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Deepa CK
Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala. India

Anju Varghese
Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala. India

Ajith Kumar KG
Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala. India

Dinesh CN
Department of Animal Breeding and Genetics, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala, India

Sanis Juliet
Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala, India

Sindhu OK
Department of Clinical Veterinary Medicine, Ethics and Jurisprudence, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad. Kerala, India

Gatchanda Shravan Kumar
Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala, India

Prabodh Kumar Hembram
Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala. India

Reghu Ravindran
Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala. India

Corresponding Author
Deepa CK
Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala Veterinary and Animal Sciences University, Pookode, Lakkidi P.O., Wayanad, Kerala. India

Detection of *Babesia gibsoni* in dogs of Northern Kerala by amplification of heat shock protein 70 gene and 18S rRNA gene

Deepa CK, Anju Varghese, Ajith Kumar KG, Dinesh CN, Sanis Juliet, Sindhu OK, Gatchanda Shravan Kumar, Prabodh Kumar Hembram and Reghu Ravindran

Abstract
Babesia gibsoni is an important intraerythrocytic haemoprotozoan parasite causing babesiosis in canines. The present study was conducted to detect the presence of *B. gibsoni* in dogs using Polymerase chain reaction (PCR) targeting*18srRNA* and *Hsp 70* gene. Fifty blood smears were stained by Giemsa's staining technique. On microscopical examination, five samples were positive for *B. gibsoni* piroplasm. Polymerase chain reaction (PCR) revealed *Babesia* genus specific ~1665 bp fragment of 18S ribosomal RNA gene fragment amplified in 15 out of 50 samples, while the ~1938 bp *B. gibsoni* specific heat shock protein gene (*BgHSP 70*) could be amplified in ten samples. Hence PCR targeting 18S rRNA was more sensitive, however, it may not differentiate the species of canine *Babesia* in blood samples.

Keywords: *Babesia gibsoni*, amplification, 70 gene and 18S rRNA gene

Introduction
Babesiosis, ehrlichiosis, anaplasmosis, hepatozoonosis and haemotropic Mycoplasma infections are the important canine tick-borne diseases (TBD) prevalent in India (Rani *et al*., 2011) [12]. *Babesia gibsoni* and *Babesia canis* are the two important intraerythrocytic haemoprotozoans of genus *Babesia* under the phylum Apicomplexa causing babesiosis in canines. *Babesia gibsoni* is naturally transmitted by *Rhipicephalus sanguineus* ticks. However, apart from the tick bite, blood transfusion, direct contact between dogs through wounds (fighting dogs), saliva or blood ingestion were also described as methods for *B. gibsoni* transmission (Stegeman *et al*., 2003; Birkenheuer *et al*., 2005; Jefferies *et al*., 2007) [16, 2, 6]. The major clinical signs noticed in babesiosis infected dogs includes thrombocytopenia, febrile syndrome (fever, anorexia, depression, dehydration) and haemolytic syndrome (anaemia, bilirubinuria, haemolysis) in acute cases while the chronic form revealed prolonged convalescence characterized by depression (Bourdoiseau, 2006; Solano-Gallego *et al*., 2008, Vishwakarma and Nandini, 2019) [3, 15, 17].
Microscopical examination of blood smear using Giemsa or Wright's stain is the simplest and most accessible and reasonably sensitive method during the diagnosis of acute babesiosis (Solano-Gallego *et al*., 2011) [14], but small piroplasms like *B. gibsoni*, are hard to observe by light microscopy, which has a relatively poor to moderate sensitivity (Miro *et al*., 2015) [10], and expertise is essential. Moreover, due to very low, often intermittent parasitaemias, identification of piroplasms in chronically infected and carrier dogs remains a significant challenge, since chronic carriers can transmit the disease via ticks to other animals. Therefore, to control *B. gibsoni* infection, proper diagnosis followed by effective treatment is essential.
The polymerase chain reaction (PCR), is a highly sensitive diagnostic technique, amplifies the specific gene sequences of infectious organisms. The present study focusses on the comparison of PCR targeting *18srRNA* and *Hsp 70* for the specific detection of *B. gibsoni*.

Materials and Methods
Peripheral blood smears were collected from 50 dogs suspected for *B. gibsoni* infection having symptoms like anaemia and fever from northern zone of Kerala. Whole blood samples in ethylene diamine tetra acetic acid (EDTA) vials were also collected from these dogs. Genomic DNA was isolated from the blood samples using DNeasy ®blood and tissue kit (Qiagen,

Germany) accor ding to the manufacturer's protocol. The isolated genomic DNA was stored at -20 ºC deep freezer. Genomic DNA (~20 ng) was used as a template for polymerase chain reaction (PCR). The 18S rRNA gene specific for *Babesia* spp. was targeted using the forward primer 5' TGGTTGATCCTGCCAGTA 3' and the reverse primer 5' CTTCTCCTTCCTTTAAGTGA 3' (Jefferies *et al*., 2007) [6]. 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.

The present study also targeted *B. gibsoni* specific heat shock protein 70 *(BgHSP70)* using the forward primer 5'ATGACAGGTCCTGCTATAGGTATTGACTTGGG 3' and the reverse primer 5' CATGTGATTAGTCAACCTCCTCAA 3' with cycling conditions, initial denaturation at 94 ºC for 5 min followed by 30 cycles, each consisting of denaturation at 94 ºC for 1 min, annealing temperature of 2 min at 55 ºC and an extension step of 3 min at 72 ºC. The final extension was at 72 ºC for 7 min (Yamasaki *et al*., 2002) [18].

Results

The peripheral blood smears (n=50), were stained using Giemsa's stain and were examined microscopically under 100X objective of a compound microscope (Leica, Germany). Five samples were positive for *B. gibsoni* organisms (Fig. 1). *Babesia* genus specific ~1665 bp fragment of 18S ribosomal RNA gene was amplified in 15 out of 50 samples (Fig. 2), while the ~1938 bp *B. gibsoni* specific heat shock protein gene (*BgHSP 70*) could be amplified in ten samples (Fig.3).

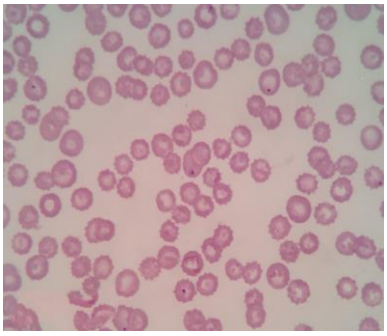

Fig 1: Blood smear of dog showing the *B. gibsoni* piroplasm

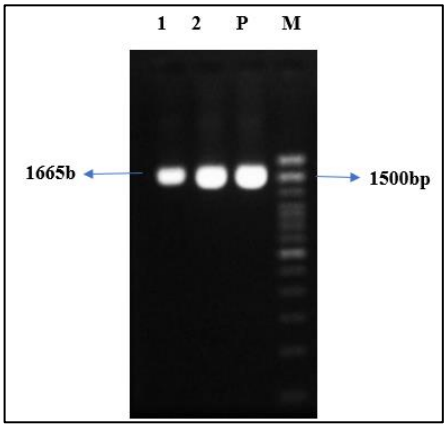

Fig 2: PCR amplification of 18S rRNA gene specific for *B. gibsoni*. Lane M: 100 bp plus ladder, Lane 1,2: Sample, Lane P: Positive control

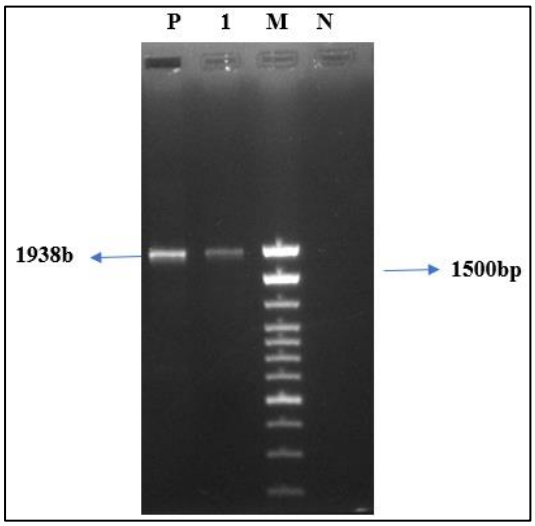

Fig 3: PCR amplification of *Hsp 70* gene specific for *B. gibsoni*. Lane M: 100 bp plus ladder, Lane 1: Sample, Lane N: Non template control Lane P: Positive control

Discussion

Global climate change and adaptation of ticks to new environmental conditions can introduce and influence the vector-borne diseases (Leschnik *et al*., 2008). Canine babesiosis caused by the apicomplexan parasites of the genus *Babesia*, is a clinically significant tick-borne disease distributed worldwide including India. The conditions in which dogs are maintained may affect the development of babesiosis (Bourdoiseau, 2006) [3]. The wide range of clinical manifestations and severity depend on different factors like species of *Babesia* causing infection, age and immune status of host, splenectomy and concomitant infection or disease (Irwin, 2009) [4]. Globally, *B. gibsoni* was the most commonly identified canine *Babesia* species (48.8 per cent of the positive results), then *B. canis* (35.2 per cent), followed by *B. vogeli* (15.3 per cent). In Asian countries, *B. gibsoni* was more common (Birkenheuer *et al*., 2020) [1]. It was also inferred that *B. gibsoni* was identified as the most prevalent haemoprotozoan organism in south India, but rare in other parts of northern and central India (Rani *et al*., 2011; Mittal *et al*., 2019) [12, 11].

Blood smear examination is the simplest and the most accessible diagnostic test for the detection of *Babesia* infection in acute phase of infection. In chronic and carrier forms of the disease, the diagnosis of babesiosis remains challenging due to the low number of parasites. The species identification of the parasite is also essential in identifying the exact therapeutic agent for instituting proper treatment for the ailing animal. The polymerase chain reaction (PCR), is a sensitive and specific diagnostic technique targeting specific gene sequences, and is most useful for the detection of the infection in dogs with low parasitaemia levels and differentiation of species of the parasites (Irwin, 2009) [4]. In the present study, PCR revealed more positive cases compared to the microscopy. These findings were in accordance with (Laha *et al*., 2014; Jain *et al*., 2018) [7, 5].

The 18S ribosomal RNA gene is one of the most frequently used molecular marker in the diagnostic and epidemiological studies of *Babesia* parasites (Figueroa *et al*., 1993). However, the analysis of 18 S rRNA sequences of *Babesia* spp. from different geographical locations revealed very low diversity (Lin *et al*., 2017). Sometimes 18S rRNA gene may not be able to differentiate the parasite at species level. Further

differentiation between species is possible either by targeting the variable region (V4) of 18S rRNA by nested PCR or RFLP of the primary PCR product (Samantaray *et al.*, 2008) [13].

The *Hsp70* form of *B. gibsoni* is also highly conserved (60–90 per cent) with similar proteins from other apicomplexan parasites. Previously, the Nanjing isolates of *B. gibsoni* from China revealed 99.79–99.9 per cent homology with each other and 99.41 to 99.57 per cent nucleotide sequence homology with the Japanese isolate (Lin *et al*., 2017). In the present study, more number of samples could be detected positive when 18S r RNA gene was targeted using PCR. This may be due to the high copy number and lesser amplification length of the target gene fragment compared to Hsp gene. In addition, the 18 S rRNA specific primers may detect the *Babesia* parasites to a genus level, while the HSP is specific for *B. gibsoni*.

Conclusion

The present study compared the molecular detection of *B. gibsoni* using *BgHsp70* gene with 18S rRNA gene. The later was identified as more sensitive, however, it may not differentiate the species of canine *Babesia* in blood samples.

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