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Comparison of polymerase chain reaction assays targeting 18S ribosomal RNA and secreted antigen1 genes for the detection of *Babesia gibsoni* in dogs

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Abstract
The haemoprotozoan parasite, *Babesia gibsoni*, a small intra-erythrocytic apicomplexan parasite is responsible for causing canine babesiosis, which is manifested as hyperacute, acute or chronic infections with clinical symptoms that vary from sub-clinical infection to extensive organ failure and death. The present study was conducted to compare the relative effectiveness of two Polymerase chain reactions (PCR) targeting*18SrRNA* and *BgSA1* genes to detect the presence of *B. gibsoni* in dogs. The polymerase chain reaction (PCR) assay targeting 18S ribosomal RNA gene fragment revealed *Babesia* genus specific ~1665 bp fragment amplified in 15 out of 50 samples. Similarly, the polymerase chain reaction for the amplification of *B. gibsoni* specific secreted antigen 1(*BgSA1*), revealed the ~1500 bp amplicon in eight samples. The comparison of results of PCRs based on McNemar's Test, revealed that the relative effectiveness of PCR targeting *BgSA1* and Bg18S rRNA genes were almost similar.

Keywords: polymerase chain, 18S ribosomal RNA, antigen1, *Babesia gibsoni*

Introduction
Babesiosis, one of the important canine vector-borne diseases, is globally drawn-out and quickly scattered due to the expansion of tick habitats and increased mobility of animals. The causative agents for the disease include both large babesial forms (*Babesia canis* 2.5-5.0 µm) or small forms (*Babesia gibsoni* 1.0-2.5 µm) (Boozer and Macintire, 2003) [3]. *Babesia gibsoni*, the small piroplasm in the erythrocytes of dogs is mainly transmitted naturally by ticks. The other modes of transmission include dog fighting, blood transfusion and transplacental (Birkenheuer *et al*., 2005; Jefferies *et al*., 2007; Schnittger *et al*., 2012) [1, 7, 6] transmission. Canine babesiosis can range from chronic or subclinical forms to per acute and fatal (Schoeman, 2009) [17] infections. There are several reports of the incidences of canine babesiosis from different zones of India, including Kerala (Jain *et al*., 2018; Bora *et al*., 2021). Microscopic examination of peripheral blood smears, indirect fluorescent antibody test (IFAT), enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are the common diagnostic methods for detection of *Babesia* spp. (Qurollo *et al*., 2017) [14]. After the advent of PCR, diagnosis of canine babesiosis became more sensitive and specific than traditional methods (Birkenheuer *et al*., 2003) [2]. The 18S ribosomal RNA gene is one of the most frequently used molecular marker for the diagnostic and epidemiological studies of *Babesia* parasites (Figueroa *et al*., 1993) [5]. The secreted antigens are associated with the erythrocyte invasion of parasite. The gene encoding *B. gibsoni* secreted antigen 1 (*BgSA1*) was identified as a marker of the active *B. gibsoni* infection (Jia *et al*., 2006) [8]. Hence, the present study was carried out to compare efficiency of PCR assays targeting two different genes for the sensitive and specific detection of *B. gibsoni*.

Materials and Methods
The whole blood samples (~1 mL) were collected in EDTA vials from 50 dogs presented to the Veterinary hospitals of Kerala with the symptoms like fever and anaemia. Genomic DNA was isolated from the blood samples using DNeasy ®blood and tissue kit (Qiagen, Germany) according to the manufacturer's protocol. The isolated genomic DNA was stored at -20 ºC deep freezer. The genomic DNA (~20 ng) was used as a template for the polymerase chain reaction (PCR). The 18S rRNA gene specific for *Babesia* spp. was targeted for amplification

using the forward 5' TGGTTGATCCTGCCAGTA 3' and the reverse 5' CTTCTCCTTCCTTTAAGTGA 3' (Jefferies *et al*., 2007) [7] primers. The cycling conditions for the reaction included, initial denaturation at 94 ºC for 5 min followed by 35 cycles, consisting of a denaturation step of 1 min at 92 ºC, an annealing temperature of 45 sec at 52 ºC and an extension step of 2 min at 72 ºC. The final extension was at 72 ºC for 10 min.

Babesia gibsoni specific PCR was also performed by targeting secreted antigen 1 (*BgSA1)* gene based on the protocol described by Liu *et al*. (2016) [12] with the forward (5' TATGGTGGTCGTTGGTTCT 3') and reverse (5' AATGATGCCTCCTTCGC 3') primers for the amplification of ~1500 bp fragment. The cycling conditions were as follows: initial denaturation at 94 ºC for 5 min followed by 30 cycles, each consisting of denaturation at 94 ºC for 45 sec, annealing temperature of 45 sec at 58 ºC and an extension step of 2 min at 72 ºC. The final extension was at 72 ºC for 7 min.

The non-parametric test, McNemar's test was used for statistical analysis for the comparison of the effectiveness of two PCR methods.

Results

In the present study, *Babesia* genus specific ~1665 bp fragment of 18S ribosomal RNA gene was amplified in 15 out of 50 samples (Fig. 1). The species-specific *B. gibsoni* secreted antigen (*BgSA1*) gene was amplified in 8 samples with an amplicon size of ~1500 bp (Fig. 2). The statistical analysis using McNemar's test revealed that the efficacies of the PCRs targeting two genes of *B . gibsoni* were comparable. The Bg18SrRNA is genus specific and hence, this PCR assay may not always predict the presence of *B. gibsoni*. However, *BgSA1* PCR is highly specific and accurate for *B. gibsoni* (Table 1)

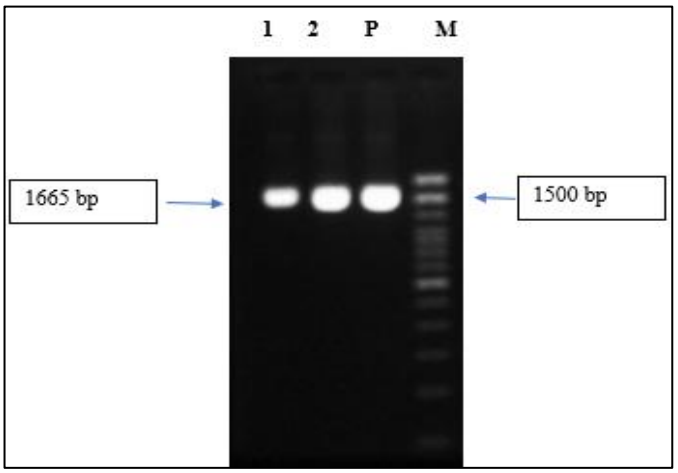

Fig 1: PCR amplification of 18S rRNA gene specific for *B. gibsoni*. Lane M: 100 bp plus ladder, Lane 1,2: Sample, Lane P: Positive control

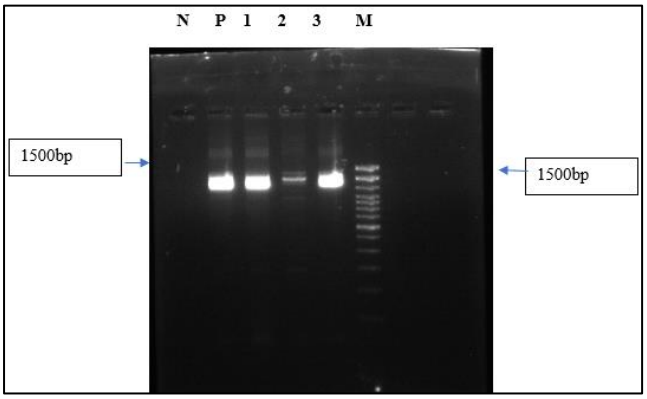

Fig 2: PCR amplification of *SA1* gene specific for *B. gibsoni*. Lane M: 100 bp plus ladder, Lane 1,2,3: Sample, Lane P: Positive control

Table 1: Comparison of PCRs targeting*BgSA1* with Bg18srRNA based on McNemar's Test

BgSA1	Bg18srRNA				Total	
	Positive (n=15)		Negative (n=35)			
	Count	Per cent	Count	Per cent	Count	Per cent
Positive	6	40.0	2	5.7	8	16.0
Negative	9	60.0	33	94.3	42	84.0
Total	15	100	35	100	50	100
P-value for McNemar Test = 0.065^{ns} Kappa value = 0.396** (<0.002)						
Sensitivity = 40.0; Specificity = 94.29; Accuracy = 78.00 Positive Predictive Value = 75.0; Negative predictive value = 78.6						
** Significant at 0.01 level *(P<0.01);* ns non-significant *(P>0.05)*						

Discussion

Microscopy fails to detect the organism in animals with low parasitaemia especially in subclinical and carrier animals. Moreover, the identification of the species of piroplasms based on morphology requires expertise. Hence, the molecular techniques such as PCR and sequencing will help in the proper detection of organisms at the species level as well as in animals with low parasitaemia (Solano-Gallego *et al*., 2016) [19].

The prevalence of canine babesiosis based on microscopical examination of Giemsa's-stained peripheral blood smears of dogs has been reported from various parts of India (Singh *et al*., 2014, Kumar *et al*. 2015) [18, 10]. However, the higher number of positive cases detected by PCR based assays when compared to microscopy has also been reported from many parts of India. This indicated the higher sensitivity of PCR compared to the conventional methods (Laha *et al*., 2013, Singh *et al*., 2014, Mahalingaiah *et al*., 2017, Kalaivanan *et al*., 2018) [11, 18, 13, 9]. Currently, there is a need for the development of a quick, highly specific, and sensitive detection system for the accurate diagnosis of babesiosis in canines.

The ribosomal DNA sequences contain hypervariable regions, which are frequently used for the species-specific amplification and are flanked by highly conserved regions used for broad-range genus amplification (Qurollo *et al*., 2017) [14]. Hence, molecular differentiation based on 18S rRNA gene sequences are more commonly used in the diagnosis of canine babesiosis, whereas further species differentiation will be possible either by targeting the variable region (V4) of 18S rRNA by nested PCR or RFLP of the primary PCR product (Samantary *et al*., 2008) [15]. Targeting a

more specific gene of *B. gibsoni* like *BgSA1* will help in quick differentiation of species of *Babesia*.

Conclusion

Comparison of results of PCR targeting 18srRNA and *BgSA1* genes based on McNemar's Test, revealed that the relative effectiveness of PCRs was almost similar.

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References

1. Birkenheuer AJ, Correa M, Levy MG, Breitschwerdt EB. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000–2003). J Am. Vet. Med. Ass. 2005;227**:**942-947.
2. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *Babesia canis* DNA in canine blood samples. J Clin. Microbiol 2003;41:4172-4177.
3. Boozer AL, Macintire DK. Canine babesiosis. Vet. Clin. Small Anim. Pract 2003;33:885-904.
4. Bora CAF, Varghese A, Deepa CK, Nandini A, Malangmei L, Kumar KGA *et al*. Sequence and phylogenetic analysis of the thrombospondin-related adhesive protein gene of *Babesia gibsoni* isolates in dogs in South India. Parasitol. Int. 2021 doi: 10.1016/j.parint.2021.102477
5. Figueroa JV, Chieves LP, Johnson GS, Buening GM. Multiplex polymerase chain reaction-based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Vet. Parasitol 1993;50:69-81.
6. Jain J, Lakshmanan B, Nagaraj HV, Praveena JE, Syamala K, Aravindakshan T. Detection of *Babesia canis vogeli*, *Babesia gibsoni* and *Ehrlichia canis* by multiplex PCR in naturally infected dogs in South India. Vet. Arh. 2018;88**:**215-224.
7. Jefferies R, Ryan UM, Jardine J, Broughton DK, Robertson ID, Irwin PJ. Blood, bull terriers and babesiosis: further evidence for direct transmission of *Babesia gibsoni* in dogs. Aust. Vet. J 2007;85:459-463.
8. Jia H, Zhou J, Ikadai H, Matsuu, Suzuki H, Igarashi I, Fujisaki K *et al*. Identification of a novel gene encoding a secreted antigen 1 of *Babesia gibsoni* and evaluation of its use in serodiagnosis. Am. J Trop. Med. Hyg 2006;75:843-50.
9. Kalaivanan M, Ponnudurai SSG. Identification of *Babesia gibsoni* in dogs from Namakkal region by polymerase chain reaction. J Entomol. Zool. Stud 2018;6:1146-1148.
10. Kumar P, Kumar V, Gopal H, Sharma H, Wadhawan VM. Prevalence of canine babesiosis in and around Jalandhar (Punjab), India. Vet. Clin. Sci 2015;3:1-3.
11. Laha R, Bhattacharjee K, Sarmah PC, Das M, Goswami A *et al*. *Babesia* infection in naturally exposed pet dogs from a north-eastern state (Assam) of India: detection by microscopy and polymerase chain reaction. J Parasit. Dis 2014;38:389-393.
12. Liu M, Cao S, Zhou M, Wang G, Jirapattharasate C, Moumouni PF. *et al*. Genetic variations of four immunodominant antigens of *Babesia gibsoni* isolated from dogs in southwest Japan. Ticks Tick Borne Dis 2016;7:298-305.
13. Mahalingaiah MKC, Asoor M, Thimmaiah RP, Narayanaswamy HD, Mukartal SY, Elattuvalappil AM. *et al*. Prevalence of canine babesiosis in different breeds of dogs in and around Bengaluru. Adv. Anim. Vet. Sci. 2017;5:140-144.
14. Qurollo BA, Archer NR, Schreeg ME, Marr HS, Birkenheuer AJ, Haney KN *et al*. Improved molecular detection of *Babesia* infections in animals using a novel quantitative real-time PCR diagnostic assay targeting mitochondrial DNA. Parasit Vectors 2017;10:1-13.
15. Samantaray S, Barari SK, Kumar S, Kumar A. Shekhar P. Molecular tools for diagnosis of latent haemoprotozoan diseases of livestock. Intas Polivet. 2008;9:388-394.
16. Schnittger L, Rodriguez AE, Florin-Christensen M, Morrison DA. *Babesia*: a world emerging. Infect. Genet. Evol. 2012;12:1788-1809.
17. Schoeman JP. Canine babesiosis. Onderstepoort J. Vet. Res 2009;76:59-66.
18. Singh A, Singh H, Singh NK, Singh ND, Rath SS. Canine babesiosis in northwestern India: molecular detection and assessment of risk factors. Biomed Res. Int 2014. https://doi.org/10.1155/2014/741785
19. Solano-Gallego L, Sainz Á, Roura X, Estrada-Peña A, Miró G. A review of canine babesiosis: the European perspective. Parasit. Vectors 2016; 9:1-18.