SHORT COMMUNICATION

Molecular characterization of *Babesia gibsoni* present in dogs in Sri Lanka

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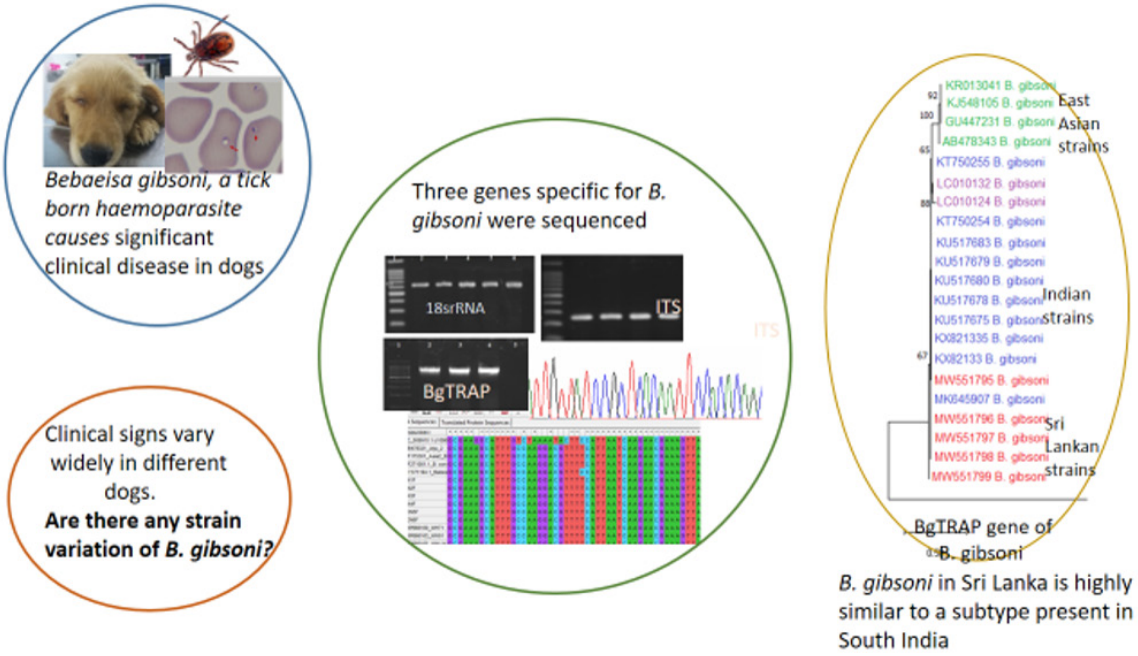

Highlights

- Sequences of 18s rRNA gene of B. *gibsoni* in Sri Lanka were highly similiar to Wayanad isolate 8 from India.
- ITS1 genes were highly similar to those of *B. gibsoni* from India.
- *B. gibsoni* in Sri Lanka is genetically similar to a strain present in South India.

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Molecular characterization of *Babesia gibsoni* present in dogs in Sri Lanka

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Abstract: Certain *Babesia* species that were known as a single species are now re-classified into subspecies or different species. To identify any strain variation in *B. gibsoni* species which infect dogs in Sri Lanka, sequences of the three genes; 18S rRNA, internal transcribed spacer 1 (ITS1), and *B. gibsoni* thrombospondin related adhesive protein (BgTRAP) were analyzed in dogs with babesiosis from three geographical locations. Analysis of the sequences of the 18S rRNA gene confirmed that the samples contained *B. gibsoni* Asian genotype. Analysis of the BgTRAP gene revealed that the *B. gibsoni* present in Sri Lanka is genetically different from the species present in East Asian countries.

***Keywords*:** *Babesia gibsoni*; 18S rRNA; ITS1; BgTRAP; variant, Sri Lanka.

INTRODUCTION

Canine babesiosis caused by the intra-erythrocytic apicomplexan parasite is a life-threatening disease (Wozniak *et al*., 1997). Two morphologically distinct forms named large and small *Babesia* are causing these infections in dogs (Kjemtrup and Conrad, 2006). The larger form with pear-shaped trophozoites belongs to three sub-species: *Babesia canis* (*B. canis*), *Babesia rossi (B. rossi)*, and *Babesia vogeli* (*B. vogeli*) (Irwin, 2009). The smaller form which appears as an oval signet ring body in the erythrocytic stage, may represent one of the three different species namely *Babesia gibsoni* (*B. gibsoni*), *Babesia conradae* (*B. conradae*) or *Babesia vulpes* (*B. vulpes*) (syn *Babesia microti* like/*Theileria annae*) (Irwin, 2009; Baneth *et al*., 2019) and of those species, *B. gibsoni* is highly prevalent in Asian countries (Irwin, 2009). Canine Babesiosis was reported in Sri Lanka as early as 1953 (Seneviratna, 1953; Seneviratna and Jayawickrama 1961; Seneviratna, 1965) and several recent studies have confirmed the high prevalence of the disease in the country (Weerathunga *et al*., 2019; Jayathilake *et al*., 2020; Neelawala *et al*., 2021). As the severity of the clinical signs caused by *B. gibsoni* shows significant variations, the possibility of the presence of different strains or sub-species of the organism has become a concern, and sequencing of various genes to identify any genetic differences has been attempted (Bostrom *et al*., 2008; Mandal *et al*., 2014; Terao *et al*., 2015; Singh *et al*., 2016).

Sequencing of 18S rRNA is widely used to differentiate the genotypes or subspecies of *Babesia* and the three distinct species of small babesia were identified based on the characterization of this gene (Kjemtrup and Conrad, 2000; Mandal *et al*., 2014). In addition, sub-species of *Babesia* are reported to contain dissimilarities in the Internal Transcriber 1 (ITS1) region as well (Baneth *et al*., 2004). Sequencing of the BgTRAP gene of *B. gibsoni* species from India and Bangladesh has shown that *B. gibsoni* species present in these two countries are genetically distinct from those present in East Asian countries (Terao *et al*., 2015; Singh *et al*., 2016). The small *Babesia* species affecting dogs are invariably called *B. gibsoni* and only two studies conducted recently have attempted to confirm the organism affecting dogs in Sri Lanka as *B. gibsoni* by molecular methods (Jayathilake *et al*., 2020; Ranatunga *et al*., 2020). In this study, we attempted to characterize *B. gibsoni* which infect dogs in Sri Lanka by analyzing partial sequences of the 18S rRNA, ITS1, and BgTRAP genes.

MATERIALS AND METHODS

Genomic DNA was extracted from blood samples of ten dogs who were confirmed to be infected with small *Babesia* species by blood smear examination. The dogs were from three different provinces in Sri Lanka namely Central (n = 6), Western (n = 2), and Sabaragamuwa (n = 2) and presented to Veterinary Teaching Hospital, Peradeniya with mild (n = 2), moderate (n = 4) or severe clinical signs (n = 4) of babesiosis in 2017. Three different genes namely 18S rRNA, ITS1, and BgTRAP were amplified using the primers, and the PCR conditions given in Table 1.

The amplicons were separated by electrophoresis on 2.0% agarose gel, stained with Diamond nucleotide dye (Promega, USA) and visualized under a gel imaging system (Geneflash, India).

The PCR products of all three genes (18S rRNA, ITS1 and BgTRAP) were sequenced using forward and or reverse primers at the sequencing facility of the Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya. The quality of the sequences was analyzed by visualizing the chromatogram on MEGAX. Sequences were BLAST

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searched at BLASTn at NCBI GenBank. Sequences were analyzed using MEGAX software. Reference sequences for each gene were downloaded from the NCBI database. Sequences were aligned by MUSCLE, best-fitted models were identified (Hasegawa–Kishono–Yano model with invariant sites -HKY+I for distance calculation) and Maximum likelihood trees with bootstrap values were generated by the MEGAX.

RESULTS

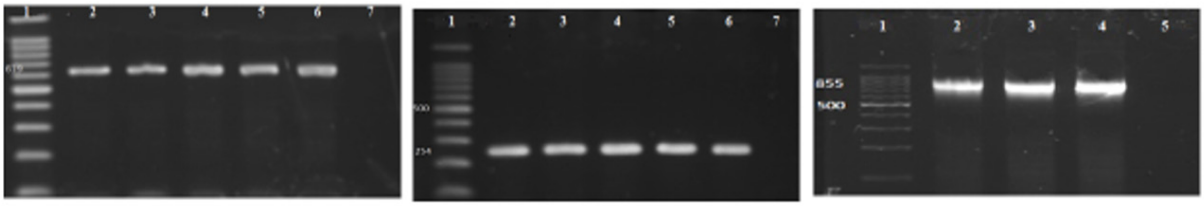
All three genes were successfully amplified and the PCR amplicons of 619 bp (18s rRNA), 254 bp (ITS1), and 855 bp (BgTRAP) were detected in gel electrophoresis (Figure 1). However, three PCR amplicons of BgTRAP contained non-specific amplification and hence were not sequenced.

Sequence similarity search showed that all 18S rRNA genes of the *B. gisoni* of the present study were highly similar to a *B. gibsoni* Wayanad isolate 8 (Accession MN134517) with 99.6% identity.

ITS1 genes of the *B. gibsoni* strains of this study were highly similar to a *B. gibsoni* strain from India (Accession KF815078) with 98.0% identity. Gene sequences of the ITS-1 region of *Babesia* species were aligned and the Maximum Likelihood tree was constructed using *Plasmodium falciparum* as an out-group. As shown in Figure 2, *B. gibsoni* isolated from Sri Lanka (GenBank accession: MW517166-MW517675) formed a clade with two Indian strains. However, the bootstrap value of the clade (49%) was not supportive to confirm any genetic difference between the two groups.

Table 1: Primer sequences and cyclic conditions used to amplify 18S rRNA, ITS1, and BgTRAP genes.

Gene	Primer Sequences and PCR conditions	Size	Reference
18srRNA	Ba103FCCAATCCTGACACAGGGAGGTAGTGAC Ba721RCCCCAGAACCCAAAGACTTTGATTTCTCTCAAG Reaction mixture (50 µl): 5 µl of PCR buffer (x 10), 2 µl of $MgCl_2$, 0.2 µl of *Taq* DNA polymerase (Promega, USA), 10 pmol of each primer and 0.2 mM concentration of each deoxynucleotide triphosphate, and 1 µl of template DNA. Cyclic conditions:Initial denaturation at 95 ºC for 5 min. followed by 30 cycles 94 ºC for 45 s, 65 ºC for 45 s and 72 ºC for 90 s with a final extension step of 10 min. at 72 ºC.	619 bp	Inokuma *et al*., 2004
ITS1	BgITS1F-ACATTGAAACTTGTCGAGCTGCG BgITS1R-AGATCCCGCACCCAGCCA Reaction mixture (50 µl): 5 µl PCR buffer (x 10), 1.5 µl of $MgCl_2$, 0.2 µl of *Taq* DNA polymerase, 10 pmol of each primer and 0.2 µl of dNTP, and 1 µl of template DNA. Cyclic conditions: Initial denaturation: 95 ºC for 3 min, followed by 34 cycles of 95 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 1 min, and a final extension step at 72 ºC for 15 min.	254 bp	Terao *et al*., 2015
BgTRAP	(F - 5 ' - A C C C A G C G A A T A C A A T G C A C C A C A) (5'-AGCCCTGCAAAACCGGCAAGTA TG-3') Reaction mixture (50 µl): 5 µl PCR buffer (x 10), 1.5 µl of $MgCl_2$, 0.2 µl of *Taq* DNA polymerase, 10 pmol of each primer and 0.2 µl of dNTP, and 1µl of template DNA Cyclic conditions: Initial denaturation. 94 ºC for 5 min., followed by 35 cycles of 94 ºC for 30 s. 60 ºC for 45 s, 72 ºC for 1 min. and final extension at 72 ºC for 7 min.	855 bp	Kjemtrup and Conrad, 2000



a: 18SrRNA gene b: ITS1 gene c: BgTRAP gene

Figure 1: (a) Agarose gel electrophores of PCR amplicons of 18S rRNA gene (b) ITS1 gene (c) BgTRAP gene of *B. gibsoni*. The first lane contains the 100 bp molecular weight marker and the last two lanes of each gel contain positive and negative controls.

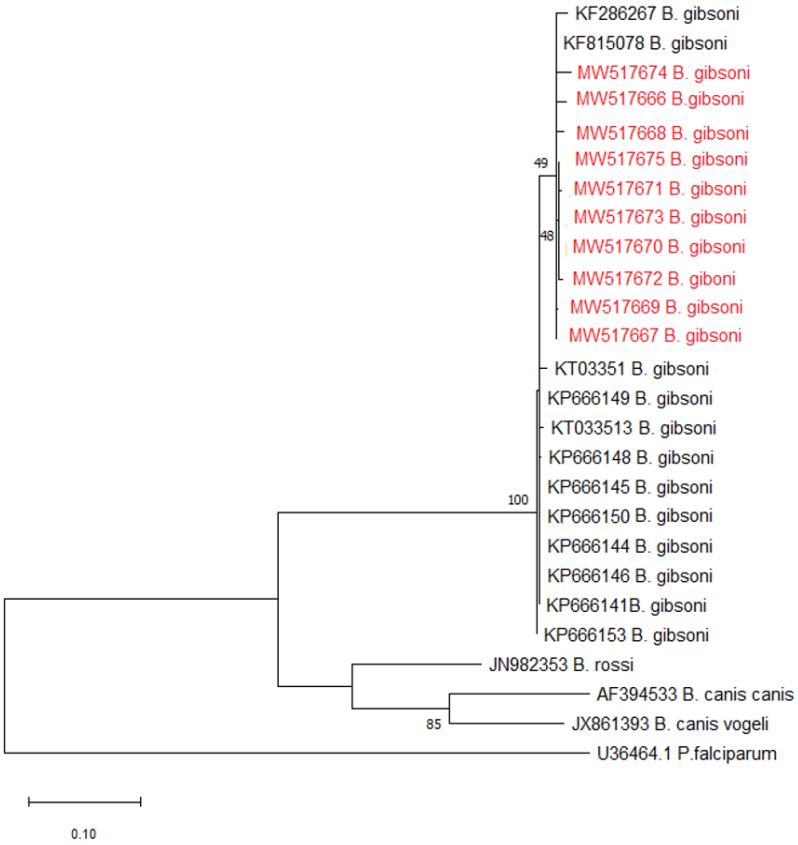


Figure 2: The Maximum Likelihood (ML) tree of ITS1 region of *Babesia gibsoni*. (*Babesia gibsoni* from Sri Lanka are in red).

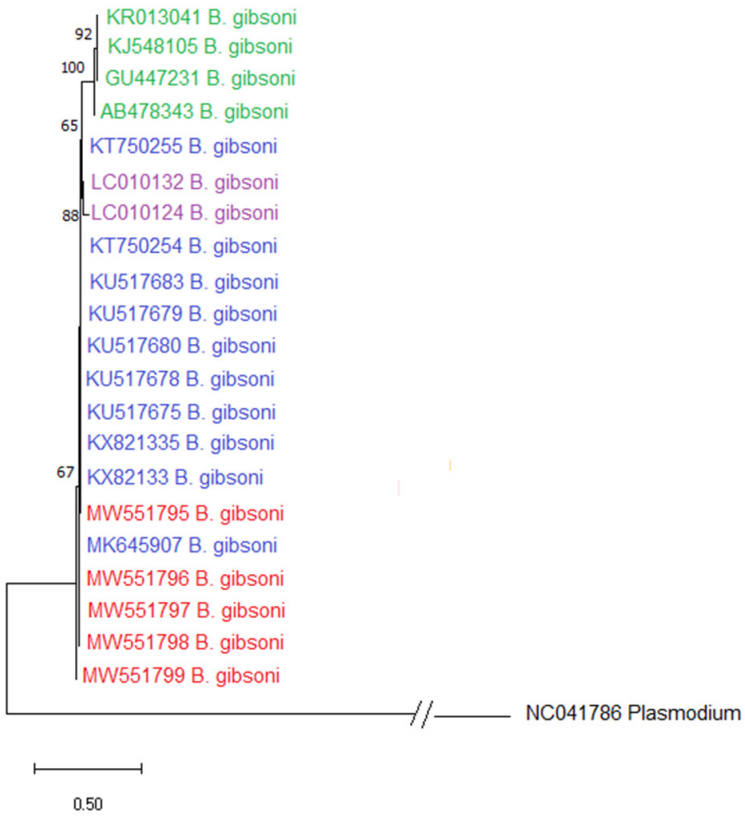


Figure 3: The Maximum Likelihood (ML) tree of the BgTRAP gene of *Babesia gibsoni.* (Green: East Asia, Blue: India, Purple: Bangladesh, Red: Sri Lanka).

The Maximum Likelihood tree based on the 855 bp region of the BgTRAP gene is presented in Figure 3**.** As shown in the figure, *B*. *gibsoni* strains from East Asia, including Taiwan Malaysia, Japan, and Korea formed a different clade with 100% bootstrap support. All *B*. *gibsoni* strains from India (KT, KU, and KX isolates), Bangladesh (LC010132 and LC010124), and Sri Lanka (GenBank accession: MW551795-MW551799) clustered on a separate clade. Further, the Sri Lankan strains clustered with a *B*. *gibsoni* strain from Trivandrum India.

DISCUSSION

The present study reports the genetic characterization of the 18S rRNA gene, ITS1 region, and BgTRAP gene of *B*. *gibsoni* from dogs in Sri Lanka. Molecular analysis of *B*. *gibsoni* strains present in several Asian countries including India, Bangladesh, Japan, Korea, Taiwan, and Malaysia has been reported previously (Inokuma *et al*., 2004; Miyama *et al.,* 2005; Terao *et al*., 2015; Singh *et al*., 2016). Homology of the sequences and phylogenetic clustering of the 18S rRNA and ITS1 sequences confirmed that the small *Babesia* species present in Sri Lanka is *B*. *gibsoni* Asian genotype-2. However, both 18S rRNA and ITS regions of *B*. *gibsoni* strains from different Asian countries did not show much genetic diversity and appeared to be highly conserved among small *Babesia* species. Bostrom *et al*. (2008) and Mandal *et al*. (2014) have made similar observations and our results re-confirmed their findings. Analysis of the 18S rRNA sequences was useful in differentiating *B*. *gibsoni* from *B*. *vulpes*, another small *Babesia* species affecting dogs. We only sequenced 619 bp fragment of the 18S rRNA gene and a previous study has shown that the sequencing of 1655 bp fragment of this gene was helpful in differentiating *B*. *gibsoni* from *B*. *conradae* (Mandal *et al*., 2014). Liu *et al*. (2016) have analyzed 54 sequences of ITS1 of *B*. *gibsoni* from different geographical regions in Japan. Authors have also reported that those isolates were found interspersed with isolates from Australia, India, Taiwan, and the USA.

The TRAP gene has been used to identify the genetic diversity of the *Babesia* and the regions between TSR and the CTD have shown a variation (Terao *et al*., 2015). The construction of a phylogenetic tree using the sequences of the BgTRAP gene revealed that the *B*. *gibsoni* species present in the Indian subcontinent is genetically different from the strains present in East Asian countries. Terao *et al*. (2015) analyzed the BgTRAP gene sequences of the *B*. *gibsoni* from Bangladesh and reported that the Bangladesh strains are genetically different from the strains present in East Asian countries. In our phylogenetic analysis also, the Bangladesh strains clustered separately with 88% bootstrap support but that cluster contained a *B*. *gibsoni* strain from India (KT750255). It is important to note that all five *B*. *gibsoni* isolates from Sri Lanka were on different genetic clades together with a strain from Trivandrum, India. The number of gene sequences of the BgTRAP gene available in the Genbank is still limited and these gene sequences representing different geographical regions will help to understand the true phylogenetic relationship of the organism in different regions of the world. It warrants further studies to understand whether these genetic differences have caused any difference in the antigenicity and the pathogenicity of the organism.

CONCLUSION

B. gibsoni present in Sri Lanka is genetically different from the species present in East Asian countries and closely related to strain present in South India.

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DECLARATION OF CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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