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Identification of *Babesia gibsoni* in dogs from Namakkal region by polymerase chain reaction

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Abstract
Dogs brought to Teaching Veterinary Clinical Complex, Veterinary College and Research Institute, Namakkal were screened for *Babesia gibsoni* by conventional microscopic examination and polymerase chain reaction. The parasite could not be detected by microscopy, however, polymerase chain reaction targeting 18S rRNA gene of *B. gibsoni* revealed a positivity of 2.0 per cent. The amplicon of *B. gibsoni* had complete homology with the isolates from India and other countries. Male, adult dogs of non-descript breeds were found to be highly susceptible for babesiosis. The PCR was found to be useful in the epemiological survey of *B. gibsoni* in dogs.

Keywords: *Babesia gibsoni*, polymerase chain reaction, phylogeny, epidemiology

1. Introduction
Worldwide, dogs are continuously exposed to a spectrum of haemoparasitic diseases [1] and amongst which babesiosis is the most widespread canine vector borne disease in the recent years in India [2]. However, there is a relative paucity of studies into canine Babesia and the epidemiology of canine babesiosis in India is still poorly understood [1]. *Babesia canis* and *B. gibsoni* are the common parasites because of frequent infestation by the tick *R. Sanguineus*. They cause mortality in dogs with hyperacute to sub-clinical or inapparent infection depending on the virulence of the species and the susceptibility of the host [3]. The acute course is the most common, and is characterized by fever, lethargy, haemolytic anaemia, thrombocytopenia, lymphadenopathy and splenomegaly [4].
Most of the cases have so far been diagnosed only by microscopic examination of peripheral blood smears which do not permit reliable detection of the parasite in clinical and asymptomatic carriers [5]. Further, serological approaches also have their limitations owing to their inability in the species-specific diagnosis, false positive and false negative results [1]. For better understanding of the disease, the knowledge on distribution and epidemiology of the disease in dogs from local regions is rather important which would help to implement rational and effective control strategies. Hence, this paper reports the molecular prevalence and characterisation with the epidemiology of *B. gibsoni* in dogs in Namakkal region of Tamil Nadu.

2. Materials and Methods
Dogs (n=150) including clinical cases (anorexia, pyrexia and lymph node enlargement) and apparently healthy, irrespective of their age, sex, breed and health status presented to Teaching Veterinary Clinical Complex (TVCC), VC&RI, Namakkal of Tamil Nadu were screened for *B. gibsoni*. Thin peripheral blood films, whole blood samples in EDTA and tick samples were collected from the study population along with relevant epidemiological data and no ethical committee approval was needed to carry out this study. The blood films were stained by Giemsa staining technique and the blood samples in EDTA were subjected to DNA extraction by using Dneasy blood kit (Qiagen, Netherland) as per the manufacturer's recommendation.
The primers specific to the parasitic genome targeting the 18S ribosomal RNA gene of *B. gibsoni* as recommended by [6] were custom synthesized (Eurofins and Sigma, India) with the following nucleotide sequences and amplicon size of 671 bp (Figure 1): Forward primer –5'-CTCGGCTACTTGCCTTGTC-3' and reverse primer-5' GCCGAAACTGAAATAACGGC-3'. The extracted DNA was amplified using the selected primers with the following reaction mixture and cycling conditions. Reaction mixture: DNA template –3µl, master mix-13 µl, forward and reverse primer (10 pico moles, each) - 1 µl each and molecular grade water -7 µl. Cycling conditions: Initial denaturation -95 °C/5min, denaturation- 95 °C/30sec, annealing-

55 °C/30sec and extension -72 °C/90sec with 35 cycles; and final extension- 72 °C/5min. The gel was visualized under UV transilluminator and the bands of appropriate size were identified by comparison with the 100 bp ladder. The images were documented using the gel documentation system (Vilber Lourmat, France). One of the amplified PCR products were sequenced and characterized by phylogenetic analysis for its homology and divergence with the isolates of *B. gibsoni* available in the GenBank. The ticks collected from the positive cases were identified and predisposition to the parasitic infection by epidemiological factors was statistically analysed by Chi-square test.

3. Results

Microscopic examination of Giemsa stained peripheral blood smears could not reveal *B. gibsoni*, whereas a low prevalence of 2.0 per cent was recorded by PCR targeting 18S rRNA of *B. gibsoni at* 671 bp (Figure 1). The sequence of *B. gibsoni* 18S rRNA gene identified was found to possess a complete homology (100.0%) with Indian isolates followed by China, Korea, Thailand, Turkey and USA isolates (Figure 2). Statistically, no significant difference between ages, sex and breeds was observed in the occurrence of babesiosis, however, statistically significant difference (P<0.05) could be recorded between seasons (Table 1).

4. Discussion

The absence of positivity by conventional microscopic examination could be attributed to the low sensitivity of the light microscopy, though the technique is being the simplest, reasonably sensitive during acute infections [7] and this is similar to the finding of [5] who recorded a very low prevalence of 0.1 per cent in Chennai (Tamil Nadu) by microscopic examination. However, a low to high prevalence of 8.26, 7.84, 47.72 and 84.9 per cent for *B. gibsoni* in dogs in Ludhiana, Jalandhar (Punjab), Guwahati (Assam) and Chennai Tamil Nadu), respectively in India were recorded by [8], [16], [2] and [18], respectively by microscopic examination of peripheral blood smears.

In comparison to microscopy, PCR could detect *B. gibsoni* in dogs, as it is the promising tool for diagnosis with specificity and sensitivity of 100.0 per cent [9]. This finding is in agreement with that of [5] who recorded a very low prevalence of 0.2 per cent for *B. gibsoni* by PCR in dogs in India, where as [6] recorded a high prevalence of 48.64 per cent by PCR for *B. gibsoni* in dogs in India. Previously, [10] and [11] also employed the 18S rRNA gene-based PCR assay for detection of *B. gibsoni* in dogs. The homology of the sequence of *B. gibsoni* observed in this study is in accordance with that of [12].

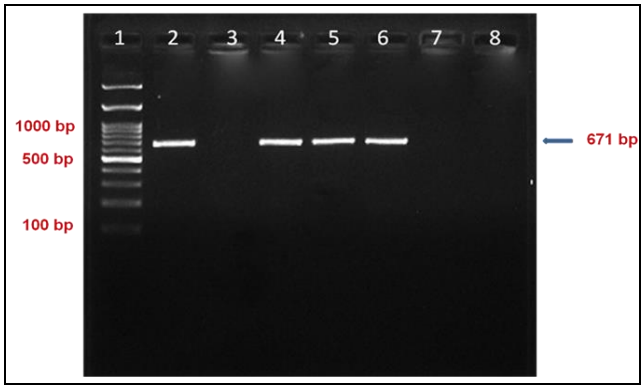
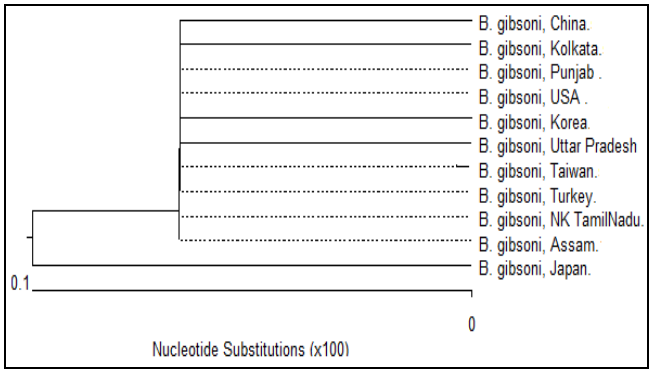


Fig 1: PCR amplified products of 18S rRNA gene of *H. canis* in 1.5 per cent agarose gel showing bands at 671 bp

Lane 1 - Ladder, lane 2-Positive control, lane 3- Negative control an lane 4 to 8- Test samples



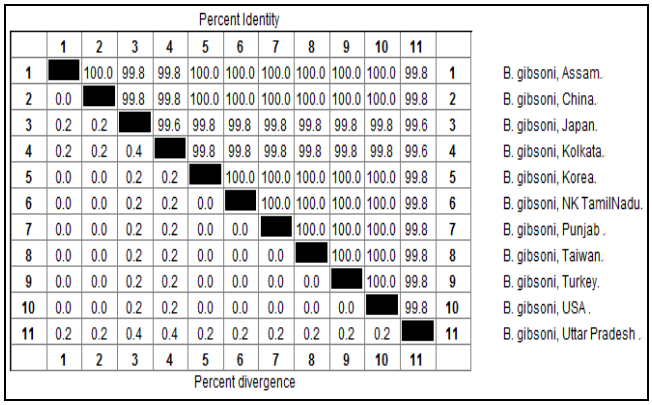


Fig 2: Phylogenetic relationship of 18S rRNA gene of *B. gibsoni* with other published isolates (GenBank) and its homology and divergence

The proven vector for babesiosis was *R. sanguineus* which had been the common tick of dogs in India, as reported by [5] and [2]. The prevalence of *B. gibsoni* was found to be high in the age group of 2-4 years (Table 1) and this in agreement with that of [13]. In contrast, [14] and [15] stated that though dogs of all age groups can be affected, dogs younger than 1 year are the most susceptible. The high prevalence of *B. gibsoni* recorded in males (Table 1) is in agreement with that of [16], whereas [17] recorded a high prevalence in females. However, [14] concluded that no predilection could exist to sex. The higher prevalence of *B. gibsoni* detected in non-descript dogs than any other breeds (Table 1) is in accordance with that of [18]. The high prevalence in non-descript dogs might be due to the poor management and health status associated with them and also increased exposure to the tick vectors in these dogs with non-hairy coat. The high prevalence of *B. gibsoni* recorded in winter (Table 1) is similar to that of [19] who observed a high prevalence in monsoon. The high prevalence during winter might be due to the prolonged subclinical period in chronic cases or recrudescence when exposed to stress or corticosteroid therapy.

5. Conclusion

The PCR, therefore was found to be a useful tool in the detection of subclinical babesiosis in dogs when compared to conventional blood smear examination due to it's poor sensitivity in detecting a very low parasitaemia. Hence, with this above view, molecular surveillance by employing techniques like PCR would help in the early identification of the subclinical carriers as sources of infection to susceptible dog population and their elimination by specific treatment

Table 1: Age, sex, breed and season-specific prevalence of *B.gibsoni* in dogs

Epidemiological determinants		Total (n=150)	No. of positives (n=3)	Per cent positives	P value
	<1year	34	-	-	0.808[NS]
	1-2 years	40	1	2.5	
	2-4 years	32	1	3.12	
	>4years	44	1	2.27	
Sex	Male	108	3	2.77	0.275[NS]
	Female	42	-	-	
Breed	Pure	106	1	0.94	0.180[NS]
	Cross	10	-	-	
	ND	34	2	5.88	
Season	Southwest monsoon	12	-	-	0.029*
	Northeast monsoon	82	-	-	
	Winter	41	3	7.31	
	Summer	15	-	-	

[NS] Non-significant and * Significant at 95.0 per cent level

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