Original Research

Molecular Diagnosis of *Babesia gibsoni* Infection

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Abstract

In the present investigation, sixty-eight blood samples collected from the dogs suspected for Babesia gibsoni infection were subjected for examination of sensitivity of PCR in diagnosis of the disease. The present study was carried out at Rajiv Gandhi Institute of Veterinary Education and Research, Puducherry. Dogs presented to the Small Animal Unit, Teaching Veterinary Clinical Campus were taken for the study. Dogs suspected for Babesia gibsoni infection were subjected for diagnosis using Giemsa stained blood smear examination and Polymerase Chain Reaction. Samples turned out positive were custom sequenced and analyzed. More number of samples were found to be positive for babesiosis by Polymerase chain reaction. Isolate identified was found to be Babesia gibsoni (Asian Genotype). Polymerase chain reaction was superior to blood smear examination in diagnosing babesiosis and the incidence of disease was significantly higher in young age group.

Key words: Asian Genotype, *Babesia gibsoni*, BLAST, Dogs, Diagnosis, PCR

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Introduction

Canine babesiosis was considered as a very common and clinically significant disease among various vector borne diseases in dogs. *B. gibsoni* was found to be more prevalent when compared to *B. canis*, in Asiatic countries with size of 1.0–2.5 µm (Solano-Gallego and Baneth, 2011). The organism appears as oval or signet ring form inside the erythrocyte (Beugnet and Moreau, 2015). Young dogs of age less than 3 months were reported to be more frequently affected by babesiosis (Selvaraj *et al.*, 2010; Selvi *et al.*, 2011) and also most of the haemoprotozoal infections (75%) were reported to be occurred below the age of one year (Samradhni *et al.*, 2005). Birkenheuer *et al.* (2005) reported that *B. gibsoni* has been identified to occur in various breeds of dogs, whereas Ayoob *et al* (2010) opined that *B. gibsoni* is commonly found in American Staffordshire and American Pit Bull Terrier (APBT) breeds. Diagnosis of the disease includes identification of the parasites in stained blood smears or infected tissue like lymph nodes or spleen where paired or unpaired piroplasms could be existing. Even though direct microscopic examination of a stained blood smear was the conclusive, feasible, common and cost-effective method to diagnose *Babesia* species (Amritpal *et al.*, 2014) molecular diagnosis was considered to have higher sensitivity to detect *Babesia* protozoa from peripheral blood (Bashiruddin *et al.*, 1999).

Materials and Methods

Total 68 dogs were selected for the study based on clinical signs suggestive of *B. gibsoni* infection and were subjected to blood smear examination by Giemsa staining technique and PCR. Details like breed, age and sex were also taken for all dogs. Thin blood smears were made from peripheral blood collected aseptically from ear tips which were later stained with Giemsa staining solution as described by Fukumoto *et al.*, 2001. Parasitemia was determined, by observing blood smears under light microscopic examination with 100x magnification. Whole blood from saphenous / cephalic vein was collected into EDTA coated vials and were stored at – 20ºC for DNA isolation. Two milliliters for blood was used for DNA extraction by HiPurA™ SPP Blood DNA Isolation Kit (Himedia®). The extracted DNA (50 µl) was stored at – 20ºC for PCR amplification. PCR was set up in 25 µl reaction mixture which consisted 12.5 µl of PCR Master Mix, 1.0 µl of forward and reverse primer each, 5 µl of template DNA, 2.0 µl of Dimethyl sulfoxide (Genie®) and 5.5 µl of autoclaved triple-distilled water. PCR amplification was done as per Inokuma *et al.* (2004) with initial denaturation of 95 ºC for 5 min, 35 cycles of denaturation (30 sec), annealing (30 sec), extension (90 sec) at 95 ºC, 58 ºC and 72 ºC respectively. Final extension was done for 5 min at 72 ºC. For thermocycling, Eppendorf® Master Cycler Gradient was used.

Species specific primers specific for 18S rRNA gene Gib599 forward and Gib1270 reverse as described by Inokuma *et al.* (2004) were used for PCR amplification with expected product size of 672 bp (Table 1).

Table 1: Showing forward and reverse primers used for the molecular diagnosis of *Babesia gibsoni* infection in dogs

Gene	Primer	Primer Sequence 5' → 3'	Expected Product Size
18S rRNA	Gib599 Forward	TCCGTTCCCACAACACCAGC	672 bp
	Gib1270 Reverse	TCCTCCTCATCATCCTCATTCG	

The amplified products were checked on 2% agarose gel electrophoresis. The amplified product was purified and subjected for sequencing at Eurofins Genomics India Private Limited, Bengaluru. The specificity of the sequence was determined by subjecting sequence to Basic Local Alignment Search Tool (BLAST). Neighbor Joining method (MOLE BLAST) was used to create phylogenetic tree for and analyze

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the similarity between isolates found in the present study and isolates reported earlier. The collected data was statistically analyzed and interpreted as described by Snedecor and Cochran (1994) and discussed.

Results and Discussion

In the present study, 68 dogs were suspected for *B. gibsoni* infection, in which seven were positive for *Babesia gibsoni* infection by blood smear examination (Fig. 1) whereas, 9 dogs were found to be positive by PCR. Agarose gel electrophoresis of PCR products revealed the amplification at 672 bp (Fig. 2).

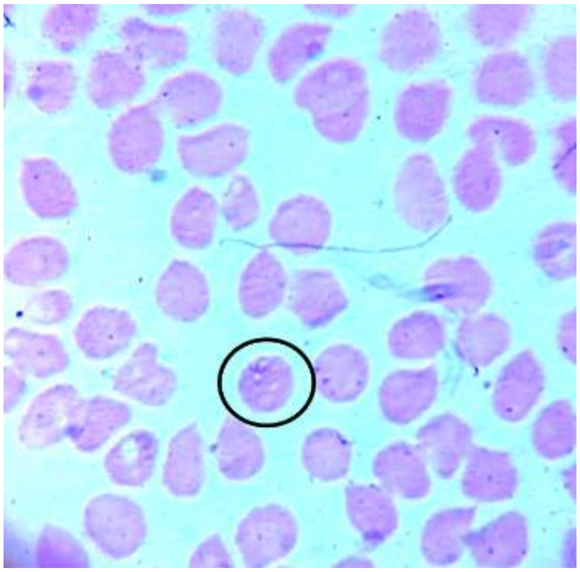

Fig. 1: Infected RBC with paired Babesia gibsoni piroplasms

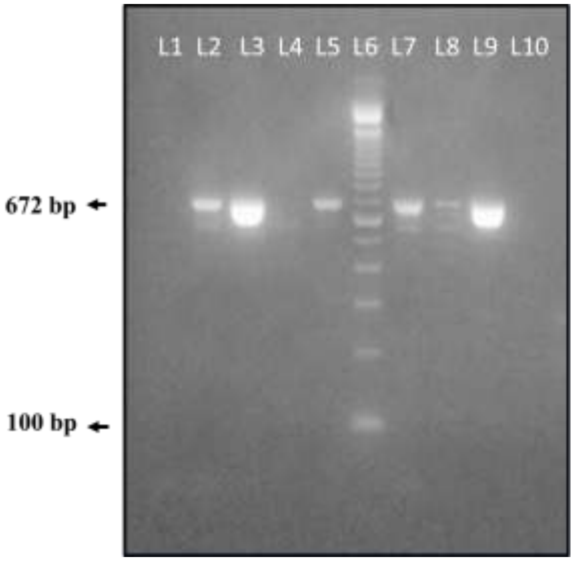

Fig. 2: Agarose gel electrophoresis of test samples showing positive results at L3, L5, L7, L8 AND L9; L1 and L2 being negative and positive controls respectively.

All the seven dog which were positive by blood smear examination were also shown positive by PCR. The reason behind false negativity of two samples with blood smear examination was assumed to be low parasitemia. The present study shows that PCR analysis was superior to the blood smear examination in diagnosis of the infection confirming the reports of Birkenheuer *et al.*, 2003; Garcia de Sa *et al.*, 2006 and Laha *et al.*, 2013, who found out that molecular analysis by PCR was superior to the routine blood smear examination in diagnosing *B. gibsoni* infection. Blood smear examination was found to be feasible and quick but the reliability was comparatively lower than that of PCR.

The amplified product was sequenced (Genbank accession number: MG708223) and was found 100% similar with the sequence of *B. gibsoni*, Asian strain (Fig. 3) available in the Genbank using BLAST (www.ncbi.nlm.nih.gov). Phylogenetic tree (Neighbor Joining method; MOLE BLAST) had shown phylogenetic relationship of the isolate with the other Asian isolates reported earlier. Similar results on *B. gibsoni*, Asiatic strain were obtained by several authors and the most similarity was found to be reported in

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north eastern region of India (KF606874.1 and KF606873.1) and Malaysia (KU500918.1 and KU500919.1).

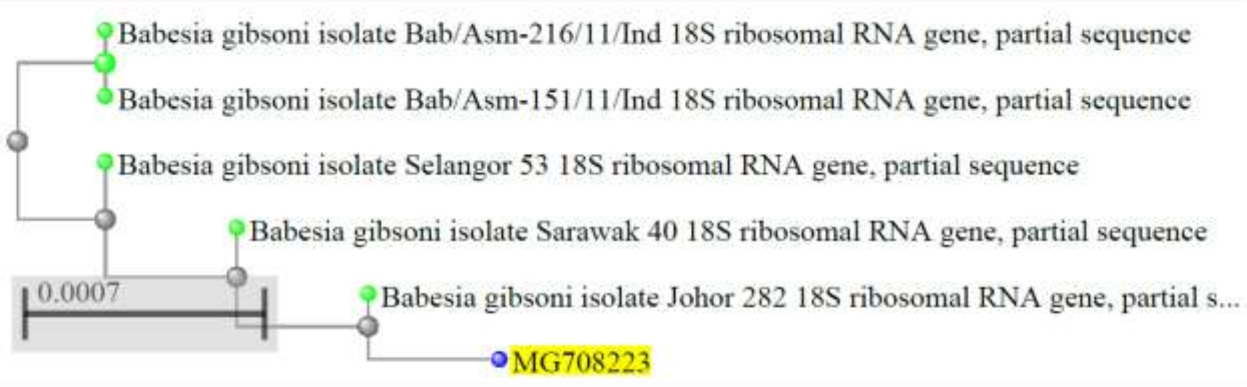

Fig. 3: Phylogenetic relationship of the isolate MG708223 with other asian isolates reported earlier

Incidence of babesiosis caused by *B. gibsoni* was found to be high in puppyhood stage. Present study revealed positivity in nine dogs were all of them were under three years of age. Out of nine, six dogs were under 1-year age group and three were in 1 – 3 years age group. In the present study Fisher's exact test revealed relative risk of 3.4 in less than one-year age group, which indicates dogs in the age group of dogs less than one year were 3.4 times being infected by *B. gibsoni* when compared to 1 – 3 years old age group (P value - 0.04) (Table 2).

Table 2: Age wise incidence of *Babesia gibsoni* infection in dogs

S. No.	Age Group	No. of Animal's Positive	No. of Animal's Negative	Total	P value	Relative Risk
1	0 - 1 year	6	8	14	0.043*	3.4
2	1 – 3 years	3	21	24		
3	< 3 years	0	30	30		
4	Total	9	59	68		

P value ≤ 0.05 was found to be significant. Incidence of the disease was found to be high in young age group more than the adult dogs and the results were in concordance with the results of Selvaraj *et al.* (2010). The present study shows similar reports with high incidence of disease in young animals. Tizard (2004) opined that, underdeveloped immunity in the young dogs when compared to elder dogs was a possible factor for occurrence of babesiosis in young age group. Various breeds of dogs like Great Dane, Doberman, Spitz, Terrier, Irish setter, Pug and Non-descript dogs were found to be positive in the present study. Out of 68 dogs suspected for babesiosis, 47 were found to be male dogs and 21 were female dogs, in which seven male and two female dogs got infected by *B. gibsoni* where no statistical difference was present by Fisher's exact test. The observation correlates with Hornok *et al.* (2006) and Amritpal *et al.* (2014), who

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reported that, statistically nonsignificant difference was noticed in the prevalence of *B. gibsoni* among various breeds and nondescript dogs.

In the present study conducted on 68 dogs, nine were found to be positive for *Babesia gibsoni* infection by blood smear examination and / or by PCR. The *Babesia* species identified in the present study was found to be of Asiatic strain by sequencing and BLAST analysis.

Conclusion

It can be concluded that PCR technique was found to be sensitive in diagnosing *B. gibsoni* infection in canines when compared to the conventional methods. Incidence of *Babesia gibsoni* infection was found to be affecting significantly in young age group. No significant difference was present in incidence of the disease sex-wise or among various breeds of the dogs.

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