



Development of Multiplex PCR for Detection of Babesiosis in Dogs along with Molecular Characterization of Pathogens

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ABSTRACT

Background: In India, canine Babesiosis has been emerged as a serious canine haemoprotozoan disease caused by protozoa namely *Babesia vogeli* and *Babesia gibsoni* which owe their sustainment and transmission to the tick vectors. Reliable, rapid and efficient molecular detection technique is necessary for choosing the effective treatment protocol and planning of control strategies against this emerging canine health problem.

Methods: *Babesia gibsoni* and *Babesia vogeli* were detected by molecular means and characterized from extracted DNA of canine blood samples from 8 selected districts of Odisha, India, by an internally controlled multiplex PCR targeting the 18S rRNA gene. The assay was standardized and in-house validated using positive controls and random field samples (n=61) with history of tick infestation. The kappa values were analysed for diagnostic specificity and sensitivity of multiplex PCR assay for detection of *naplasma. platys*, *B. gibsoni*, *Erlichia canis*, *Hepatozoon canis* and *B. vogeli* considering singleplex PCR counterparts as standard test. The PCR products found positive, were subjected to sequencing and phylogenetic analysis. The epidemiological survey (n=280) was performed using both microscopy and the standardized mPCR and risk factors were analyzed.

Result: The mPCR assay generated amplicons of 602 bp and 489 bp corresponding to *B. vogeli* and *B. gibsoni* along with amplicon of 218 bp targeting canine β -actin gene as IAC to avoid false negative result. The kappa values for diagnostic specificity and sensitivity of multiplex PCR assay in detection of *B. gibsoni* and *B. vogeli* revealed very good agreement with their singleplex PCR counterparts (n=61). The epidemiological survey (n=280) revealed 17.14% prevalence of Babesiosis with high prevalence of *B. gibsoni* in Khordha districts and incidence of infection were high in summer season. Presence of ticks and limited outdoor activity were found to be significantly associated with rise in incidence of Babesiosis. The Odisha isolates aligned well within the established genetic boundaries for these species, solidifying the utility of gene markers for epidemiological tracking and comparative studies.

Key words: Babesiosis, Internal control, mPCR, Risk factor, Sensitivity.

INTRODUCTION

Babesia species are protozoan parasites transmitted by ticks, classified under the phylum Apicomplexa, class Piroplasmata, order Piroplasmida and family Babesiidae. They invade and multiply within the red blood cells of humans, domestic animals and wildlife. Canine babesiosis occurs globally and multiple *Babesia* species have been identified as causative agents in dogs (Irwin, 2009). In India Canine Babesiosis is accounted to two major species viz *B. vogeli* and *B. gibsoni* (Mittal *et al.* 2019). Coming under large forms of babesia (2.5-5.0 μ m), *B. vogeli* usually causes subclinical infections with a low parasitemia in adult dogs, but a take a clinical course due to severe anemia in puppies and grey hounds (Singla *et al.*, 2016). On the other hand, *B. gibsoni*, one of the small forms of Babesia (1.0-2.5 μ m). The disease is considered highly pathogenic and the disease is, characterized by high fever, mucous membrane shows paleness, jaundice, enlargement of the spleen, weakness, thrombocytopenia, pigmenturia and, in severe cases, collapse. These clinical manifestations are linked to both intra- and extravascular hemolysis, tissue hypoxia and systemic inflammatory responses (Jain *et al.*, 2018). The tropical climate, high

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humidity and abundant rainfall, as seen in coastal states of eastern India, provide a conducive environment for growth and propagations of tick vectors (Thomas *et al.*, 2022). *Rhipicephalus sanguineus* is the most prominent canine tick vector found in India followed by *Haemaphysalis* ticks (Abd Rani *et al.*, 2011). *Babesia vogeli* is transmitted by *Rhipicephalus* ticks and whereas the putative vectors for transmission of *Babesia gibsoni* are both *Haemaphysalis* and *Rhipicephalus* ticks (Manoj *et al.*, 2020).

The diagnosis of tick borne haemoparasitic infections in dogs are mostly done by peripheral blood smear examination through microscopy and serological testing. Despite of being labeled as gold standard test, microscopy has the major limitations for its inability to detect parasite under low and intermittent parasitemia, mostly in case of chronically affected and carrier animals (Kordick *et al.*, 1999). Likewise, many serological assays and diagnostic kits lack reliability due to antibody cross-reactivity among species, failure to differentiate between past and present infections and high cost (Waner *et al.*, 1998). In this regard, nucleic acid-based detection of parasites in clinical samples, which are more sensitive and specific *viz.* polymerase chain reaction (PCR) assay, along with sequence and phylogenetic analysis have been employed globally for detection of active and ongoing infections and speciation of the isolates (Pirata *et al.*, 2015). Though, nested PCR followed by sequence characterization as standard molecular modality for species identification is considered for canine Babesiosis (Mittal *et al.*, 2019), however, this assay is time-consuming and labor-intensive, because it requires a large number of reagents and disposable consumables, as at least three separate PCRs are needed to segregate the two species, thus it is better suited for clinical sample. Therefore, to overcome these limitations and challenges, a straight forward single-step multiplex PCR system for simultaneous identification of the two prevalent *Babesia* spp. was developed.

Being convenient, rapid, cost-effective and highly efficient in diagnostic performance, multiplex PCR assay has proven to be beneficial than that of conventional methods, involving detection of pathogens and co infections in a single reaction (Liu *et al.*, 2015), thus better suited for screening of large number of samples and epidemiological studies. However, a lower sensitivity of mPCR assays for canine hemoparasites in comparison to conventional PCR has been reported previously, which needs further studies for improvement (Azhahianambi *et al.*, 2018; Kaur *et al.*, 2020).

Though multiplex PCR based assays including sensitivity comparison was reported for detection and epidemiology of Babesiosis in dogs (Singh *et al.*, 2024), however, amplification inhibitors and the ligase chain reaction (LCR) make validation of the assay challenging especially causing false negative results which can be eliminated by using internal amplification control (IAC) that monitor the overall effectiveness of the amplification in each

tube (Rosenstraus *et al.* 1998). Till date, no multiplex PCR assay has been reported for the concurrent detection and differentiation of *B.gibsoni* and *B. vogeli* along with internal control. Molecular based prevalences of Babesiosis in east cost of India is also a prerequisite for effective disease control strategy and monitoring surveillance. The present study assessed the molecular epidemiology of above canine tick-borne haemoparasitic infections by a novel internally controlled mPCR method and identified the biological origins by comparing with the existing strains recorded from different geographical areas.

MATERIALS AND METHODS

Study area

Odisha (17°49" to 22°34"N and 81°27" to 87°29"E) is one of the states located in the east coast regions of India, having 1,55,707 km² geographical area. The coastal belt of Odisha falls under the Tropical Savanna (Aw) climate type as per the Koppen-Geiger climate classification, characterized by distinct wet and dry seasons, high temperatures and significant rainfall during the monsoon season. (Kottek *et al.*, 2006). Eight numbers of coastal districts were selected for the current study namely Khordha (20°11'N, 85°40'E), Cuttack (20°28'N, 85°54'E), Puri (19°48'N, 85°52'E), Ganjam (19°22'N, 85°06'E), Nayagad (20°08'N, 85°08'E), Bhadrak (21°03'N, 86°33'E), Jajpur (20.83'N, 86.33'E) and Jagatsingpur (20.2549°N, 86.1706°E) (Fig 1).

Sampling of animal

The investigation spanned from July 2024 to June 2025. The sample were collected from dogs with history/presence of tick infestation and with or without clinical signs of Babesiosis, such as fever, lymphadenopathy, generalized or limb weakness, respiratory distress, Jaundice, petechiae on skin and visible mucous membrane and bleeding disorder. A total of 280 blood samples presented to Teaching Veterinary Clinical Complex, Odisha University of Agriculture and Technology, Bhubaneswar from eight districts of Odisha were collected aseptically from the cephalic/saphenous vein of dogs in EDTA vials and utilized immediately for preparation of the thin blood smears and hematological analysis and then kept at -80°C for further use for DNA extraction. The consent for blood collection was obtained from the dog owners for the diagnostic purposes by registered professionals following the guidelines for blood collection stipulated by Committee for the Control and Supervision of Experiments on Animals (CCSEA) and the approval was obtained from Institute of Animal Ethical Committee, College of Veterinary Sc. and Animal Husbandry, OUAT, Odisha (833/IAEC, dated- 7-10-2023) for conducting the study. The data about location, sex, age, breed, tick infestation, acaricidal treatment and presence of outdoor activity were recorded and analyzed as possible risk factors.

Microscopy

Thin blood smears were prepared and subsequently subjected to Giemsa staining and then viewed under oil immersion objective lens to detect the presence of large globular piroplasm of *Babesia vogeli* and small dot shaped piroplasm of *Babesia gibsoni* in the erythrocyte for evidence of infection.

Molecular analysis

The PCR analysis was carried out from genomic DNA, extracted from blood sample of individual dogs using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) following manufacturer's instructions with slight modification in terms of quantity of blood used (200 µl) and final elution volume (50 µl). The quality of elutes was examined using a nano spectrophotometer and samples stored at -80°C until further studies.

Individual singleplex PCR assays were initially optimized for each hemoparasite using species-specific primer sets. Subsequently, a multiplex PCR was developed to enable the simultaneous detection and differentiation of the targeted organisms (*B. vogeli* and *B. gibsoni*) along with IAC and co-infections in a single tube PCR test. Highly infected microscopically positive clinical samples for

individual parasites, confirmed by PCR assay, served as positive control while sample obtained from an infection free neonatal puppy was used as negative control. For each PCR, negative control was run together. The details of the oligonucleotide primers used in the study are depicted in Table 1.

A 20 µl volume of singleplex PCR reaction mixture was standardized containing 0.5 µl of each primer (10 µM), 10 µl of multiplex Master Mix (2×) (Qiagen, Germany), 8 µl nuclease-free water and a 1 µl aliquot of extracted DNA. The cycling condition of the PCR was set as initial heat activation (95°C for 15 min), denaturation (94°C, 30 sec) - 35 cycles, annealing (59-62°C, 90 sec) and extension (72°C, 90 sec) and final extension (72°C, 10 min).

For multiplex PCR, the PCR reaction mixture was standardized containing 0.6 µl of each primer (2 pairs each 10 µM) along with another primer pair targeting canine β-actin gene (β-actin-F: CTGTCCCTGTATGCCTCTG, β-actin-R: ATGTCACGCACGATTTC) of 218 bp (Chen *et al.*, 2014), 15 µl of multiplex master Mix (2×) (Qiagen, Germany), a 3 µl aliquot of isolated DNA and nuclease-free water was added to make up the final volume to 30 µl. Concentration of primers and annealing temperature were adjusted to reach the optimum conditions. The thermal profile for PCR

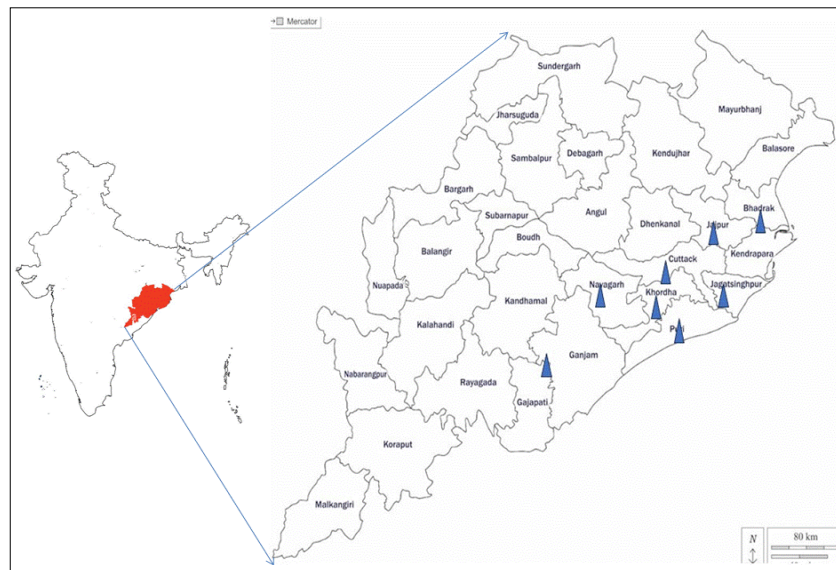


Fig 1: Map of India depicting Odisha and map of Odisha with districts included in the investigation marked by blue triangle.

Table 1: The primer sequences used in singleplex conventional and multiplex PCR assay.

Parasite	Sequence	Size	Target gene	Reference
<i>B. vogeli</i>	BCV F5'GTGAACCTTATCACTTAAAGG 3' BCV R5'CAACTCCTCCACGCAATCG 3'	602 bp	18S ribosomal RNA gene	Kaur <i>et al.</i> , 2020
<i>B. gibsoni</i>	BAG F5'TGGGGTTTTCCCTTTTAC 3' BAG R5'TTC AGC CTT GCG ACC ATAC 3'	489 bp	18S ribosomal RNA gene	Sathish <i>et al.</i> , 2021
<i>ACTB</i>	β-actin F5'CTGTCCCTGTATGCCTCT 3' β-actin R5'ATGTCACGCACGATTTC 3'	218 bp	canine β-actin gene	Chen <i>et al.</i> , 2014

amplification is used as initial heat activation (95°C , 15 min) denaturation at (94°C , 30 sec)- 35 cycle, annealing (60°C, 90 sec) and extension (72°C , 90 sec) and final extension (72°C , 10 min).

Thermal Cycler (T100TM Thermal Cycler, Bio-Rad, USA) were used for all the amplifications. PCR products were subjected to horizontal electrophoresis on a 1.5% agarose gel containing ethidium bromide dye in Tris-acetate-EDTA (TAE) buffer at 85V for 60 min along with 100 bp DNA ladder. The amplifications were checked and documented under UV light gel documentation system (Gel Doc TM EZ Imager, Bio-Rad, Hercules, CA, USA).

Validation of developed m-PCR assay

DNA samples isolated from the 3 numbers of positive control dogs for each parasite were used to validate the diagnostic efficiency of the multiplex PCR assay. Further, 61 random clinical samples from field with history of tick infestation, were used for singleplex PCR assays targeting the specific parasite as well as for the standardized multiplex PCR assay to evaluate the sensitivity and specificity of the developed multiplex assay. Later, selected mPCR amplified products from field samples (2 each for a parasite species) were also sequenced for further confirmation.

Sequencing and phylogenetic analysis

The positive samples, which showed a distinct bright band in multiplex PCR, were purified from gels by QIAquick® Gel Extraction Kit (Qiagen, GmbH, Germany) as per the manufacturer's instructions and subsequently sequenced by Eurofins Laboratories, Bangalore. The obtained sequences were aligned using MEGA version 12 and the representative sequences were submitted to the GenBank database under the accession number (PV329704- *B. gibsoni*), (PV329651- *B. vogeli*). The partial 18S rRNA gene sequence was compared with previously reported sequences available in GenBank. Multiple sequence alignment was performed using the MUSCLE algorithm and similarity with homologous sequences was assessed using nucleotide (nBLAST) Basic Local Alignment Search Tool. Sequence identity analysis was carried out with the Clustal V method, while phylogenetic relationships were inferred from the nucleotide alignments by applying the maximum-likelihood method with 1000 bootstrap replications in MEGA 12. (Kumar *et al.*, 2024).

Statistical analysis

The statistical analysis was implemented through SPSS version 22 statistical windows software program. The developed multiplex PCR was validated through calculation of McNemar's test p value, Kappa value, specificity and sensitivity with 95% confidence interval by comparing the field DNA samples with its singleplex PCR counterpart. The risk factors association with prevalence of haemoparasitic infections was determined by the Chi-square test, with acceptable probability of error up to 5% ($p < 0.05$). The Chi-square value (χ^2), degrees of freedom and *P*-values (≤ 0.05)

were evaluated to determine the strength of association between variables. Analyzed risk factors included locality (Khordha, Cuttack, Puri, Ganjam, Nayagad, Bhadrak, Jajpur and Jagatsingpur), sex (male and female), age groups (<1, 1-6 and >6 years), breeds (Non descrypt, Golden retriever, Labrador, Spitz and others), tick infestation and outside activity (nil, limited, active).

RESULTS AND DISCUSSION

Comparison of singleplex and multiplex PCR assay and validation

In the present study, for the very first time an internally controlled multiplex PCR assay was developed and validated for the detection and discrimination of *B. gibsoni* and *B. vogeli* including an IAC to avoid false negative result. The singleplex PCR assays revealed amplicons of 602 bp for *B. vogeli* and 489 for *B. gibsoni* respectively. Similar amplicons were also produced in the standardized multiplex PCR assay for the respective parasites along with an amplicon of 218 bp for IAC and without any non-specific amplifications (Fig 2 A-C). Validation of the standardized multiplex PCR assay on positive control samples from each 2 catagories revealed 100% sensitivity and 100% specificity. Later, field validation on randomly screened 61 blood samples, for mPCR taking singleplex PCR as standard revealed the diagnostic sensitivity (95% CI) of multiplex PCR assay in the detection of *B. gibsoni* and *B. vogelias* 80%

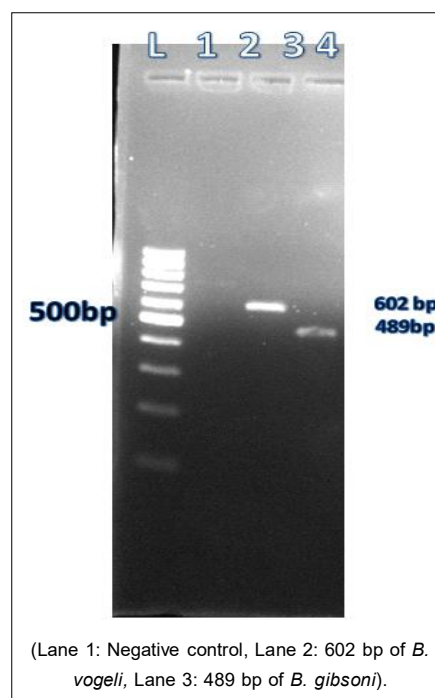


Fig 2A: Gel image showing singleplex PCR assays for the detection of fragments of 489 bp of *Babesia gibsoni*, 602 bp of *Babesia vogeli* L: Gene ruler 100 bp DNA ladder (Thermo scientific).

(78.71% to 81.28%) and 100% (98.04% to 100%) respectively. The diagnostic specificity (95% CI) of multiplex PCR assay in the detection of *B. gibsoni* and *B. vogeli* was detected as 100% (98.04% to 100%). Statistical analysis using McNemar's test indicated that there was no significant difference between singleplex and multiplex PCR assays in detection of *B. gibsoni* ($p = 0.125$) and *B. vogeli* and ($p=1.000$). The kappa values of multiplex PCR assay in detection of *B. gibsoni* and *B. vogeli* revealed very good agreement with that of singleplex PCR (Table 2).

The optimization of multiplex PCR assay becomes challenging due to competition between multiple primers for a finite amount of reagents, the chance of cross hybridization of multiple primer pairs and amplicon size (Elnifro *et al.*, 2000). In this study, the assay was optimized to obtain maximum sensitivity and specificity comparable to its singleplex assay counterparts without any significant difference. Despite the use of three primer sets in one reaction (including one set for IAC), the multiplex PCR was able to detect and differentiate the *Babesia* spp. at a higher resolution and to yield amplified products of comparable amplicon sizes. Moreover, the co-amplification of the internal control alongside the target gene confirmed overall integrity of the reaction and further validated the result. Further, the sequencing of PCR products also confirmed the specific amplification.

Microscopy and field evaluation of multiplex PCR

Among the 280 dogs screened by both microscopy and mPCR from 8 selected districts, total 7.14% dogs found to be microscopically positive for Babesiosis with higher incidence of *Babesia gibsoni* infection (6.1%) than that of *Babesia vogeli* (0.3%) and instances of concurrent/mixed infection were microscopically detected. Whereas Multiplex PCR assay of the screened samples from the specified areas revealed 17.14% (48/280) prevalence of Babesiosis among which 15.35% (43/280) dogs were found to be infected with single pathogen and 10.62% (5/280) dogs were infected by both the pathogen. Among the single infections, the prevalence of *B. gibsoni* infection found to be higher (13.21%) in the examined dog population compared to the prevalence recorded for *B. vogeli* (2.14%) infection. The diagnostic sensitivity is significantly ($p<0.05$) higher in case of mPCR compared to microscopy in detection of Babesiosis (Table 3). This significant difference in this study, suggests the superiority of the standardized mPCR assay in terms of its ability to discriminate and detect co-infection carrier animals as well as during subclinical and latent infection in diseased animal (Kaur *et al.*, 2020). A higher sensitivity of molecular screening compared to the microscopy also previously reported from Punjab (Singh *et al.*, 2014; Singla *et al.*, 2016); Kerala (Jain *et al.*, 2017); Rajasthan (Choudhary *et al.*, 2025); Andhra Pradesh (Kopparthi *et al.*, 2021); Northeastern and Western India (Mittal *et al.*, 2019).

Multiplex PCR analysis with respect to various risk factors associated with the prevalence of babesiosis

The association of risk factor with prevalence of various tick-borne pathogens has been summarized in Table 4. The variations of prevalences of Babesiosis among

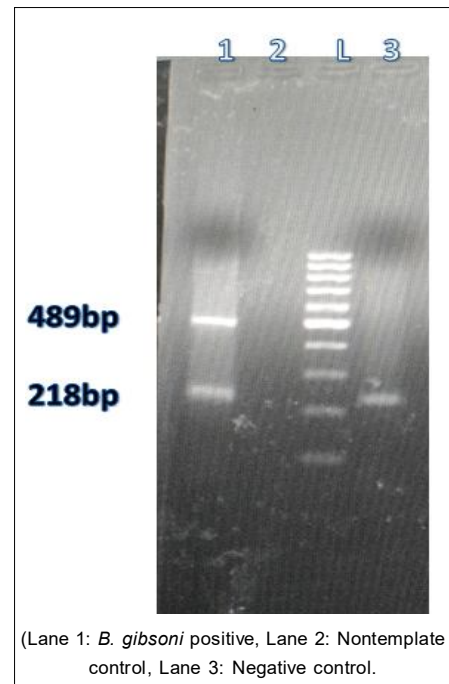


Fig 2B: Multiplex PCR with canine β -actin gene of 218 bp for detection of *B. gibsoni*.

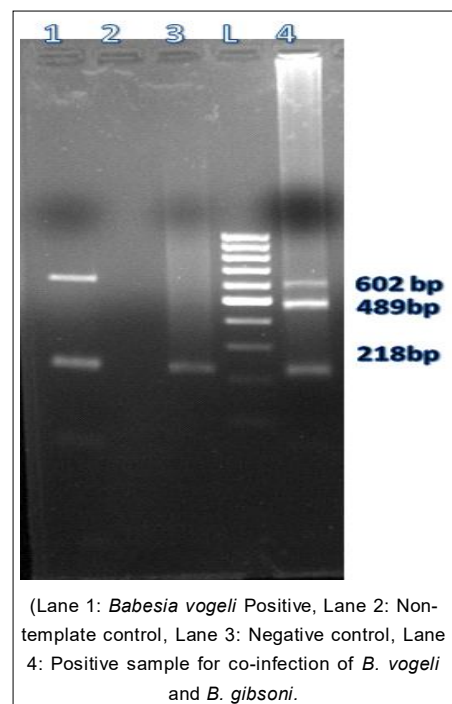


Fig 2C: Multiplex PCR with canine β -actin gene of 218 bp for detection of *B. vogeli* and co-infection.

various districts were non-significant except for Khordha districts which was found to be significantly harbouring *B. gibsoni* positive dogs. Higher prevalence of *Rhipicephalus* and *Haemaphysalis* ticks were recorded in this area previously Sahu *et al.* (2013) which might have led to high circulation of this pathogen among them and to the naïve dogs, though vectorial role of these ticks in this area needs further investigation.

A seasonally influenced pattern of incidence of *B. gibsoni* and *B. vogeli* infections was implied from the statistically significant association ($p < 0.05$) and the disease incidence was highest in summer season. The climate of Odisha is characterized by three main seasons namely summer (March-June), rainy (July- October) and winter (November-February) having variable ambient air temperature (19°C - 31°C), 76.9% average relative humidity and average rainfall of 1400-1500 mm annually with abundance of ticks in summer season (Sahu *et al.*, 2013). So, the significant higher prevalence of Babesiosis in summer season, found herein, may be due to higher vector activity in dogs. However, there was no significant risk of infection with breed predisposition, sex and age of the dogs which was also previously reported (Singla *et al.*, 2016; Manoj *et al.*, 2020).

Presence of ticks rather than history of tick infestation was significantly related to Babesiosis similar to previous reports Manoj *et al.* (2020) as the tick, acts as putative

vector for *Babesia* infection, though vertical transmission, blood transfusion and direct transmission through fighting injury are also possible modes of transmission (Karasová *et al.*, 2022).

The risk of infection was significantly associated with outside activity of dogs. Dogs with no outside activity revealed lowest rate of incidence and dogs with limited activity revealed highest rate of incidence. Although strict in-house domestication was found to have significant role in minimizing the infections due to minimal tick exposure, still dogs with limited outdoor activity unexpectedly revealed higher prevalence than feral dogs. This might be due to the fact that outdoor activity of pet dogs near vegetation during dusk and dawn increases the chance of tick exposure (Noden *et al.*, 2007). Although feral dogs have greater exposure to tick vectors and more frequent contact, their immune system appears to be better adapted to hemoparasitic infections than that of owned dogs. The notably higher expression of the granzyme B gene in stray dogs, compared with owned dogs, contributes to the commencement of apoptotic pathways within their immune defense (Temizkan *et al.* 2022).

Phylogenetic analysis

Phylogenetic analysis was performed based on partial sequences of 18s rRNA gene of *B. gibsoni* and *B. vogeli* retrieved from the NCBI database. The nucleotide

Table 2: Comparative analysis of singleplex and multiplex PCR assay.

<i>Babesia gibsoni</i>	Singleplex PCR			McNemar's test (Two tailed) p value	Kappa value $\pm$ S.E. (agreement)	Sensitivity	95% CI	Specificity	95% CI
	Negative	Positive	Total						
Multiplex PCR				0.125	0.843 $\pm$ 0.075 (very good)	80%	78.71% to 81.28%	100	98.04 to 100%
Negative	41	4	45						
positive	0	16	16						
Total	41	20	61						
<i>Babesia vogli</i>	Singleplex PCR								
Multiplex PCR	Negative	Positive	Total	1	1 $\pm$ 0 (very good)	100	98.04 to 100%	100	98.04 to 100%
Negative	59	0	59						
positive	0	2	2						
Total	59	2	61						

CI: Confidence interval.

Table 3: Microscopy and multiplex PCR assay of field samples.

Parasite	Microscopy			mPCR		
	SI	MI	Total	SI	MI	Total
<i>B. gibsoni</i>	18 (6.4%)	0	18 (6.4%)	37 (13.21%)	5 (1.8%)	42 (15%)*
<i>B. vogeli</i>	3 (1.07%)	0	3 (1.07%)	6 (2.14%)	5 (1.8%)	17 (6.07%)*
BG+BV	0	0	0	0	5 (1.8%)	5 (1.8%)*

SI: Single infection, MI: Mixed infection; BG+ BV- *Babesia gibsoni* with *Babesia vogli*.

The figures in percent in parenthesis indicates prevalence (n=280).

* $p < 0.05$; McNemar's test of sensitivity between microscopy and mPCR (Two tailed) p value.

sequence identity study of the Odisha isolates also revealed 97-100% similarity between various global isolates of these pathogens from dogs.

The phylogenetic analysis revealed that the *B. gibsoni* Odisha isolate (PV329704) fell within a well-supported clade alongside other Indian and Asian isolates, which implied an endemic lineage with shared evolutionary origins. The Odisha isolate of *B. gibsoni* found to be closely related with Trivandrum isolate (MN134509), Ludhiana isolate (MN080887) and Indian isolate from Jackel (OR512622). The *B. vogeli* Odisha isolate (PV329651) clustered within a separate clade consistent with global *B. vogeli* sequences and was found to be closely related

with nucleotide sequences of *B. vogeli*, isolated from dogs of Chandigarh isolate (MN398960) and Mannuthy isolate of India (JX831363) and Brazil isolate (FJ588003). Both clades exhibit high bootstrap support (>90%) and distinct interspecies separation from the clades containing other species of Babesia including *B. rossi* (KC453992), *B. bovis* (EF601930) and *T. annae* (JX454779), confirming accurate species identification (Fig 3). Branching patterns between species are pronounced, confirming the marker's resolution power for species discrimination. These findings collectively implied recent common ancestry, stable epidemiological cycles and conserved genetic evolution in these pathogens in Odisha which might contribute crucially

Table 4: Distribution of canine Babesiosis with respect to various risk factors.

Risk factor	Parameter	n	Prevalence		
			<i>B. gibsoni</i>	<i>B. vogeli</i>	BG+BV
Place	Khordha	100	22 (7.9%)	2 (0.7%)	2 (0.7%)
	Cuttack	37	4 (1.4%)	0	1 (0.4%)
	Puri	28	1(0.4%)	1 (0.4%)	0
	Ganjam	27	2 (0.7%)	0	0
	Nayagad	23	2 (0.7%)	1 (0.4%)	0
	Bhadrak	20	2 (0.7%)	1 (0.4%)	0
	Jajpur	23	1 (0.4%)	0	2 (0.7%)
	Jagatsingpur	22	3 (1.1%)	1 (0.4%)	0
Irrespective of sampling districts		280	37 (13.2%)	6 (2.1%)	5 (1.8%)
Pearson χ^2 value (P value)			12.151 (0.054)*	4.108 (0.768)	8.647 (0.279)
Season	Summer	98	20 (7.1%)	3 (1.1%)	3 (1.1%)
	Rainy	144	11 (3.9%)	2 (0.7%)	1 (0.4%)
	Winter	38	6 (2.1%)	1 (0.4%)	1 (0.4%)
Pearson χ^2 value (P value)			8.545 (0.014)*	7.441(0.024)*	2.042 (0.36)
Breed	Nondescript	113	14 (5%)	4(1.4%)	3 (1.1%)
	Golden retriever	30	3 (1.1)%	0	2 (0.7%)
	Labrador	61	5 (1.8%)	1(0.4%)	0
	Spitz	49	8 (2.9%)	1(0.4%)	0
	Other	20	7 (2.5%)	0	0
Pearson χ^2 value (Pvalue)			5.895 (0.207)	2.376 (0.667)	7.053 (0.133)
Sex	Female	141	22 (7.9%)	2 (0.7%)	3(1.1)%
	Male	139	15 (5.4%)	4 (1.4%)	2 (0.7%)
Pearson χ^2 value (P value)			1.413 (0.235)	0.711 (0.399)	0.189 (0.663)
Age	<1 year	52	5 (1.8%)	3 (1.1%)	2 (0.7%)
	1-6 year	174	22 (7.9%)	2 (0.7%)	1(0.4%)
	>6year	54	10 (3.6%)	1 (0.4%)	2 (0.7%)
Pearson χ^2 value (P value)			1.961 (0.375)	4.1 (0.129)	3.846 (0.146)
Tick infestation	Yes	67	16 (5.7%)	5 (1.8%)	4 (1.4%)
	No	213	21 (7.5%)	1 (0.4%)	1 (0.4%)
Pearson χ^2 value(P value)			8.738 (0.003)*	6.178 (0.03)*	8.762 (0.003)*
Outside activity	Nil	110	6 (2.1%)	0	0
	Limited	81	19 (6.8%)	4 (1.4%)	3 (1.1)%
	Active	89	12 (4.3%)	2 (0.7%)	2 (0.7%)
Pearson χ^2 value (P value)			13.191 (0.001)*	10.963 (0.002)*	8.76 (0.012)*

The figures in per cent in parenthesis indicates prevalence (n=280), "n" denotes number of dogs.

*Significant at $p \leq 0.05$.

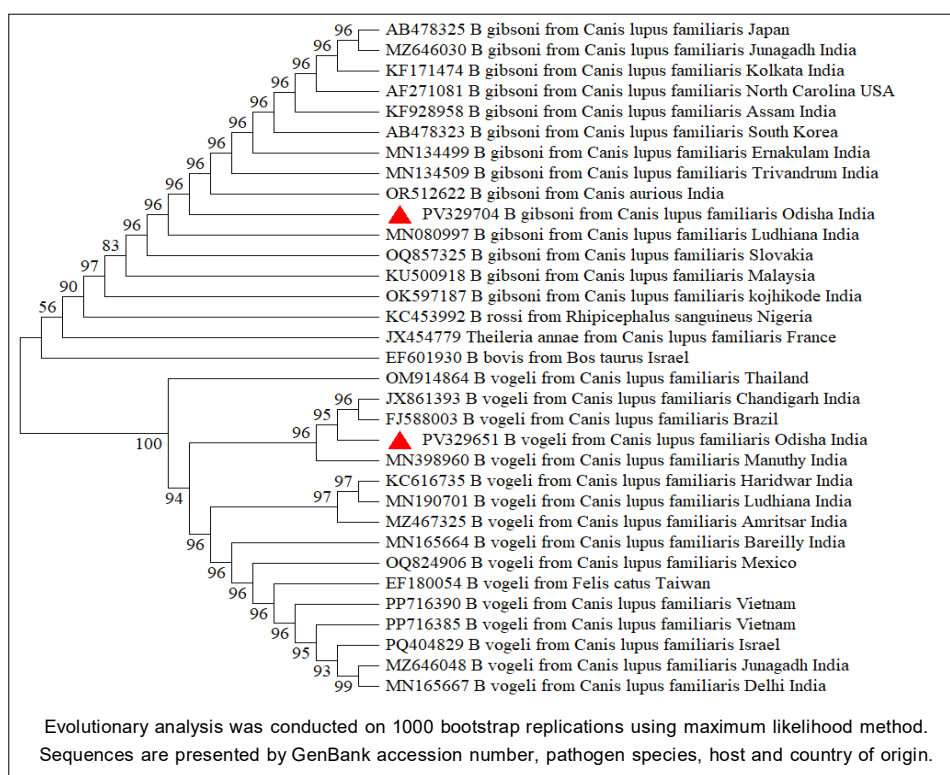


Fig 3: Phylogenetic analysis of *Babesia* spp. based on partial sequence of 18S rRNA gene.

to surveillance, diagnosis and epidemiology of Babesiosis, guiding region-specific interventions and understanding of their transmission ecology. Future studies might utilize more variable genomic regions or whole genome sequencing to uncover any rare or cryptic genetic diversity that lies below the resolution threshold of these markers.

CONCLUSION

In the present study, a multiplex PCR was developed for detection and discrimination of *Babesia gibsoni*, *Babesia vogeli* along with amplification of canine β -actin gene for validation. Absence of nonspecific amplification and high degree of sequence homology between generated sequence and published sequence data of respective pathogens revealed high specificity of the assay. The epidemiological study revealed high prevalence of *B. gibsoni* in Khorda districts and high prevalence of Babesiosis in summer season. Further study may be employed to assess the carrier status of ticks in this particular geographical area. The evolutionary analysis of *Babesia* spp. revealed high genetic homology to corresponding isolates from India and neighboring countries, underlining regional pathogen connectivity and evolutionary stability in key molecular markers. Conclusively, the developed mPCR assay might be promising as a rapid, reliable and sensitive diagnostic tool for processing of large no of clinical samples and for epidemiological studies for planning of pragmatic control strategies against canine Babesiosis.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by Institutional Animal Ethical Committee Regd. No- 433/ CPCSEA, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar vide reference no 833/IEAC dated 7.10.2023. The consent for blood collection was obtained from the dog owners for the diagnostic purposes by registered professionals following the guidelines for blood collection stipulated by Committee for the Control and Supervision of Experiments on Animals (CCSEA).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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