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DEVELOPMENT OF *Cytochrome b* BASED PCR AND EPIDEMIOLOGY OF *B. gibsoni* IN DOGS

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ABSTRACT

Canine babesiosis is emerging vector-borne disease of dogs and wild canids. The study aimed to develop PCR assay for detection of *Babesia gibsoni* and understanding epidemiology of disease in dogs. The cytochrome b gene base PCR assay was developed with analytical sensitivity of detection up to 10⁻² (0.5 ng/μl) of DNA. The specificity was confirmed by testing DNA from *B. vogeli*, *Ehrlichia canis*, *Hepatozoon canis*, and *Dirofilaria repens*. The thin blood smears examination revealed prevalence of 9.75% (32/328) for *B. gibsoni*. Whereas, with PCR the prevalence rate of 10.97% was recorded. There was significant association between vomition and dark yellow coloured urine with *B. gibsoni* infection. While age, breed, sex of the host and presence of ticks on animal body were not significantly associated with the occurrence of *B. gibsoni* infection. The sequence analysis showed 99.16-99.63% identity among themselves and 98.41 to 98.69% similarities with the published sequences of *B. gibsoni*. The phylogenetic analysis of Indian *B. gibsoni* isolates formed a single major group with other Asian countries indicating the monophyletic nature of *B. gibsoni* compared to other *Babesia* spp. Even though the vomition and yellow coloured urine combined with microscopic examination may help in diagnosis of *B. gibsoni* infection. Considering high analytical sensitivity and specificity of the *cytochrome b* based PCR, we recommend confirmatory diagnosis with PCR before undertaking the treatment in suspected cases of *B. gibsoni* infection.

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1 Introduction

Canine babesiosis is an emerging vector-borne haemoprotozoan disease caused by *Babesia gibsoni* and *B. vogeli*, in India. The disease is transmitted mainly by tick bite and rarely by dog bite, blood transfusion, transplacental route (Fukumoto et al., 2005). The acute form of the disease is typically associated with remittent fever, lethargy, haemolytic anemia, vomiting, thrombocytopenia, haemoglobinuria, marked splenomegaly and hepatomegaly (Conrad et al., 1991; Boozer & Macintire, 2003). In contrast, the chronic form may either lead carrier stage without clinical signs or recurrence of disease (Suh et al., 2017).

Routinely, the diagnosis of *B. gibsoni* infection is carried out by clinical signs combined with history and light-microscopic examination of blood smear (Solano-Gallego et al., 2016). But it's difficult to differentiate from other haemoprotozoan diseases with similar clinical picture (Suh et al., 2017). On the other hand, serological tests are used to detect circulating antibodies (Beugnet & Moreau, 2015). However, due to high cross-reactivity and inability to differentiate acute from chronic infections limited their diagnostic application (Solano-Gallego et al., 2016). The more sensitive and specific diagnostic assay such as polymerase chain reaction (PCR) is being used for detection of *B. gibsoni* infection by targeting 18S rRNA gene (Augustine et al., 2017).

In India, most of the *B. gibsoni* epidemiological studies were based on blood smear examination (Senthil Kumar et al., 2009; Wadhwa et al., 2011; Sahu et al., 2014) and very few references are available regarding the using of PCR assay targeting 18S rRNA gene (Abd Rani et al., 2011; Laha et al., 2014; Singh et al., 2014). The aim of the present study was to understand the risk factors and to evaluate the sensitivity and specificity of a *cytochrome b* gene based PCR assay to detection of *B. gibsoni* and to understand the genetic diversity of *B. gibsoni* in dogs.

2 Materials and methods

2.1 The study area

The samples were collected from urban areas of Bengaluru (12.97°N 77.56°E; area: 741km²; elevation: 900m) and Hassan districts (13.012°N 76.068°E; area: 26.52km²; elevation: 980m). They lie in the southeast of the South Indian state of Karnataka, India. Both districts have a tropical savanna climate (Koppen climate classification Aw) with distinct wet and dry seasons. Bengaluru climate presents an average low temperature of 15.1 °C (January) and with an average high temperature of 35 °C (April).

2.2 Samples

A total of 328 dogs of different breeds and age groups from Bengaluru urban (n=316) and Hassan districts (n=12) were

included in the present study. The blood samples were collected along with clinical history and risk factors. Two thin blood methanol fixed smears were prepared for each sample and stained with Giemsa for light microscopic screening of the *B. gibsoni* organisms (Bilwal et al., 2017).

2.3 Primer design

The primers were designed based on the *B. gibsoni* mitochondrial *cytochrome b* (cytb) gene sequences available in GenBank database (AB685190; AB215096; AB685187; DQ287953; AB499087; KP666169; AB685184; AB685183 and AB685182) using Vector NTI advance 11 software. The primers BgcytbF (ATG TTG TCC TAT TTG GTT CC) and BgcytbR (TAT AAA CGC ATT CTA GCG C) were then evaluated for similarities against the GenBank database by means of BLASTn algorithm.

2.4 Genomic DNA isolation

The genomic DNA was isolated from the 200 µl of anticoagulated blood of each dog using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) as per manufacturer's protocol. The purity of genomic DNA was checked with spectrophotometer (Nano drop 2000C, ThermoScientific, USA).

2.5 Standardisation of Bgcytb based PCR

Three blood samples which were confirmed positive for *B. gibsoni* by microscopic examination as well as by 18S rRNA based seminested PCR (Birkenheuer et al., 2003) were used as positive controls. The Gradient PCR for bgcytb gene was performed to optimize the annealing temperature by using known positive and negative DNA. An analytical sensitivity of the assay was determined based on the amplification of serially 10-fold diluted DNA (23.5ng/µl) of positive control showing parasitemia of 3.17%. In order to compare the sensitivity of bgcytb based PCR with 18S rRNA based PCR, the same serially diluted DNA (10⁻¹ to 10⁻⁶) were subjected for genus specific PCR targeting 18s rRNA (Birkenheuer et al., 2003). In order to check the analytical specificity, genomic DNA of *Ehrlichia canis*, *Hepatozoon canis*, *Dirofilaria repens* and *B. vogeli* were used as reference.

2.6 Protocol

PCR was performed in a final volume of 25 µl, containing 12.5 µl of PCR master mix (DreamTaq™ Green PCR Master Mix (2x), Thermo Fisher Scientific), 7.5 µl of nuclease free water (Thermo Fisher Scientific, Lithuania), 1 µl of each forward and reverse primers (10 pmol/µl) and 3µl of template DNA. A non-template and negative control were included in each run. The amplification was performed with the following thermal profile; initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (95°C for 1 min), annealing (57°C for 1 min), and

extension (72°C for 1 min), and a final extension at 72°C for 7 min (Mastercycler@nexus, Eppendorf, Germany). All PCR products obtained were confirmed for amplification by electrophoresis in 1% agarose gel containing 0.2µg of ethidium bromide/ml. The gels were visualized using gel documentation system (Syngene, UK).

2.7 Sequencing and phylogenetic analysis

After gel electrophoresis, the specific 1070bp size amplicons were excised and the PCR product was purified using gel extraction kit (Thermo Fisher Scientific, Lithuania) by following manufactures protocol. The final elution's were sequenced from both ends by sanger's dideoxy chain termination method using forward and reverse primers (Eurofins Pvt. Ltd., Bengaluru). The sequences were subjected for alignment and phylogenetic tree was constructed using MEGAX (Kumar et al., 2018).

2.8 Statistical analysis

Association between the prevalence of *B. gibsoni* by PCR with various risk factors such as, age, breed, gender, fur length, tick infestation and clinical signs like fever, vomition and dark yellow colour urine carried out by Pearson's Chi-square (χ^2) test. The data was analysed by using statistical software program (Graphpad Prism). Values of $p < 0.05$ were considered significant.

3 Results

3.1 Blood smear examination

The Giemsa-stained blood smear examination revealed 9.75 % prevalence rate (32/328) for the *B. gibsoni* organisms. During examination of blood smears, the different forms such as signet ring, rod and anaplasma like forms of *B. gibsoni* were observed. Interestingly, atypical forms of *B. gibsoni* such as large ovoid to

circular forms of *B. gibsoni* i.e., about half the diameter of the host cell were found and these were rare forms. Dividing stage and multiple organisms in one individual RBC has also been recorded.

3.2 Molecular diagnosis (PCR)

In analytical sensitivity, distinct amplicons were observed up to 10^{-2} (0.5ng/µl) dilution of template DNA (Figure 1). Further, in analytical specificity no amplification was observed with positive *B. vogeli*, *H. canis*, *E. canis* and *D. repens* DNA, which confirms the specificity of designed primers in the present study. After standardization, a total of 328 blood samples were screened by in-house developed PCR assay targeting the complete coding sequence of *B. gibsoni* cytochrome *b* gene. A total of 10.97% of dogs ($n=36/328$) were positive for *B. gibsoni* as revealed by the amplification of 1070bp size amplicon, which were later confirmed by sequencing.

3.3 Sequencing and phylogenetic analysis

Three complete coding sequences of the cytochrome *b* gene obtained were edited and submitted to GenBank (MK840539, MK840542 and MK840550). BLAST analysis of the sequences revealed 98.41 to 98.69% similar with the published sequences of *B. gibsoni* Asian genotype. The three sequences shared at 99.16 to 99.63% identities among themselves. The phylogenetic analysis showed all the three Indian isolates grouped with *B. gibsoni* isolates from Japan and China forming single group (Gr-I Babesia of dogs). Within the Gr-I, the Indian isolates formed separate subgroup (Figure 2). The other babesia species affecting dogs formed separate group (Gr-II babesia of dogs) compared to other animal babesia species (Gr-III babesia of other animals). The genus babesia formed separate clade compared to genus theileria of the order piroplasmida (Figure 2). The nucleotide variations

Figure 1 Analytical sensitivity of *B. gibsoni*-specific cytb (A) and 18S rRNA (B) PCR: Lane1: No template control, Lane2: DNA from uninfected dog, Lane3: DNA from infected dog, Lane 4-9: Serial 10- fold dilutions of positive DNA (10^{-1} to 10^{-6}) and Lane M: DNA ladder.

Figure 2 Maximum likelihood tree generated by complete *cytochrome b* gene sequences showing the genetic relationship of the *B. gibsoni* isolates from India with other countries. Isolates for which the *cytb* gene obtained in this study are indicated by a triangle (Red)

were observed at positions 69 (A-G), 96 (G-T), 360 (A-G), 363 (T-G), 430 (C-T), 504 (A-G), 615 (C-T), 658 (A-G), 726 (G-A), 810 (A-T), 907 (G-A) and 939 (A-G) when compared to standard (AB685190). Whereas, at amino acid level the variations were observed at 220 (I-V) and 303 (V-I) compared to standard (AB685190).

3.4 Risk factors analysis

The prevalence of *B. gibsoni* infection was not significantly associated with risk factors like age ($X^2=8.497$, $p=0.5804$), breed ($X^2=17.31$, $p<0.5018$), gender ($X^2=1.564$, $p<0.2111$), fur length ($X^2=0.040$, $p<0.8409$) and presence of tick ($X^2=2.919$, $p<0.0875$) in present study population. However, the prevalence of *B. gibsoni* was higher in >5-6 years (17.85%) age group dogs, followed by >1-2 years (16.66%) and lower in above 10 years

(2.5%) age group dogs (Table 1). The prevalence of *B. gibsoni* was highest in golden retriever (25.8%) breed of dogs and lowest in beagle (0.25%). The infection rate was higher in male (13.11%) compared to female (8.27%) dogs. The *B. gibsoni* infection significantly correlated with clinical signs of vomiting ($X^2=4.673$, $p=0.0306$) and yellow coloured urine ($X^2=3.937$, $p=0.0472$).

4 Discussion and Conclusions

Canine babesiosis is an emerging vector-borne haemoprotozoan disease of domestic and wild canids caused by *Babesia gibsoni* and *B. vogeli*. The disease is being reported from different states of India and is mainly based on clinical and microscopic examination. Even though few studies based on PCR have been carried out by targeting 18S rRNA gene. The disadvantage is due to its very low copies of 18S rRNA in the piroplasm genomes

Table 1 Descriptive and statistical analysis of various characteristics related to dogs infected with *B. gibsoni*, detected by PCR

Attributes		N	N+	N-	%	X ²	P
Sex	Male	183	24	159	13.11	1.564	0.2111
	Female	145	12	133	8.27		
Fur length	Small	197	21	176	10.65	0.0403	0.8409
	Medium	131	15	116	11.45		
Fever	Yes	167	24	143	14.37	3.228	0.0724
	No	161	12	149	7.45		
Vomition	Yes	130	21	109	16.15	4.673	0.0306
	No	198	15	183	7.57		
Yellow coloured urine	Yes	49	10	39	20.40	3.937	0.0472
	No	279	26	253	9.31		
Presence of ticks	Yes	125	19	106	15.2	2.919	0.0875
	No	203	17	186	8.37		

N: Number of dogs tested by PCR; X²: Chi-square test; P: P-value

(Criado et al., 2006) which makes sensitivity of the assay low. Therefore, the present study was carried out to develop PCR assay by targeting *cytochrome b* of *B. gibsoni* as a diagnostic target, which is present in more copies in an organism. The primers were designed based on available sequences and PCR was first standardized with microscopic positive *B. gibsoni* samples.

The sensitivity of the *cytb* gene based PCR to detect *B. gibsoni* was 10⁻² (0.5ng/μl) for blood (50μl) with a parasitaemia of 3.17%. Whereas, with 18S PCR assay the specific amplification was observed at lower template dilution of 10⁻¹ (2.5ng/μl), which confirming the greater sensitivity of the *cytb* based PCR method than 18S. Similar results have been reported for *B. bovis* and *B. bigemina* (Salem et al., 1999) and for *Theileria annulata* detected to the dilution representing a parasitaemia of 10⁻⁴ based on PCR of *cytb* gene (Bilgic et al., 2010).

The prevalence of *B. gibsoni* by microscopic examination was reported at 9.75%, which is more compared to earlier study in pet and stray dogs at the rate of 4.81% (Sahu et al., 2014). Whereas, few studies have reported higher prevalence rate of 31.37% (Mahalingaiah et al., 2017) and 35.13% (Laha et al., 2014). The varied prevalence rates might be attributed to vector and immune status of host and climatic conditions in different geographical regions (Mahalingaiah et al., 2017). In addition, the variation in sample size and type of case enrolled for study (Laha et al., 2014) might have contributed to higher prevalence rate compared to present study.

The PCR based prevalence (10.97%) was higher compared to microscopic examination based prevalence of *B. gibsoni* in dogs. Similar finding have reported in the past (Inokuma et al., 2004;

Cardoso et al., 2016) PCR targeting 18S rRNA gene in comparison to microscopic examination. In contrast, studies by Jain et al. (2017) and Mahalingaiah et al. (2017) have reported 47.33% and 49.09% prevalence by PCR in India. Along with vector, environmental conditions, case definition and sample size, the lower prevalence rate in the study might be attributed to good immune status of the host, body condition and other management practices like hygienic rearing of pets, deworming, regular use of ectoparasiticides etc., as observed during clinical examination. The higher prevalence has been reported in young than old age dogs (Sahu et al., 2014; Mahalingaiah et al., 2017), similarly due to more number of old age dogs being enrolled during the study period might have also contributed to overall lower prevalence rate. However, there was no significant correlation between age and *B. gibsoni* infection. Similar to present finding, no association was established between presence of infection with breed and sex in many other studies (Senthil Kumar et al., 2009; Singh et al., 2014; Roopesh, 2017). The higher prevalence in male dogs is reported (Sahu et al., 2014) and authors attributed to hormonal changes and behaviour changes like roaming in search of mates, food and territory. But in this study even though there were no stray dog cases even then there was higher prevalence in males (13.11%) which needs further investigations.

During the study period the overall prevalence of tick presence was recorded at 38.1%. Even though there was no significant correlation between presence of ticks and disease, it is observed that the high prevalence in proportion to presence of tick (Abd Rani et al., 2011; Mahalingaiah et al., 2017; Roopesh, 2017). The clinical signs of vomition and yellow coloured urine correlated with *B. gibsoni*

infection, whereas no association was found with fever as reported earlier (Miyama et al., 2005; Varshney et al., 2008).

To understand the molecular diversity of *B. gibsoni* in the region, three representative isolates were subjected for sequencing and phylogenetic analysis, which confirm all the three isolates as *B. gibsoni* (MK840539, MK840542 and MK840550). The *B. gibsoni* isolates from Indian origin grouped with isolates from Japan and China forming single group (Gr-I *Babesia* of dogs) indicating circulation of same genotype in Asian region. The similar grouping of all *B. gibsoni* isolates irrespective of country of origin and morphology and absence of differential biologic and immunologic features were observed in other studies targeting 18S rRNA and ITS1 (Criado-Fornelio et al., 2003; Bostrom et al., 2008; Mandal et al., 2014). The Indian isolates formed separate subgroup within the *B. gibsoni* group of Asian genotype indicating circulation same clonally expanded single strain as was observed with 18S rRNA and ITS1 gene based phylogenetic analysis (Mandal et al., 2014).

In conclusion, *B. gibsoni* infection is highly prevalent in this region of country. The cytochrome b gene based PCR found to be potential confirmatory diagnostic assay compared to conventional microscopy. None of clinical signs and host factors studied significantly correlated with presence of infection except vomiting and yellow coloured urine, which make differential diagnosis difficult for clinician with other disease conditions. The *cytb* gene sequencing and phylogenetic revealed genetically similar *B. gibsoni* isolates are circulating in the region. The *cytb* gene can be useful molecular diagnostic and genetic marker, which needs further studies with more number of isolates from different regions of India.

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Conflict of interest

All Authors declare that there is no conflict of interests that could possibly arise.

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