>	Uttar Pradesh, India	Dog (<i>Canis lupus familiaris</i>)	2025	1,664
13	FJ769386	<i>Babesia gibsoni</i>	Taiwan	Dog (<i>Canis lupus familiaris</i>)	2008	1,262
14	MN134508	<i>Babesia gibsoni</i>	Kerala, India	Dog (<i>Canis lupus familiaris</i>)	2019	1,525
15	PX745188	<i>Babesia gibsoni</i>	Maharashtra, India	Dog (<i>Canis lupus familiaris</i>)	2025	1,599
16	PV054096	<i>Babesia gibsoni</i>	Brazil	Dog (<i>Canis lupus familiaris</i>)	2022	1,517
17	KF878947	<i>Babesia gibsoni</i>	Uttar Pradesh, India	Dog (<i>Canis lupus familiaris</i>)	2013	1,079
18	AB478328	<i>Babesia gibsoni</i>	Korea	—	2010	1,663
19	PX644966	<i>Babesia gibsoni</i>	Uttarakhand, India	Dog (<i>Canis lupus familiaris</i>)	2025	1,665
20	AB478329	<i>Babesia gibsoni</i>	Korea	—	2009	1,663
21	MT752610	<i>Babesia gibsoni</i>	Italy	Dog (<i>Canis lupus familiaris</i>)	2019	1,662
22	OQ396762	<i>Babesia gibsoni</i>	Sri Lanka	Dog (<i>Canis lupus familiaris</i>)	2018	1,539
23	OQ727057	<i>Babesia gibsoni</i>	Malawi, Africa	Dog (<i>Canis lupus familiaris</i>)	2022	1,658
24	MT752609	<i>Babesia gibsoni</i>	Italy	Dog (<i>Canis lupus familiaris</i>)	2019	1,601
25	LC830842	<i>Babesia gibsoni</i>	Nepal	Dog (<i>Canis lupus familiaris</i>)	2024	1,419
26	DQ184507	<i>Babesia gibsoni</i>	Texas, USA	Dog (<i>Canis lupus familiaris</i>)	2005	1,708
27	EU084677	<i>Babesia gibsoni</i>	Texas, USA	Dog (<i>Canis lupus familiaris</i>)	2006	1,707
28	KC811802	<i>Babesia gibsoni</i>	Assam, India	Dog (<i>Canis lupus familiaris</i>)	2013	1,665
29	KP666160	<i>Babesia gibsoni</i>	China	Dog (<i>Canis lupus familiaris</i>)	2015	1,708
30	KC811803	<i>Babesia gibsoni</i>	West Bengal, India	Dog (<i>Canis lupus familiaris</i>)	2013	1,665
31	OR512917	<i>Babesia gibsoni</i>	Odisha, India	Jackel (<i>Canis aureus indicus</i>)	2016	1,548
32	OQ727056	<i>Babesia gibsoni</i>	Malawi, Africa	Tick	2023	1,662
33	AB478323	<i>Babesia gibsoni</i>	Korea	—	2009	1,664
34	AB478320	<i>Babesia gibsoni</i>	Korea	—	2009	1,660
35	PV018515	<i>Babesia gibsoni</i>	Uttar Pradesh, India	Dog (<i>Canis lupus familiaris</i>)	2024	787
36	PV018516	<i>Babesia gibsoni</i>	Delhi, India	Dog (<i>Canis lupus familiaris</i>)	2024	787
37	PV018517	<i>Babesia gibsoni</i>	Delhi, India	Dog (<i>Canis lupus familiaris</i>)	2024	787
38	PV018518	<i>Babesia gibsoni</i>	Haryana, India	Dog (<i>Canis lupus familiaris</i>)	2024	787
39	PV018519	<i>Babesia gibsoni</i>	Tamilnadu, India	Dog (<i>Canis lupus familiaris</i>)	2024	787
40	PV018520	<i>Babesia gibsoni</i>	Tamilnadu, India	Dog (<i>Canis lupus familiaris</i>)	2024	787
41	KT844899	<i>Babesia canis</i>	Poland	Dog (<i>Canis lupus familiaris</i>)	2013	500

Conclusion

This study confirmed the higher sensitivity of PCR over microscopy for detecting *B. gibsoni* and showed that Indian isolates belong to a genetically conserved Asian lineage with low genetic diversity. Future studies should include larger sample sizes, wider geographic coverage, and more variable molecular markers or whole genome sequencing to better understand parasite evolution, transmission, and epidemiology.

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