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


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 18S rRNA 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; Schnitter 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. (Quollo 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 °C 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’ TGGTTGATCCTGCAGTA 3’ and the reverse 5’ CTTCCTTTCTTTAAATGT 3’ (Jeffries 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’ TATGTTGCTGGTTGTCT 3’) and reverse (5’ AATGATCCTCTTCTGC 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)

**Table 1:** Comparison of PCRs targeting *BgSA1* with *Bg18SrRNA* based on McNemar’s Test

<table>
<thead>
<tr>
<th><em>BgSA1</em></th>
<th><em>Bg18SrRNA</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive (n=15)</strong></td>
<td><strong>Negative (n=35)</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>Count</strong></td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
</tr>
</tbody>
</table>

P-value for McNemar Test = 0.065**<sup>10</sup>**
Kappa value = 0.396**<sup>10</sup>** (<0.002)

Sensitivity = 40.0; Specificity = 94.29; Accuracy = 78.00
Positive Predictive Value = 75.0; Negative predictive value = 78.6

**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, 19]. 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, 19]. 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) [44]. 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) [13]. Targeting a

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<sup>10</sup> Significant at 0.01 level (P<0.01); ns non-significant (P>0.05)
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.

**Acknowledgements**

This work was supported financially by Kerala Veterinary and Animal Sciences, University RKVY-RAFTAAR 2019-20 Project (KE/RKVY-ANHB/2019/1422), Government of India and Kerala and State Plan project 2021-2022 (RSP/21-22/VI-7).

**References**