



Molecular Occurrence and Characterization of Canine Babesiosis in Naturally Infected Pet Dogs in Jaipur, Rajasthan, India

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ABSTRACT

Background: Canine babesiosis is an important tick-borne, haemolytic disease caused by intra-erythrocytic protozoan belonging to the genus *Babesia*, comprising two main species *B. canis* and *B. gibsoni*. In dogs, infection by *Babesia spp.* causes a wide range of clinical manifestations, from subclinical disease to serious illness characterised by fever, pallor mucous membrane, jaundice, splenomegaly and weakness.

Methods: This study was conducted from March 2022 to September 2022 on the dogs presented at the government veterinary polyclinic, Jaipur and Veterinary Clinical Complex of Post- Graduate Institute of Veterinary Education and Research (PGIVER), Jaipur. The present study aims to diagnose canine babesiosis based on history, clinical signs and detect the organisms in the blood of infected dogs by using light microscopy and polymerase chain reaction (PCR).

Result: A total of 100 blood samples were collected from different breeds, sex and age group of dogs suspected for canine babesiosis in Jaipur. Out of 100 samples screened, 7 (7%) and 24 (24%) were found positive by blood microscopy and PCR, respectively. PCR was carried out for detection of *Babesia canis* and *B. gibsoni* species, using appropriate primers. Samples were found positive for *Babesia canis* species in PCR and product amplified at 671bp. None of the samples was found positive for *B. gibsoni*. One PCR-positive sample was randomly selected and sequenced, then BLAST analysis revealed that the sequence is very closely related to that of the *B. canis vogeli* strain. When diagnosing canine babesiosis in dogs, it is advised to use a combination of blood microscopy as a screening test and PCR as a confirming test.

Key words: Canine babesiosis, Dogs, PCR.

INTRODUCTION

Canine babesiosis is a tick-borne protozoal haemolytic disease with global distribution and significance. Global distribution, including India, poses an important veterinary medical problem. It is a serious clinically significant tick-borne haemoprotozoan disease of dogs (Kjemtrup and Conrad, 2006). Hard-bodied ticks of various genera and species, including *Rhipicephalus sanguineus*, *Dermacentor reticulatus*, *D. marginatus*, *Haemaphysalis ellipticum*, *H. longicornis* and *H. leachi* transmit the infection to dogs. Canine babesiosis caused by two organisms *Babesia gibsoni* and *B. canis* causing disease in ocular, neural and anaemic forms of clinical manifestation (Senthil and Chakravarthi, 2023). In India, ticks *Rhipicephalus sanguineus* and *Haemaphysalis longicornis* are found to be transmitted *Babesia vogeli* and *B. gibsoni*, respectively (Shaw *et al.*, 2001).

Clinical signs of babesiosis are haemolytic anaemia, pyrexia, anorexia, pale mucous membrane, anaemia, tachypnoea, tachycardia, splenomegaly, icterus, malaise and apathy. Many approaches are currently being used to diagnose canine babesiosis, with varying sensitivity and specificity. Babesiosis is diagnosed through clinical signs and symptoms, simple microscopy, in-vitro culture, animal inoculation, serological tests (CFT, ELISA and IFAT) and molecular methods such as Polymerase Chain Reaction (PCR). For the diagnosis of babesiosis most common method is the direct microscopic examination of Giemsa

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or Wright-stained blood smears because it is conclusive, feasible and cost-effective, but it cannot always detect inapparent or chronic infection in dogs because the level of parasitaemia is low (Caccio *et al.*, 2002, Kushwaha *et al.*, 2018). Recent advances in molecular biology techniques such as PCR have enabled piroplasm to be identified with far greater sensitivity and specificity than traditional methods. PCR can also be used to detect and differentiate infections caused by various *Babesia* species that cannot be identified morphologically by blood smear examination (Duarte *et al.*, 2008). Objective of this study is standardisation

of PCR and diagnose the babesiosis disease with the help of light microscopy and PCR.

MATERIALS AND METHODS

Selection of dogs and blood samples collection

Dogs of different ages, sex and breeds presented at the government veterinary polyclinic, Jaipur and Veterinary Clinical Complex of Post- Graduate Institute of Veterinary Education and Research (PGIVER), Jaipur with clinical signs such as high fever above 104°F, inappetence, anaemia, lethargy, congested/pale/icteric mucous membranes, lymphadenopathy, haematuria, epistaxis and a tick infestation history were randomly selected for the blood sample collection. The duration of the study was from March 2022 to September 2022. Blood samples were collected from the cephalic or saphenous veins and preserved in EDTA as a blood anti-coagulant for smear observations and PCR to detect *Babesia spp.*

Thin film blood smear

Thin blood smears were made with each blood sample and was fixed with methanol and then stained with Giemsa solution. The Giemsa-stained blood smear was examined under the oil immersion objective of a microscope. *Babesia* organisms were blue, rod/piriform-shaped found in erythrocytes. After evaluating at least 500 oil immersion fields in 30 minutes, failure to detect the parasite in a smear was declared microscopically negative.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from 200 µl of each blood sample using a commercial DNA extraction kit, (DNASure® Blood Mini kit; NP 61107) as per the manufacturer's protocol. DNA samples were stored at -20°C until further use. A conventional PCR was conducted to detect the presence of piroplasm in the blood samples. The PCR for the amplification of *B. gibsoni* and *B. canis* in the blood

samples were performed based on the method of Inokuma *et al.* (2004) and Foldvari *et al.* (2005), respectively with a few modifications.

Species-specific set of primers for *B. canis* PIRO-A1 (F5 AGGGAGCCTGAGAGACGGCTACC3) and PIRO-B (R5 TTAAATACGAATGCCCCCAAC 3) was used to amplify 450 bp gene fragment of 18S rRNA. For *B. gibsoni* Gib599 (F5 CTCGGCTACTTGCCTTGTC 3) and Gib1270 (R5 GCCGAAACTGAAATAACGGC3) was used to amplify 671 bp gene fragment of 18S rRNA. Known positive and negative control was used in PCR.

Briefly, reactions were conducted in a total of 10 µl, including 1.0 µl of 10 × PCR buffer (included 10 mM MgCl₂), 1.0 µl of dNTP (2.5 mM each), 0.4 U of Taq DNA polymerase, 2.0 µl of template DNA, 0.5 µl of each primer (10 pmol) and 4.6 µl of double distilled water.

To standardize PCR for *Babesia gibsoni*, various temperature and time settings were applied during each stage of the reaction. The denaturation step was performed at either 94°C or 95°C for 40 seconds. Annealing temperatures ranged from 52°C to 64°C, with durations of 30 to 45 seconds: 52°C, 53.8°C and 55°C for 30 seconds each; 58°C and 59.5°C for 40 seconds each; 60°C for 45 seconds and 61°C, 62°C and 64°C for 40 seconds each. The extension step occurred at 72°C for 30, 40, 45, or 60 seconds, followed by a final extension step at 72°C for either 5 or 7 minutes. Likewise, for *Babesia canis*, the PCR was standardized using similar but slightly different conditions. The denaturation phase was carried out at either 94°C or 95°C for 40 seconds. Annealing temperatures were tested at 58°C for 30 seconds and 60°C and 62°C for 40 seconds each. The extension step was performed at 72°C for either 40 or 60 seconds. The final extension step also took place at 72°C for either 40 or 60 seconds.

After standardisation, the ideal cycling conditions for the 18S PCR assay of *Babesia gibsoni* begin with an initial denaturation stage at 94°C for 5 minutes, followed by 35

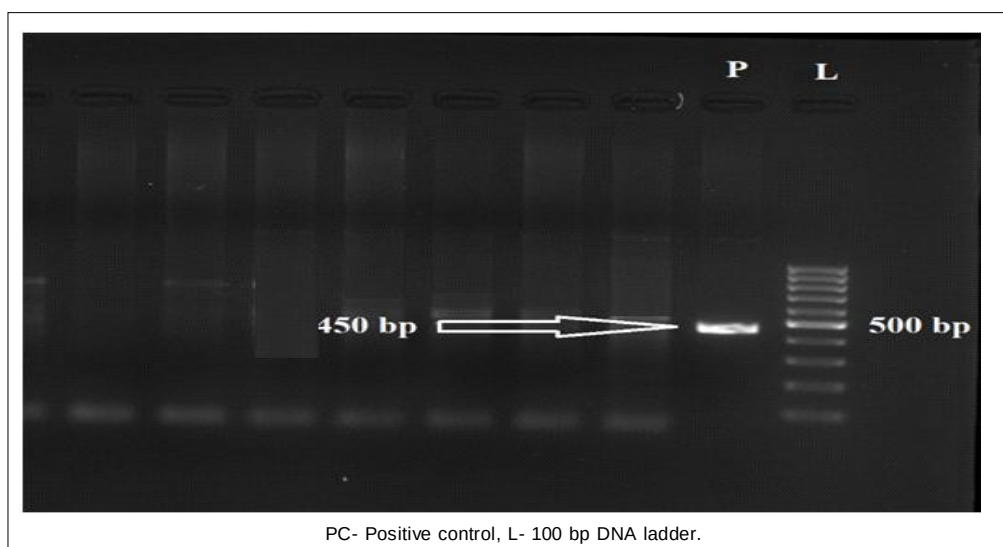


Fig 1: Standardisation of PCR for *Babesia canis*.

cycles of three steps: denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds. The process concludes with a final extension at 72°C for 7 minutes. For *Babesia canis*, the conditions are similar but with slight adjustments. The initial denaturation is set at 95°C for 5 minutes. This is followed by 35 cycles that consist of denaturation at 95°C for 40 seconds, annealing at 60°C for 40 seconds and extension at 72°C for 40 seconds. The final extension step is also conducted at 72°C for 7 minutes (Fig 1).

The PCR products were electrophoresed on a 1.5% agarose gel containing 5 µl of ethidium bromide in Tris-acetate-EDTA buffer at 100 V for 1.5 hours and then examined under UV light. Purified DNA from one representative sample was sequenced by Eurofins Scientific India Pvt. Ltd. Bengaluru, Karnataka, India, using the same PCR primers to prime the sequencing reaction. PCR-purified amplicons were sequenced in both directions, using the forward and reverse PCR primer.

Sequences analysis

The 18S rRNA gene sequences were subjected to blast analysis on the NCBI website using the BLAST program. The obtained sequence was submitted to GenBank NCBI have accession number OP954492.

Animals ethics

The permission for sampling and other procedures was duly approved by the Institutional Animal Ethics Committee (IAEC) vide memo no. IAEC Approval No: PGIVER / IAEC 12022 - 19.

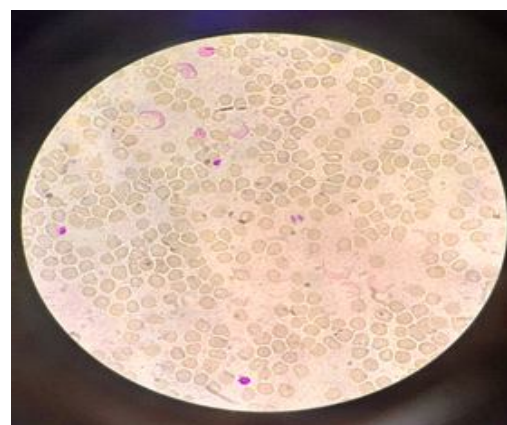
Statistical analysis

Data obtained were subjected to analysis by chi-square test using the statistical package SPSS software 20 version.

RESULTS AND DISCUSSION

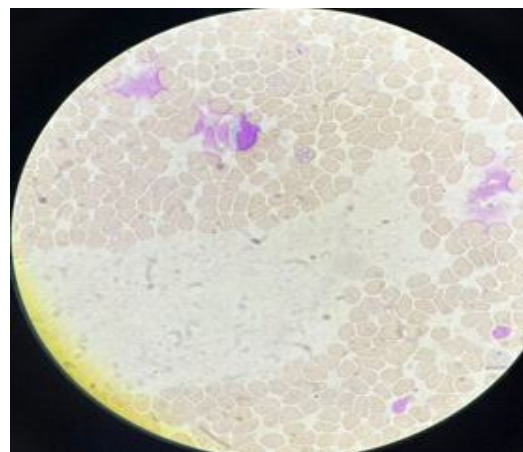
Incidence of Babesiosis through light microscopy and PCR

The microscopic blood smear examination of 100 suspected cases revealed 7% incidence of canine babesiosis. The PCR was performed for all 100 cases using both *B. canis* and *B. gibsoni* primers, which revealed 24% incidence of



(*B. canis* appears as larger, often paired, pear-shaped organisms within erythrocytes).

Fig 2a: Demonstration of Babesia spp. by blood smear examination; 100X oil immersion.



(*B. canis* shows "Maltese cross" or "tetrad" formation within erythrocytes).

Fig 2b: Demonstration of Babesia spp. by blood smear examination; 100X oil immersion.

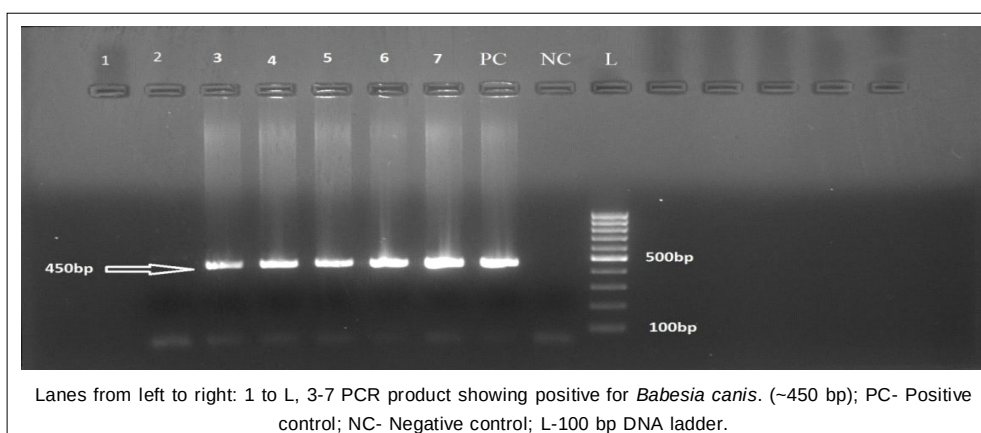


Fig 3: Electrophoresis gel (1.5% agarose, stained with ethidium bromide).

canine babesiosis. In the present study, out of 100 suspected samples, 7 (7%) blood samples show parasitaemia through microscopic blood smear examination and 24 (24%) sample were found positive for *B. canis* infection through PCR and no case was found positive for *B. gibsoni* infection.

Comparative efficacy of light microscopy and PCR assay

In this study, Pearson's chi-square test was used to assess the statistical difference between diagnostic methods. A chi-square value of 11.03 with a p-value of 0.0008 indicates a highly significant difference ($p < 0.01$) between the results obtained by light microscopy and PCR assay. Specifically, while only 7 out of 100 samples tested positive with light microscopy, PCR detected 24 positive cases from the same sample set, demonstrating that PCR is notably more sensitive than conventional blood smear examination.

However, the PCR assays were able to detect parasitaemia in dogs with sub-clinical or chronic disease and a total of 24 cases were found positive for Babesiosis through the PCR technique. DNA amplification has been stated to be far more sensitive than conventional methods because lower parasitaemia cannot always be detected by Giemsa staining, but because PCR assays are known to have extremely high sensitivity, they can amplify even minute amounts of parasitic DNA.

Diagnosis of Babesiosis by blood smear examination and PCR

In the present study, Giemsa-stained blood smear examination through light microscopy, of 100 suspected canine babesiosis cases revealed 7 (7%) cases positive for babesiosis. After the standardisation of PCR, all 100 samples were subjected to PCR for both *B. gibsoni* and *B. canis*. PCR revealed a total of 24 (24%) positive clinical cases of *B. canis*, out of 100 suspected cases (Fig 2a, 2b and 3).

Sequence analysis

BLAST analysis of 18s small subunit rRNA gene was performed for the *B. canis* sequence to obtain sequence similarity and per cent identity with other available sequences of dogs viz. with Thailand, Junagadh, West Bengal, Kolkata, China, Chile, Brazil, Ludhiana, France etc.

Our study revealed that the 18S small subunit rRNA gene of *B. canis* has 100% query cover with Junagadh, West Bengal, Kolkata, Chile, Brazil, Ludhiana's *B. canis vogeli* strain as well as *B. canis* strain of Thailand, France and Brazil (Fig 4). This is an important finding of our study as this is not reported in earlier studies and also the strain present in Rajasthan is *B. vogeli* which is symptomatically same as *B. canis*, which needs to be characterized at a large scale for the diagnosis purpose. This shows 57.07% similarity with *B. vogeli* Junagadh strain, 56.54% with West Bengal, Kolkata, Chile and Brazil of *B. canis vogeli* strain and *B. canis* strain of Thailand, France and Brazil whereas 48.17% Ludhiana *B. canis vogeli* strain (Fig 4). Maximum likelihood phylogenetic analysis showed that our sequence is very closely related to that of *B. canis vogeli* strain of Ludhiana followed by *B. canis vogeli* strain of Kolkata and distantly related with *B. canis* of China and *B. canis vogeli* of Junagadh (Fig 4). The possible reason for this variation is that some addition and deletion is existing in our population which are not present in any other studied sequence and there are some substitutions also there in our population (Fig 5).

The results emphasize the heightened sensitivity of PCR as a diagnostic tool for detecting *Babesia* infections, especially in subclinical cases where low parasitaemia might escape detection through light microscopy. Traditional methods of diagnosis of babesiosis in dogs are based on the morphologic appearance of intra-erythrocytic piroplasm seen in peripheral blood smears. Microscopic examination of *Babesia* is effective in the acute phase, but in chronic and/or subclinical infection, the disease tends to be associated with cryptic infection, which often limits the sensitivity of microscopic examination. Intra-species differentiation is also not possible through microscopic examination (Shaw *et al.*, 2001). Whereas light microscopy has a detection limit of approximately 0.001% parasitaemia, PCR could even detect parasite loads in the range of 50 organisms/ml (Birkenheuer *et al.* 2003) and 9 parasites/μl (Matsuo *et al.*, 2005).

A previous study conducted by Godara *et al.* (2010) in the Jaipur region showed a 13.1% prevalence of canine

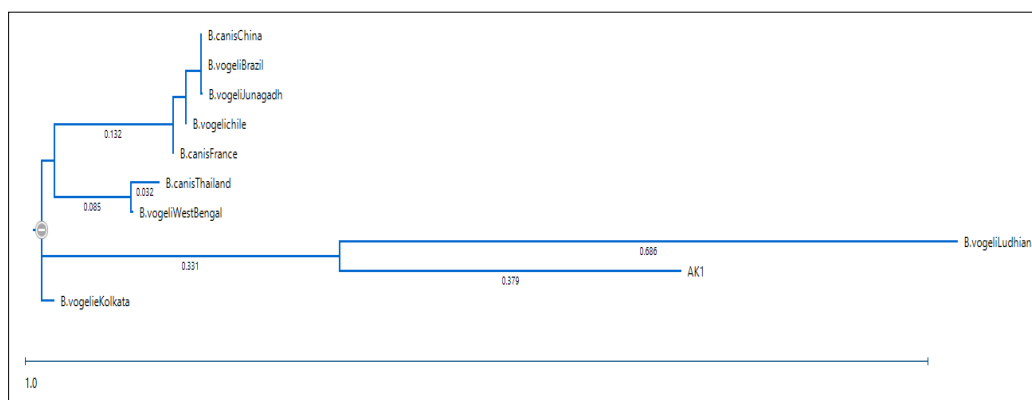


Fig 4: Findings of phylogenetic tree analysis.

babesiosis. Another study conducted by Yoak *et al.*, 2014 showed a 10% prevalence of *B. canis* in Jaipur. According to Jumde *et al.* (2011), Shrivastava and Shukla (2013) and Das and Konar (2013), the prevalence of canine babesiosis was 8%, 6.93% and 8.51% in Nagpur, Jabalpur and West Bengal, respectively, which is comparable to our findings. Kopparthi *et al.* (2021), Obeta *et al.* (2020) and Chaurasia *et al.* (2022) were found 11.8%, 10.8% and 9.8% positive cases through microscopic blood smear examination.

The PCR result is in the accordance with Gonmei *et al.* (2020) and Panda *et al.* (2021) who detected 28.3% and 19.19% incidence, respectively. Higher species-wise prevalence rate of *Babesia canis* was also reported by

Bilwal and Mandali (2016), who recorded the prevalence rate of *Babesia canis* i.e., 88.89% and *Babesia gibsoni* 11.11%. The higher prevalence of *B. canis* was also recorded by Abd Rani *et al.* (2011).

The prevalence of *Babesia canis* and *Babesia gibsoni* in dogs can vary depending on the region and the breed of dog. The prevalence of *Babesia canis* infection can be influenced by the geographical distribution of its tick vectors.

The 18S rRNA gene sequencing of *Babesia canis* from Rajasthan showed a high degree of similarity to global *B. canis vogeli* strains, including those from Junagadh, West Bengal, Thailand, France and Brazil, with 100% query coverage. Phylogenetic analysis revealed a close genetic

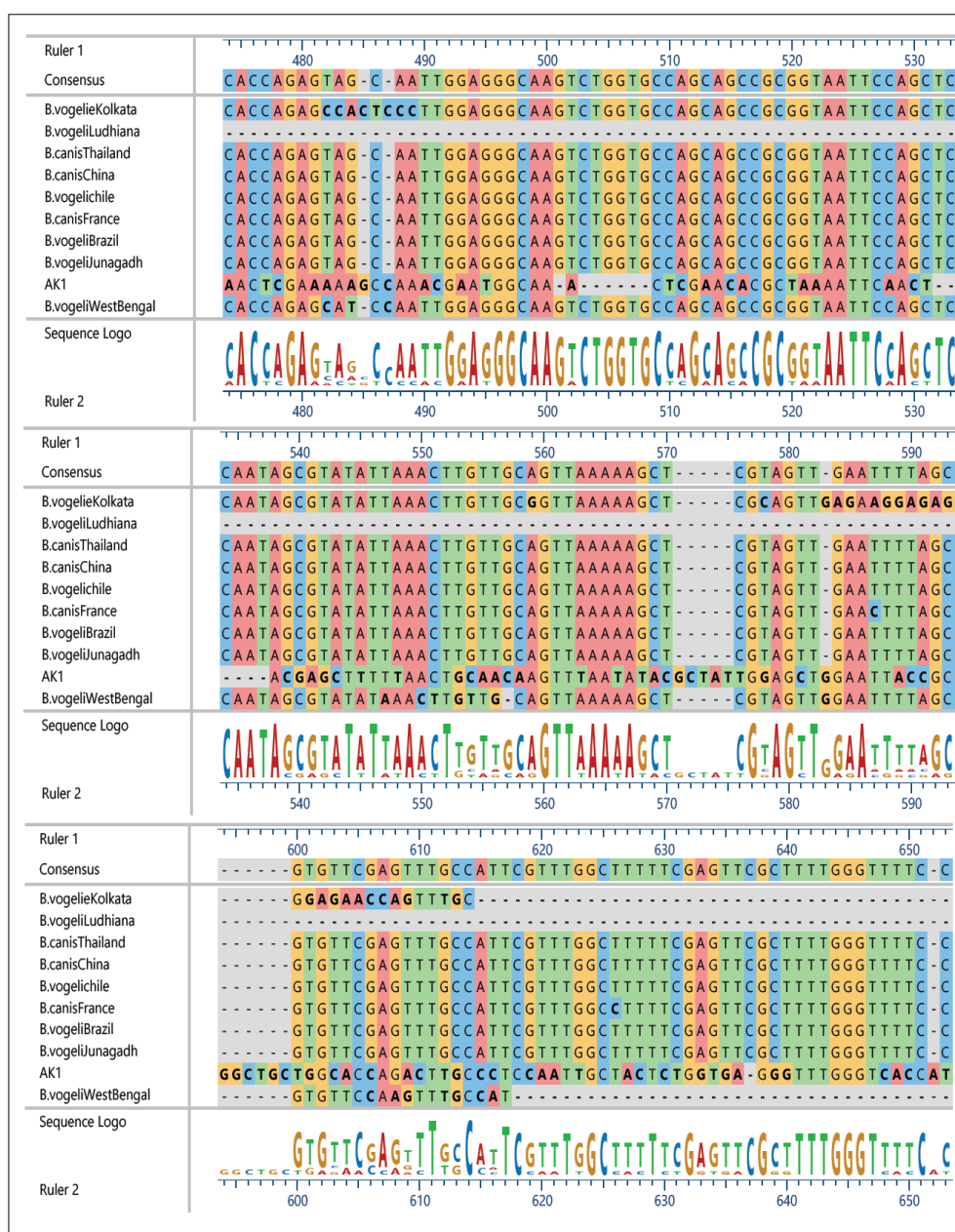


Fig 5: Sequence analysis.

relationship with Ludhiana's *B. canis vogeli* strain, followed by the strain from Kolkata, while showing greater divergence from strains in China and Junagadh. These findings suggest regional genetic variations, potentially due to unique insertions, deletions, or substitutions, highlighting the need for extensive sequencing across regions to improve diagnostics and characterization of *B. canis* strains.

CONCLUSION

In the present study, *Babesia* infection was diagnosed using conventional technique *i.e.*, light microscopy and molecular technique *i.e.*, PCR. The microscopic examination is cost-effective and rapid but the PCR is a more specific and sensitive test because it differentiates the *Babesia* species that is difficult to identified morphologically by blood smear examination. PCR also detect a low level of parasitaemia which cannot be detected by blood smear examination.

In this study, we can conclude that the occurrence of canine babesiosis was found at 7% through light microscopy and 24% through PCR technique. *Babesia canis* species was found in PCR results and none of the sample was found positive for *B. gibsoni*. More studies are further needed towards confirming the trend of different species of babesia and other blood protozoa in canines of this area.

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Disclaimers

The perspectives and conclusions presented in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. While the authors have carefully ensured the accuracy and completeness of the information shared, they disclaim any liability for direct or indirect losses that may result from the application of this content.

Informed consent

All procedures involving animals in this study were approved by the Committee for Experimental Animal Care and were conducted in accordance with the guidelines set forth by the University Animal Care Committee.

Conflict of interest

The authors affirm that there are no conflicts of interest regarding the publication of this article. This work was conducted independently, with no external funding or sponsorship influencing the study design, data collection, analysis, decision to publish, or manuscript preparation.

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