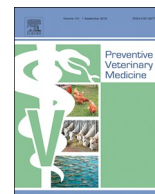




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Canine babesiosis among working dogs of organised kennels in India: A comprehensive haematological, biochemical, clinicopathological and molecular epidemiological multiregional study

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ABSTRACT

Canine babesiosis is a serious disease among tick-borne haemoprotozoan diseases, globally. The present study was envisaged for carrying out thorough investigation of the disease among working dogs of organised kennels situated in different agro-climatic zones of India as comprehensive understanding of the disease from this country was pertinently lacking. During the study period of three years (2012–2014), 330 dogs suspected for babesiosis were examined for clinicopathology by their physical examination, haematological and biochemical parameters estimation, while the detection of apicomplexan parasites was confirmed by using various diagnostic techniques i.e. by conventional microscopy, by two different *Babesia* specific 18S rRNA based PCR protocols (conventional/simple PCR and nested PCR assays) followed by sequencing of obtained PCR amplicons for *Babesia* spp. identification. Out of 330 clinical cases screened 5.15% (17/330), 9.09% (30/330) and 15.45% (51/330) were found to be positive in microscopic examination, simple- and nested- PCR assay, respectively. Comparative statistical analyses of these diagnostic assay results revealed that significant difference exists among the three diagnostic methodologies and thus it is recommended that the nested PCR technique be relied upon as a screening molecular assay and also for epidemiological studies of the disease in this country. Phylogenetic analysis based on 18S rRNA depicted the monophyletic nature and clonal expansion among all the *B. gibsoni*, under study. Sequencing results of PCR amplicons revealed that *B. gibsoni* has predominantly established itself over *B. vogeli* as former was incriminated in 47 cases while latter was confirmed in only four animals. Based on the clinical severity, these 51 affected animals were classified into three main groups¹ of 17 animals each viz., apparently healthy-, simple or uncomplicated babesiosis- and atypical or complicated babesiosis-group. Haematological and biochemical profiling of these dogs confirmed the characteristics findings of infection by both the *Babesia* spp. It was observed that the infection by small form of *Babesia* (*B. gibsoni*) is posing a significant therapeutic challenge and chemosterilization by commonly prescribed anti-protozoal drugs was not achieved as clinical relapses were often observed. The clinical signs, sequence based confirmation and severity of the infection suggested that there is a positive selection of *B. gibsoni* (smaller form) over *B. vogeli* (larger form) in this country and raises serious concerns as prognosis in former is considered to be poor compared to latter. Thus, these findings have opened new paradigms for planning of pragmatic control strategies against this emerging canine health problem.

1. Introduction

Canine babesiosis (or piroplasmosis) is a significant and potentially life threatening tick-borne protozoan disease of dog populations,

worldwide. The disease is caused by intraerythrocytic parasites of the genus *Babesia*, order *Piroplasmida*, phylum *Apicomplexa* (Irwin, 2009). Two distinct forms of *Babesia* are recognized by morphometric evaluation during microscopic examinations of blood smears:- large forms

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(2.5–5.0 μm) which consist of three species viz., *B. canis*, *B. rossi* and *B. vogeli* and the small forms (1.0–2.5 μm) comprising of the *B. gibsoni*, *B. conradae* and *B. microti* like piroplasmids. Among all the species of *Babesia* infecting dogs, *B. rossi* and *B. gibsoni* are considered to be highly pathogenic species with poor prognosis (Irwin, 2009). To date, canine babesiosis encompasses two main species in this country viz., *B. vogeli* and *B. gibsoni* (Kundu et al., 2012; Mandal et al., 2014). Both of these pathogens are well known *Babesia* species among domestic canine population and globally distributed across the continents, making them good example of ‘parasite globalization’. Increase in international travels with pet dogs as companion animals or for commercial trade and climatic/ecosystem changes favouring expansion of vector tick habitats have also immensely contributed to their ubiquitous distribution and rapid spread across the continents (Solano-Gallego and Baneth, 2011; Schnittger et al., 2012). Varying range of temperature and humidity conditions favouring vector tick survival and existence of stray canine population in large numbers supports transmission of *Babesia* spp. among canines in India (Abd Rani et al., 2011).

These apicomplexan parasites are solely restricted to erythrocytes but they cause a myriad of clinical conditions in affected dogs varying from transient anaemia to a complex syndrome involving multiple organ failure. Thus, depending on the clinical severity, canine babesiosis can be classified as uncomplicated- or complicated- form or can also be classified as peracute-, acute- and subclinical or chronic- form of the disease. In uncomplicated babesiosis, clinical signs are results of haemolytic anaemia. These include pyrexia, anorexia, pale mucous membrane, anaemia, tachypnoea, tachycardia, splenomegaly, icterus, malaise and apathy (Taboada and Merchant, 1991). Complicated form of canine babesiosis is characterized by additional organ involvement viz., acute renal failure (ARF), hepatopathy, cerebral babesiosis, coagulopathy, immune-mediated haemolytic anaemia (IMHA), acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), haemoconcentration and shock (Lobetti, 1998). Hitherto, such diverse clinical manifestations have not been studied from India. Hence, the present study has been attempted to understand varying manifestation of the disease and its outcome.

Conventionally, the diagnosis of canine babesiosis has relied mainly upon stained microscopic blood smear examination in most of the resource constrained settings in the developing countries (Abd Rani et al., 2011). A fastidious microscopic study may even lead to errors due to low sensitivity and specificity inherent to the technique. Reports of studies comparing different diagnostic techniques are limited since ‘gold standard’ assay for diagnosis of these apicomplexan parasites are not clearly established (Karagenc et al., 2006). Although, with the advancement of the nucleic acid amplification techniques like PCR, loop mediated isothermal amplification (LAMP) assay and quantitative PCR (Ikadai et al., 2004; Mandal et al., 2015; Quorollo et al., 2017) diagnostic sensitivity has increased manifold in recent years. Most of the simple/single-step PCR assays for detection of canine *Babesia* spp. have targeted 18S ribosomal RNA gene. Moreover, sequencing of 18S rRNA and internal transcribed spacer-1 (ITS-1) have also been used for molecular phylogeny studies of this parasite (Mandal et al., 2014). Serological tests including indirect fluorescent antibody test (IFAT) for diagnosis of babesiosis are less popular due to cross reactivity with other closely related piroplasmids (Irwin, 2009). Canine babesiosis still presents a significant diagnostic challenge and this is especially true for asymptomatic carriers or chronic reservoir dogs where in false negative PCR results may be observed.

The epidemiology or prevalence of canine babesiosis in this country have been studied sporadically in various isolated pockets or cities or towns by different researchers basically on stray/feral/roaming dog populations and on the family pet dogs brought to the veterinary dispensaries (Kumar et al., 2009; Abd Rani et al., 2011; Singh et al., 2014; Singla et al., 2016; Jain et al., 2017; Mittal et al., 2017). Working dog

population in this country, which are used under varied geographical terrains, climatic regions and in urban/suburb/rural habitats, runs the risks for exposure to ticks and tick borne diseases. This may be due to their exposure to feral canine and stray dog populations during their deployment. There has been no detailed study of canine babesiosis among working dogs of organised kennels from different agro-climatic zones of this country. This study is an attempt for correlating the disease with the clinical signs, anamnesis, prognosis, haematological and biochemical parameters of positive cases. Diagnosis in these cases was achieved using microscopy of stained thin smears, simple PCR-, nested PCR- assay and post sequencing phylogeny. Histopathological studies were also conducted in fatal cases encountered during the study period. Present work was thus undertaken in an extensive manner to bridge the existing knowledge gap and to provide comprehensive understanding of canine babesiosis among working dogs in organised canine establishments of this country.

2. Materials and methods

2.1. Sampling design and methodology

Special emphasis was given to include organised kennels located in different states of the country having different climatic conditions such as tropical humid, tropical dry, tropical semiarid and temperate zones. Samples from tropical humid conditions in northeast part of India included states of West Bengal, Assam, Meghalaya and Nagaland and samples from temperate Northern India conditions were collected from the state of Jammu and Kashmir. Central Indian state, Uttar Pradesh was chosen for sampling from sub tropical humid conditions while samples from tropical semiarid regions were collected from western state of Maharashtra. Three hundred and thirty working dogs suspected for canine babesiosis along with in-contact and apparently healthy dogs from these organised kennels at various locations as mentioned were included in the present study. These organised kennels have been meticulously following recommended vaccination, acaricide and deworming schedules in their dogs. Moreover, these organized kennels especially maintained Labrador Retriever and German Shepherd breeds of working dogs as these two breeds of the dogs form the major corpus of working dogs/security dogs not only in this country but also globally. The clinical samples were collected over a period of three years from Jan, 2012 to Dec, 2014. Details of the age, breed, sex and clinical manifestations of disease among 51 nested PCR positive cases have been recorded and summarized in Table 1. Collected blood or serum samples and necropsy tissue samples were transported under cold chain to Central Military Veterinary Laboratory (CMVL), Meerut, India for further processing.

Differential diagnosis of *Babesia* spp. from various viral, bacterial, rickettsial and parasitic infectious agents such as, canine parvo virus (CPV), canine adeno virus-1 (CAV-1), canine adeno virus-2 (CAV-2), canine corona virus (CCV), canine distemper virus (CDV), rabies, *Leptospira* species, *Mycoplasma*, *Streptococcus canis*, *Trypanosoma evansi*, canine heart worm (*Dirofilaria immitis*), canine *Anaplasma*, canine *Ehrlichia* and *Borrelia burgdorferi* were also carried out with suitable panel of serology and PCR based assays. These tests were carried out to ensure that canine samples being included in the present study are solely infected with *Babesia* spp. and are free of other concomitant viral, bacterial or parasitic infections/infestations.

Fresh blood drops from ear tip puncture were drawn into thin smears, air dried and methanol fixed for microscopic examination and estimation of differential leukocyte count (DLC). Whole bloods collected in BD® Vacutainer (containing EDTA as anticoagulant) from cephalic vein were used for haematological analysis and for DNA extraction for PCR assays. Blood for biochemical studies were collected in BD® Vacutainer (containing clot activator) and sera was subsequently harvested. Necropsy samples from spleen, lung, liver, heart and kidney

Table 1

Details of the dogs to be positive for canine babesiosis by different diagnostic assays along with their signalment and anamnesis (Conventional/simple PCR refer to 18S rRNA based PCR as per (Oyamada et al., 2005); Nested PCR protocol as per (Hitoshi et al., 2001); yr- Years; mo- months; F-Female dog; F*-Neutered female dog; M- Male dog; Lab-Labrador Retriever; GSD-German Shepherd; CS-Cocker Spaniel; MODS-Multiple Organ Dysfunction Syndrome; DIC-Disseminated Intravascular Coagulopathy; P-Positive; N-Negative).

| S. No | Breed | Age (in yr/mo) | Sex | Anamnesis | Microscopic Examination | Simple PCR | Nested PCR | Sequence Analysis | Accession Numbers |
|--------------------------------------------------------------------|-------|-------------------|-----|------------------------------|----------------------------|------------|------------|-------------------|-------------------|
| Group-1: Apparently healthy group (subclinical babesiosis) | | | | | | | | | |
| 1 | Lab | 9 yr | M | Apparently healthy | N | N | P | <i>B. gibsoni</i> | |
| 2 | GSD | 3 yr | F* | Apparently healthy | N | N | P | <i>B. gibsoni</i> | |
| 3 | Lab | 4 yr | F* | Apparently healthy | P | P | P | <i>B. gibsoni</i> | |
| 4 | GSD | 4 mo | M | Apparently healthy | N | N | P | <i>B. gibsoni</i> | |
| 5 | Lab | 4 mo | F* | Apparently healthy | P | P | P | <i>B. gibsoni</i> | |
| 6 | GSD | 4 mo | F* | Apparently healthy | N | P | P | <i>B. gibsoni</i> | KY563117 |
| 7 | Lab | 10 mo | F* | Apparently healthy | N | N | P | <i>B. gibsoni</i> | |
| 8 | Lab | 9 yr | M | Apparently healthy | N | P | P | <i>B. gibsoni</i> | |
| 9 | Lab | 5 yr | M | Apparently healthy | P | P | P | <i>B. gibsoni</i> | KY524482 |
| 10 | Lab | 4 yr | F* | Apparently healthy | N | P | P | <i>B. gibsoni</i> | KY524483 |
| 11 | Lab | 1yr 9 mo | F* | Apparently healthy | N | N | P | <i>B. gibsoni</i> | |
| 12 | GSD | 2 yr | F* | Apparently healthy | N | N | P | <i>B. gibsoni</i> | |
| 13 | GSD | 3 yr | F* | Apparently healthy | N | N | P | <i>B. gibsoni</i> | |
| 14 | GSD | 8 mo | M | Apparently healthy | N | P | P | <i>B. gibsoni</i> | KY563116 |
| 15 | GSD | 1 yr | F | Apparently healthy | P | P | P | <i>B. gibsoni</i> | |
| 16 | Lab | 4 mo | M | Apparently healthy | N | N | P | <i>B. vogeli</i> | KY513707 |
| 17 | Lab | 5 mo | F | Apparently healthy | N | N | P | <i>B. vogeli</i> | KY513708 |
| Total | | | | | 04 | 08 | 17 | | |
| Group-2: Simple babesiosis group (uncomplicated babesiosis) | | | | | | | | | |
| 1 | GSD | 2 yr | F* | Anaemia | N | N | P | <i>B. gibsoni</i> | |
| 2 | Lab | 11 yr | F* | Anaemia | P | P | P | <i>B. gibsoni</i> | |
| 3 | GSD | 4 yr | F* | Anaemia | N | P | P | <i>B. gibsoni</i> | |
| 4 | Lab | 5 yr | F* | Anaemia | P | P | P | <i>B. gibsoni</i> | |
| 5 | Lab | 3 yr | F* | Anaemia | N | P | P | <i>B. gibsoni</i> | |
| 6 | Lab | 7 yr 10 mo | F* | Anaemia | N | N | P | <i>B. gibsoni</i> | |
| 7 | Lab | 3 yr | M | Pyrexia | P | P | P | <i>B. gibsoni</i> | KF878946 |
| 8 | Lab | 6 yr | M | Pyrexia | N | P | P | <i>B. gibsoni</i> | KF878943 |
| 9 | Lab | 3 yr | F* | Pyrexia | N | N | P | <i>B. gibsoni</i> | |
| 10 | GSD | 2 yr 5 mo | F* | Pyrexia | P | P | P | <i>B. gibsoni</i> | KY563118 |
| 11 | GSD | 2 yr | M | Pyrexia | N | N | P | <i>B. gibsoni</i> | |
| 12 | GSD | 1yr 8 mo | F* | Pyrexia | N | N | P | <i>B. gibsoni</i> | |
| 13 | GSD | 2 yr | F* | Pyrexia, Anorexia | P | P | P | <i>B. gibsoni</i> | KP901261 |
| 14 | CS | 5 yr | M | Pyrexia, Anorexia, Vomition | P | P | P | <i>B. gibsoni</i> | KP901263 |
| 15 | Lab | 4 yr 3 mo | M | Pyrexia, Anorexia | N | P | P | <i>B. gibsoni</i> | KP901253 |
| 16 | GSD | 6 mo | M | Pyrexia, Anorexia | N | N | P | <i>B. vogeli</i> | KJ439648 |
| 17 | GSD | 6 mo | M | Pyrexia, Anorexia | N | N | P | <i>B. vogeli</i> | KJ439649 |
| Total | | | | | 06 | 10 | 17 | | |
| Group-3: Atypical babesiosis group (complicated babesiosis) | | | | | | | | | |
| 1 | Lab | 4 yr | M | Epilepsy | P | P | P | <i>B. gibsoni</i> | KP901254 |
| 2 | Lab | 5 yr | M | Epilepsy | N | P | P | <i>B. gibsoni</i> | KP901255 |
| 3 | Lab | 4 yr 7 mo | F* | Epilepsy | N | N | P | <i>B. gibsoni</i> | |
| 4 | Lab | 4 yr | F* | Epilepsy | P | P | P | <i>B. gibsoni</i> | KF878945 |
| 5 | Lab | 3 yr 10 mo | F* | Jaundice | N | N | P | <i>B. gibsoni</i> | |
| 6 | Lab | 6 yr 3 mo | F* | Jaundice | P | P | P | <i>B. gibsoni</i> | |
| 7 | Lab | 5 yr 6 mo | M | Jaundice | N | N | P | <i>B. gibsoni</i> | |
| 8 | GSD | 2 yr 8 mo | M | Renal Failure | N | P | P | <i>B. gibsoni</i> | |
| 9 | GSD | 6 yr 10 mo | M | Renal Failure | P | P | P | <i>B. gibsoni</i> | KP901262 |
| 10 | GSD | 6 yr 8 mo | F* | Renal Failure | N | N | P | <i>B. gibsoni</i> | |
| 11 | Lab | 9 yr | M | Jaundice & Renal Failure | N | N | P | <i>B. gibsoni</i> | |
| 12 | Lab | 6 yr 6 mo | F* | Jaundice & Renal Failure | N | P | P | <i>B. gibsoni</i> | KP901256 |
| 13 | Lab | 6 yr | M | Septic Shock (DIC) and death | P | P | P | <i>B. gibsoni</i> | KY524481 |
| 14 | Lab | 8yr 9 mo | F* | Septic Shock (DIC) and death | P | P | P | <i>B. gibsoni</i> | KP901258 |
| 15 | Lab | 2 yr 10 mo | M | Septic Shock (DIC) and death | N | P | P | <i>B. gibsoni</i> | KP901257 |
| 16 | Lab | 6 yr 4mo | M | MODS and death | N | P | P | <i>B. gibsoni</i> | KP901259 |
| 17 | Lab | 5yr 10 mo | F* | MODS and death | P | P | P | <i>B. gibsoni</i> | KP901260 |
| Total | | | | | 07 | 12 | 17 | | |

from the dead dogs suspected for babesiosis were collected in 10% formal saline solution and 50% glycerol saline, respectively. These clinical samples were then transported to CMVL for further analysis.

2.2. Microscopy

The air dried and methanol fixed microscopic blood smears were stained using the May Grunwald Giemsa method (Vaden et al., 2009).

The slides were examined under 100x (oil immersion) objective for presence of intraerythrocytic piroplasms.

2.3. Haematology

Anticoagulant added whole blood were used for estimation of haemoglobin (Hb), packed cell volume (PCV), total leukocyte count (TLC), total erythrocyte count/total RBC count (TEC/TRBC) and platelet count

using MS4s vet automated haematology analyser (MELET SCHLOESING Laboratories, France). Values of studied parameters were determined and compared with control values as mentioned in kits. Erythrocyte sedimentation rate (ESR) was estimated in automated ESR counter (ESR 2010) as per manufacturer's instructions. Stained thin smears were examined microscopically for estimation of DLC.

2.4. Biochemistry

Serum samples were examined for different biochemical parameters in automated clinical chemistry analyser (EM 360™, ERBA diagnostics Mannheim GmbH, Germany) using suitable internal controls. Serum samples were subjected to testing for various parameters such as- total bilirubin, total protein, albumin (A), globulin (G), albumin:globulin ratio (A/G), aspartate amino transferase (AST/SGOT), alanine amino transferase (ALT/SGPT), blood urea nitrogen (BUN) and creatinine (CRE) using standard diagnostic kit procedures for respective parameters manufacturers' recommendation for estimation of each analyte.

2.5. Histopathology

Formalin fixed, post necropsy tissue samples, received from different field veterinary clinics were processed for histopathology. Processing of these clinical specimens and staining of tissue sections with Haematoxylin and Eosin staining was performed as per standard procedure (Fischer et al., 2008) and the diagnosis of SIRS, MODS and septic shock among study cases were based on criteria as proposed by Matijatko et al (2009).

2.6. Nucleic acid amplification and sequencing

2.6.1. Nucleic acid extraction

DNA was extracted from whole blood and necropsy tissue samples using DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The quality and quantity of extracted DNA was estimated for using NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). DNA samples were stored at -20°C , until further use.

2.6.2. Primers used for PCR amplification

Two different *Babesia* genus specific PCR protocols for partial amplification of the 18S ribosomal gene (rRNA) were used for screening. Simple PCR of Oyamada et al. (2005) and nested PCR of Hitoshi et al. (2001) were used in the study. The amplicons obtained from PCRs were then used for sequence analysis and for species identification.

2.6.3. Nucleic acid amplification

PCR amplification was performed in 50 μl reaction, using 100 ng of extracted DNA, 25 μl of 2X Green Dream Taq PCR master mix, 10 μM of the forward and reverse primers combination. Final volume of reaction mixture was made up with nuclease free water. Simple PCR (Oyamada et al., 2005) and primary step of nested PCR (Hitoshi et al., 2001) were performed using stored genomic DNA. For the nested step, 1 μl products of primary reaction were used as template and amplification was carried out as described by Hitoshi et al., 2001. The PCR amplifications were performed in a thermo-cycler (Master Cycler®, Eppendorf, Germany). Cycling conditions were kept the same as reported by the respective researchers. Non-template negative controls were used in all PCR experiments. PCR amplified products were resolved on ethidium bromide stained 1.5% agarose gels in Tris acetate EDTA buffer and post electrophoresis gel documentation was carried out using Alpha Imager® EP (Alpha Innotech, USA).

2.7. Sequencing of the amplified product and bioinformatics analysis

The PCR products were gel extracted using QIAquick gel extraction

kit (Qiagen, Germany) following manufacturers' instructions. Purified PCR products were assessed for quality and quantity. Custom sequencing of purified PCR products were carried at BioServe Biotechnologies (India) Pvt Ltd, Hyderabad, India. The sequence chromatogram was annotated with BioEdit Sequence Alignment Editor software vs 7.0.5 (Isis Therapeutics, USA). The annotated sequences were identified using nucleotide BLAST analysis. Details of sequences submitted to the NCBI GenBank with their accession numbers are mentioned in Table 1. Closely matching sequences in NCBI database were used in the phylogenetic study.

Partial 18S rRNA gene sequence of nine *Babesia* isolates from the present study and 71 other sequences were retrieved from GenBank were included in the analysis. The sequences were aligned using MUSCLE module of MEGA6. Evolutionary analyses and nucleotide substitution model selection were conducted in MEGA6 package (Tamura et al., 2013). Evolutionary history was inferred by the maximum likelihood (ML) method based on the general time reversible model (GTR + G + I). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2470)). The rate variation model allowed for some sites to be evolutionarily invariable. Bootstrap analysis was carried out using 1000 replicates of the dataset.

2.8. Statistical tools

McNemar's Chi squared test was used to analyse difference in diagnostic results obtained from simple PCR assay, nested PCR assay and microscopical examination (<http://www.graphpad.com/quickcalcs/McNemar>) (Mandal et al., 2015; Mittal et al., 2017). Kappa statistics was performed using the online software (<https://www.graphpad.com/quickcalcs/kappa1>) so as to estimate the agreement between the variations in results obtained from the different diagnostic tests.

3. Results

3.1. Microscopy, simple PCR- and nested PCR- assay

Out of 330 stained blood smears examined for the presence of small and large forms of *Babesia*, only 17 smears were found positive under light microscopy for small intraerythrocytic piroplasms similar to *B. gibsoni*. DNA extracted from all the blood samples (330) were subjected to 18S rRNA based simple PCR (Oyamada et al., 2005) and nested PCR (Hitoshi et al., 2001) assays, out of which 30 and 51 samples were found positive, respectively in both assays deployed. Year wise percentage of positive samples found in 2012, 2013 and in 2014 was 10.9% (12/110), 16.36% (18/110) and 19.09% (21/110), respectively. Among 51 nested PCR positive samples 19, 16, 10 and six were from northern, eastern, central and west India, respectively. Overall prevalence among 330 dogs was 15.45% (51/330) by nested PCR, 9.09% (30/330) by simple PCR and 5.15% (17/330) by microscopy. McNemars chi square test estimated the p-values between the diagnostic assays. P-values estimated were less than 0.0001 ($p < 0.0001$), for microscopy/nested PCR and nested PCR/simple PCR comparison pairs. Results obtained from nested PCR and simple PCR were significantly distinct from microscopy at $p < 0.05$ and similarly, nested PCR results also differed significantly from simple PCR results ($p < 0.05$) too. Inter-rater agreement between the microscopy and nested PCR tests were evaluated using kappa (κ) statistics. Kappa \pm standard error value was estimated as $\kappa \pm \text{se} = 0.426 \pm 0.075$, which is a moderate agreement between the observations, obtained from microscopic examination and nested PCR (Viera and Garrett, 2005). Kappa statistics of nested PCR and simple PCR assays were estimated as $\kappa \pm \text{se} = 0.707 \pm 0.059$, which indicates a substantial agreement between the observation obtained from the two tests (Viera and Garrett, 2005).

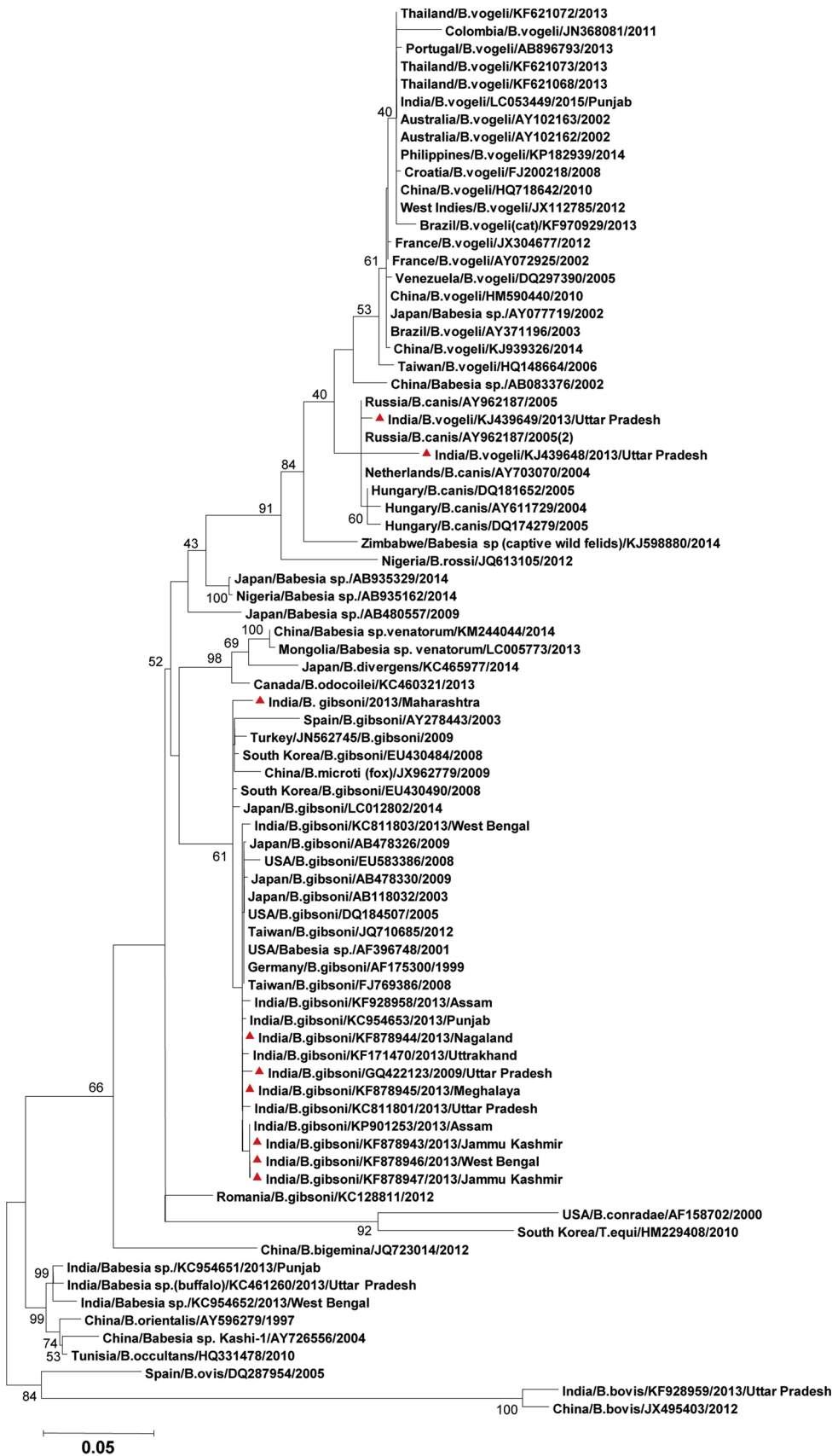


Fig. 1. Phylogenetic tree based on partial 18S rRNA gene sequence of Babesia isolates by maximum likelihood method. The tree with the highest log likelihood (-9070.8448) is shown. The percentage (only if more than 40%) of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The country of origin, species of Babesia, GenBank accession number and year of isolation are shown for each sequence. Sequences determined in this study are marked with a filled red triangle (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Haematological & biochemical analysis

On the basis of clinical severity, haematological and biochemical parameters all nested PCR positive animals were classified into three groups of 17 animals each i.e. dogs apparently healthy but were observed to be subclinical carriers (group-1), dogs manifesting mild symptoms of simple or uncomplicated babesiosis (group-2) and lastly, dogs manifesting symptoms of complicated babesiosis (group-3). Majority of the dogs in group-2 and group-3 showed common clinical signs like pyrexia, anorexia, pale mucous membrane and anaemia (Table 1).

Detailed haemogram report from PCR positive dogs are mentioned in Table 3. Haematology study showed low haemoglobin (Hb) concentration in 29 dogs, low PCV and total erythrocyte count (TRBC) among 31 dogs and lower ESR among 32 dogs. Most of the dogs with lowered haematological values also clinically manifested anaemia. Leucopenia with low TLC value was observed in 30 dogs while leucocytosis was found in five dogs. DLC study revealed neutrophilia in 17 dogs, neutropenia in 11 dogs and neutrophils were found to be within the normal range in rest of the dogs. Lymphocytosis was observed in 15 animals, while lymphopenia was noticed among two dogs.

Biochemical parameters of the serum samples obtained from 51 dogs have been tabulated in Table 4. Hyperbilirubinemia was found in 13 dogs. Elevation in total serum protein levels were observed in nine animals. None of the animals showed hyperalbuminemia while 21 animals had hypoalbuminemia. Hyperglobulinemia was observed in 29 animals while hypoglobulinaemia was seen in only two animals. Significantly, low albumin:globulin (A/G) ratio values were observed in 27 animals. High ALT and AST values were found to be higher in 22 and 24 dogs, respectively while clinical manifestation indicative of icterus was apparent in only five dogs. Increased levels of BUN and creatinine were found in 10 and nine animals, respectively indicating renal failure in these animals (of which seven animals were clinically exhibiting symptoms of renal failure).

3.3. Histopathology

Cerebral form of babesiosis with signs of epilepsy was noticed in four dogs out of total 51 dogs screened. Two dogs which were diagnosed for MODS died during the study. Two apparently healthy dogs from North India and one dog from East India died suddenly without showing any clinical symptoms. Post-mortem examinations from these three dogs were indicative of septic shock due to disseminated intravascular coagulopathy (DIC). Tissues of these three animals were analysed by histopathology and changes were indicative of endotoxemic shock and haemodynamic failure. All five fatal cases were positive for *B. gibsoni*.

Postmortem examination revealed pale mucous membranes; enlarged lymph nodes, hepatosplenomegaly, cardiomegaly, hydropericardium and prominent congestive lesions in kidneys, liver, spleen, lungs and cardiac muscles. Pulmonary congestion was associated with frothy transudate in large airways. Microscopic lesions were characteristic of haemolytic anaemia (Suppl. Fig. 1). Pulmonary haemorrhages and oedema with interstitial infiltration of inflammatory cells were evident in histopathology of lung tissues. Moderate haemorrhages in parenchyma with engorged vessels and interstitial fibrosis in kidneys were observed. Hepatocellular degeneration, parenchymal haemorrhages, hemosiderin pigment deposits, congestion of sinusoids and engorged vessels were observed in liver tissues. Congested splenic cords and sinuses along with hemosiderin pigment deposits and myofibrillar degeneration along with moderate haemorrhages and engorged vessels were observed in all the three cases.

3.4. Sequencing of 18S r RNA fragment and phylogenetic analysis

Purified PCR amplicons (327 bp) from 51 PCR positive cases were

sequenced for identification of *Babesia* species. Sequence chromatograms were visualized and annotated with BioEdit sequence alignment editor software version 7.0.5. Forty seven *Babesia* sequences matched closely with *B. gibsoni* sequences that were submitted from India and had an identity of 99–100% and only four sequences matched with *B. vogeli* (KJ439648, KJ439649, KY513707 and KY513708). Twenty four *B. gibsoni* sequences and four *B. vogeli* sequences were submitted to GenBank, NCBI. *Babesia* spp. and their accession numbers have been illustrated in Table 1.

In this study, partial 18S rRNA sequences of the Indian canine *Babesia* spp. from working dogs and phylogenetic analysis was carried out by maximum likelihood method. Phylogenetic study included sequences of various *Babesia* species from different geographical retrieved from the NCBI database. All *B. gibsoni* and *B. vogeli* isolates clustered separately as monophyletic clades in the phylogenetic tree (Fig. 1). All Indian *B. gibsoni* sequences determined in the present study or in the earlier studies clustered in the *B. gibsoni* clade and shared ancestry with *B. gibsoni* isolates from other countries such as Japan, USA, Taiwan, Germany, South Korea and Spain (accession numbers mentioned in Fig. 1). All *B. vogeli* isolates including those from India formed a cluster distinct from the *B. canis* cluster. *B. vogeli*, *B. canis* and *B. rossi*, although formed different clusters, apparently they shared the most recent common ancestor as evident from phylogenetic tree. The two Indian sequences originating from Uttar Pradesh (KJ439649/2013 and KJ439648/2013, showed best match (100% and 99% identity) with both *B. vogeli* and *B. canis* sequences available in the NCBI database in the BLAST analysis. These sequences, however clustered in the *B. canis* group being interspersed between *B. canis* sequences from Russia, the Netherlands and Hungary. Rest all other species such as *B. bovis*, *B. ovis*, *B. occlusans*, *B. orientalis*, *B. bigemina*, *B. conradae*, *T. equi*, *B. venatorum*, *B. divergens* and *B. odocoilei* formed distinct branches. Despite the overall tree topology remaining the same, minor variations compared to the earlier publications could be explained in part by the difference in the length of the 18S rRNA sequence dataset taken for comparison and in the methods adapted for evolutionary analyses.

4. Discussion

Warm weather conditions prevailing in various agro-climatic zones of tropical India favours ectoparasites lifecycle and transmission of vector-borne diseases. Few cases and limited prevalence status among pet and stray dogs have been reported from this country. Singh et al (2014) from Punjab state in North West India recorded microscopic and molecular prevalence of canine babesiosis as 6.54% and 15.42%. Singla et al (2016) reported molecular prevalence of 15.04% for *B. gibsoni* among the pet dogs brought to clinics from the Ludhiana city of Punjab state. Jain et al (2017) reported microscopic and molecular prevalence of 26.67% and 47.3%, respectively for *B. gibsoni* among the pet dogs in South Indian state of Kerala. Abd Rani et al (2011) reported molecular prevalence of *Babesia gibsoni* among the stray dogs from two cities (Mumbai and Delhi), Ladakh region of Jammu and Kashmir state and small state of Sikkim, as mere 0.2% only. Mandal et al (2015) studied 75 dog blood samples, of which 37 and 23 samples were positive by nested PCR and microscopy. Present study showed an overall prevalence of 15.45% with nested PCR, 9.09% with simple PCR and 5.15% with microscopy.

Above studies did not provide information regarding clinical scenario of disease, especially its diverse manifestations across different agro-climatic conditions of this country. Moreover, these studies also did not address the diagnostic and therapeutic challenges in backdrop of latest control/preventive measures being widely adopted in this country. Tick vector borne diseases have not been studied in working dogs from organised kennels of India. Present study was focused on getting comprehensive information about the presence of canine babesiosis from the working dogs of organised kennels located in different geographical locations using various diagnostic tools like conventional

microscopy as used as molecular assays (simple PCR, nested PCR and sequence analysis). These diagnostic assay results were correlated with clinical manifestations, haematology and biochemical parameters from these dogs.

During this study, myriad conditions and their clinical manifestations of canine babesiosis due to *B. gibsoni* were observed. These varied from mild anaemia, transient anorexia to complex syndrome of multiple organ failure and fatality. Overall, it seems that canine babesiosis in this country does not have a simple manifestation but has at least three clinically different forms. Asymptomatic form of disease was manifested by chronic or subclinical carrier dogs that were generally observed to be apparently healthy. Classical or uncomplicated forms resulted from haemolytic anaemia while complicated form of disease involved the additional organ failure (ARF, ARDS, hepatopathy, cerebral babesiosis, coagulopathy and MODS) besides haemolytic anaemia.

The variable clinical manifestations or pathophysiology of canine babesiosis may range from subclinical to severe or fatal, critically depending on the piroplasm spp. involved, on factors associated with the hosts (e.g. age and immune status) and importantly host-parasite interplay towards the insult (Solano-Gallego and Baneth, 2011). In the present study, 56.86% of the dogs presented haemolytic anaemia which is in agreement with the previous studies (Kraje, 2001; Furlanello et al., 2005). Three mechanism of haemolytic anaemia have been postulated for canine babesiosis: mechanical, immune-mediated and toxic by the production of haemolytic factor of the parasite (Onishi et al., 1990; Bourdeau and Guelfi, 1995). BUN and creatinine levels were higher in ten animals indicative of ARF. Possible reasons of this elevated level in some of the dogs may be due to non-renal accumulation of ammonia in serum as a result of haemolytic anaemia. Significant biochemical findings of hypoalbuminemia and hyperglobulinemia resulting in the decrease in the AG ratio in 52.94% animals; increase of serum activity of AST and ALT were observed as previously described (Pages et al., 1990; Bourdeau and Guelfi, 1995; Lobetti, 2000; Welzl et al., 2001).

Atypical/complicated babesiosis form and fatalities due to *B. gibsoni* infection has not been reported in previous studies conducted from India. MODS in complicated cases of canine babesiosis are characterized by involvement of different organs (Welzl et al., 2001; Jacobson, 2006). Though MODS is common with *B. rossi* infections, four dogs in the present study showed MODS. The possible mechanism of MODS may be due to the uncontrolled proinflammatory and anti-inflammatory mechanisms involved in acute phase reactions causing auto-destructive inflammation in various organs (Goris et al., 1985). Five dogs died as a result of canine babesiosis (attributed to *B. gibsoni*). Two dogs were suffering from MODS while in remaining three cases sudden death due to septic shock was observed. Mortality is common with *B. rossi* infections and may reach to around 10–15% (Matijatko et al., 2009). Our study revealed overall case fatality rate of 9.80% (05/51) among *B. gibsoni* positive cases. This was 29.41% (05/17) of complicated cases of babesiosis. Epilepsy due to cerebral babesiosis was seen in four dogs i.e. overall 7.84% (04/51) of positive cases. This was 23.53% (04/17) among atypical (complicated) babesiosis cases. This finding is contrary to previous studies (Welzl et al., 2001; Matijatko et al., 2009) where central nervous system dysfunction was observed to be the rarest complication due to canine babesiosis as it was found to be quiet significant in the present study. Coma, collapse and other neurological signs that are attributed to cerebral babesiosis may be due to hypoglycaemia and ischemia (Matijatko et al., 2009; Jacobson and Lobetti, 2005).

Previous studies from India have revealed that both *B. vogeli* and *B. gibsoni* have common ticks' vector *R. sanguineus* while *H. longicornis* is associated with transmission of *B. gibsoni* (Shaw et al., 2001; Abd Rani et al., 2011; Singla et al., 2016). Present study among working dogs of organised kennels from India revealed *B. gibsoni* and *B. vogeli* are two main *Babesia* species, former being the predominant species. *B. vogeli* usually causes a subclinical to mild or moderate clinical disease in adult dogs (Carret et al., 1999; Uilenberg et al., 1989) while severe to fatal

haemolytic anaemia has been reported in juvenile dogs and pups (Solano-Gallego et al., 2008). No fatal infection among juvenile dogs and pups was reported. Four pups (four to six months age) were found to be infected with *B. vogeli* where as one pup (eight month of age) and one juvenile dog (one year of age) was found to be infected with *B. gibsoni*. As reported elsewhere, pathogenic potential of *B. vogeli* was also observed in the present study to be mild or subclinical only as haematological and biochemical parameters in pups infected with *B. vogeli* were found to be within normal range while pup and juvenile dog infected with *B. gibsoni* were reported to have slightly low Hb concentration (9–10 g m%). This study further substantiates that *B. gibsoni* infection doesn't appear to be associated with age. While screening the blood samples, surprisingly the dogs which were apparently healthy i.e. not manifesting clinical signs were also found positive in single-step PCR and nested PCR. This finding correlates with the earlier findings where subclinical infection and chronic infection of *B. gibsoni* has been reported in adult dogs (Birkenheuer et al., 2005). The present study has shown that *B. gibsoni* predominates *B. vogeli* as a major pathogen among working dogs in this country and raises a serious concern over almost twofold increase in the prevalence of canine babesiosis in 2013 (19.82%) in comparison of prevalence of 10.24% in 2012 which is more important in the context that stray dogs were not included in this study. Stray dogs are a major chunk of domestic canine population in India and are known to act as reservoir of the same. This study pertinently emphasizes for the timely control measures to be installed at top priority for the control of canine babesiosis in India.

Microscopic, peripheral blood smears from 17 animals were positive for small piroplasms (*B. gibsoni*) revealing small signet, rod or cocci shaped usually occurring singularly and of 1-2.5 µm size. Larger intraerythrocytic piroplasms (*B. vogeli*) were not observed microscopically even in four samples which were found to be positive in nested PCR assay. These findings are contrary to the earlier reports from India as reported by Abd Rani et al. (2011) and Kundu et al. (2012) where *B. vogeli* was the predominant *Babesia* spp. found in Northern and Western India. Although, the present finding are in concurrence with Kumar et al. (2009) who reported maximum incidence of *B. gibsoni* infection in Chennai region (Southern India) by smear microscopy. Serological assays do not definitely discriminate *Babesia* spp. and due to their cross-reactivity along with persistence of antibodies for several months even after the infection make them less popular for individual diagnosis and for epidemiological studies of canine babesiosis (Irwin, 2009) and hence were not used in the present study.

Nucleic acid based amplification techniques, especially PCR have been proven to be valuable tool for the diagnosis of parasitic diseases (Gasser, 2006). PCR with a sensitivity and specificity of nearly 100% as determined by various workers (Birkenheuer et al., 2003; Inokuma et al., 2005; Martin et al., 2006) have been useful for the detection of *Babesia* DNA in the canine blood samples. Microscopy is prone to errors and is also known for its poor sensitivity. Significant difference in diagnostic results of single-step PCR and of nested PCR protocols were too found to be statistically significant (Table 2) in McNemar Chi squared

Table 2

Prevalence of *B. gibsoni* in dogs as observed using three diagnostic assays (microscopic examination of thin blood smears, conventional/simple PCR and nested PCR). Prevalence is placed as percentage in parentheses.

| Sample size (n) = 330 *nPCR | Microscopy | | | Conventional/simple PCR | | |
|--------------------------------|---------------|----------|----------------|-------------------------|----------|----------------|
| | Positive | Negative | Total | Positive | Negative | Total |
| Positive | 16 | 35 | 51 (15.45%) | 30 | 21 | 51 (15.45%) |
| Negative | 1 | 278 | 279 | 0 | 279 | 300 |
| Total | 17 (5.15%) | 313 | 330 | 30 (9.09%) | 300 | 330 |

*n PCR- nested PCR.

Table 3

Haematological parameters obtained from 51 dogs found positive for canine babesiosis in group wise depiction based on clinical disease diagnosed (Group-1- dogs with subclinical babesiosis; Group-2- dogs with uncomplicated babesiosis or simple babesiosis; Group-3- dogs with complicated babesiosis). Changes in the haematological parameters are indicated as increased, decreased or unchanged (remained within normal/reference range) and the normal/reference range* are as per the values standardised by Central Military Veterinary Laboratory for screening of dog blood samples.

| Haematology parameters with normal/reference range* in parentheses | Number of Dogs | | | | | | | | | | | |
|-----------------------------------------------------------------------|----------------|---------|---------|-------|-----------|---------|---------|-------|-----------|---------|---------|-------|
| | Increased | | | | Decreased | | | | Unchanged | | | |
| | Group-1 | Group-2 | Group-3 | Total | Group-1 | Group-2 | Group-3 | Total | Group-1 | Group-2 | Group-3 | Total |
| 1. Haemoglobin (12-18 g %) | 1 | 1 | 0 | 2 | 3 | 11 | 15 | 29 | 13 | 5 | 2 | 20 |
| 2. Packed Cell Volume (37-55 %) | 1 | 0 | 0 | 1 | 4 | 10 | 15 | 31 | 12 | 7 | 2 | 19 |
| 3. Erythrocyte Sedimentation Rate (5-25 in mm) | 0 | 0 | 0 | 0 | 3 | 12 | 16 | 32 | 14 | 5 | 1 | 19 |
| 4. Total Erythrocyte Count ($5.5-8.5 \times 10^6 \mu\text{l}$) | 2 | 1 | 0 | 3 | 5 | 11 | 15 | 31 | 10 | 5 | 2 | 17 |
| 5. Total Leucocyte Count ($6-17 \times 10^6 \mu\text{l}$) | 2 | 3 | 0 | 5 | 6 | 10 | 14 | 30 | 9 | 4 | 3 | 18 |
| 6. Platelet Count ($2-9 \times 10^5 \mu\text{l}$) | 2 | 1 | 0 | 5 | 2 | 6 | 6 | 14 | 13 | 10 | 11 | 34 |
| 7. Differential Leucocyte Count (%) | | | | | | | | | | | | |
| a. Neutrophils (60-73 %) | 4 | 6 | 7 | 17 | 4 | 4 | 3 | 11 | 9 | 7 | 7 | 23 |
| b. Lymphocytes (12-30 %) | 2 | 4 | 9 | 15 | 1 | 1 | 0 | 2 | 14 | 12 | 8 | 34 |
| c. Monocytes (3-10 %) | 1 | 2 | 3 | 6 | 2 | 2 | 2 | 6 | 14 | 13 | 12 | 39 |
| d. Eosinophils (2-10 %) | 1 | 1 | 2 | 4 | 3 | 4 | 4 | 11 | 13 | 12 | 11 | 36 |
| e. Basophils (0, Rare) | 1 | 1 | 2 | 4 | 0 | 0 | 0 | 0 | 16 | 16 | 15 | 47 |

test ($p < 0.05$) which was largely due to low parasitaemia and asymptomatic (stealth) infections of canine babesiosis observed among these dogs. Overall prevalence among 330 dogs was 15.45% (51/330) by nested PCR, 9.09% (30/330) by simple PCR and 5.15% (17/330) by microscopy. This study emphasises that results of simple PCR may be inadequate as compared to nested PCR. Hence, nested PCR is recommended as the test of choice for detection of *Babesia*. This must be followed by sequence characterization as appropriate method for species identification.

During the study, babesiosis caused by large form of *B. vogeli* was commonly treated with imidocarb dipropionate and diminazene aceturate with good prognosis while *B. gibsoni* were found to be more resistant to the anti-babesial therapy and prognosis of the cases remained poor. Clinical and parasitological cure are often not achieved in the treatment of *B. gibsoni* infections and clinical relapses are frequently noticed (Irwin, 2009). *B. gibsoni* infection is resistant to treatment with

imidocarb dipropionate and diminazene aceturate, which are two main drugs currently being widely used in India although recommended therapy is the combination of the anti-malarial atovaquone and the macrolide azithromycin for clearance/elimination of *B. gibsoni* infection (Birkenheuer et al., 2004). In this study, it was observed that the treatment regime being followed in the Indian field conditions by most of the clinicians is mainly empirical and is focused on symptomatic recovery and not on elimination of the infection. This could be one of the reasons for high number of subclinical infection diagnosed in this study and many apparently healthy dogs testing positive in nested PCR. Moreover, treatment regimen is based on tentative diagnosis of haemoprotozoan infection and comprises of use of multidrug therapy ranging from oxytetracycline, doxycycline, metronidazole, clindamycin, azithromycin, diminazene aceturate and imidocarb dipropionate along with other supportive drugs. All these drugs have been reported to have some efficacy against *B. gibsoni* but are not completely

Table 4

Biochemical parameters obtained from 51 dogs found positive for canine babesiosis with group wise depiction based on clinical disease diagnosed (Group-1- dogs with subclinical babesiosis; Group-2- dogs with uncomplicated babesiosis or simple babesiosis; Group-3- dogs with complicated babesiosis). Changes in the biochemical parameters are indicated as increased, decreased or unchanged (remained within normal/reference range) and the normal/reference range* are as per the values standardised by Central Military Veterinary Laboratory for screening of dog serum samples (A/G Ratio- Albumin/Globulin Ratio; AST- Aspartate amino transferase; ALT- Alanine amino transferase; BUN- blood urea nitrogen).

| Biochemical parameters with normal/ reference range* in parentheses | Number of Dogs | | | | | | | | | | | |
|------------------------------------------------------------------------|----------------|---------|---------|-------|-----------|---------|---------|-------|-----------|---------|---------|-------|
| | Increased | | | | Decreased | | | | Unchanged | | | |
| | Group-1 | Group-2 | Group-3 | Total | Group-1 | Group-2 | Group-3 | Total | Group-1 | Group-2 | Group-3 | Total |
| 1. Total Protein (5.5-7.5 mg%) | 3 | 4 | 2 | 9 | 3 | 3 | 3 | 9 | 11 | 10 | 12 | 33 |
| 2. Albumin (2.6-4.0%) | 0 | 0 | 0 | 0 | 1 | 9 | 11 | 21 | 16 | 8 | 6 | 30 |
| 3. Globulin (2.1-3.7%) | 3 | 11 | 15 | 29 | 1 | 1 | 0 | 2 | 13 | 5 | 2 | 20 |
| 4. A/G Ratio (0.7-1.9) | 0 | 0 | 0 | 0 | 3 | 9 | 15 | 27 | 14 | 8 | 2 | 24 |
| 5. Total Bilirubin (0.1-0.6 mg%) | 1 | 3 | 9 | 13 | 3 | 4 | 0 | 7 | 13 | 10 | 8 | 31 |
| 6. AST (8-48 IU/L) | 3 | 8 | 13 | 24 | 0 | 0 | 0 | 0 | 14 | 9 | 4 | 27 |
| 7. ALT (8-58 IU/L) | 3 | 7 | 12 | 22 | 0 | 0 | 0 | 0 | 14 | 10 | 5 | 29 |
| 8. BUN (8.8-26 mg%) | 0 | 2 | 8 | 10 | 2 | 3 | 3 | 8 | 15 | 12 | 6 | 33 |
| 9. Creatinine (0.5-1.6 mg%) | 0 | 1 | 8 | 9 | 0 | 0 | 0 | 0 | 17 | 16 | 9 | 42 |

effective in eliminating the parasitaemia. At the best, these drugs can only result in amelioration of the clinical signs. To date, a therapy comprising of anti-malarial atovaquinone (at dose rate of 13.5 mg/kg bwt PO tid with fatty meal for 10 days) and the macrolide azithromycin (at dose rate of 10 mg/kg bwt q24 h for 10 days) combination have only shown promising results in the clearance of *B. gibsoni* infection. Unfortunately, atovaquinone is not readily available in India and due to lack of awareness and high cost, this drug is not being used by veterinarians and this may have contributed in the positive selection of *B. gibsoni* infection over *B. vogeli* in the field. In the absence of recommended treatment for *B. gibsoni* infection, field veterinarians find the treatment of babesiosis to be frustrating with most of the cases relapsing after initial recovery and most of them either suffered from subclinical/chronic infection while some suffered with severe infection/MODS/septic shock. Thus, *B. gibsoni* babesiosis poses a significant and complex therapeutic challenge for the practitioners of this country as current babesiacidal drugs were found only effective in preventing the clinical disease and not in eliminating the infection. In the absence of prophylactic vaccination in this country, chemoprophylaxis against the ticks' infestations was carried out by use of acaricides applications (spot-on, sprays, neck collars, shampoos, dips and powders) and by tick removal during the regular physical inspection of these working dogs.

Phylogenetic analysis based on partial 18S rRNA revealed that the gene is highly conserved and depicted the monophyletic nature and clonal expansion of all the *B. gibsoni* isolates under study. Interestingly, two sequences (KJ439649 and KJ439648) being reported from the present study although showed best match (100% and 99% identity) with both *B. vogeli* and *B. canis* sequences available in the NCBI database in the BLAST analysis but these sequences somehow clustered in the *B. canis* group. Although it is tempting to assign these two isolates to *B. canis* species based on their genetic grouping pattern, it may be too early to draw any conclusion at this stage considering the fact that *B. canis* has so far never been reported from India and only 281 nucleotide sequence stretch was used in present analysis that could have missed relevant phylogenetically informative sites. Non-vectorial i.e. direct dog-to-dog transmission through bite (Jefferies et al., 2007) or through blood transfusion (Stegeman et al., 2003) and transplacental transmission of *B. gibsoni* (Fukumoto et al., 2005) are some of the possible explanation for its clonal expansion.

Presently, due to lack of effective point-of-care (POC) test, recommended therapeutics (atovaquinone) and prophylactic vaccines, disease prevention is majorly being relied upon by acaricides applications which raise pertinent concerns over their indiscriminate use leading to environmental pollution and development of acaricides resistant ticks. Thus, canine babesiosis with its continuously changing distribution is posing a significant global threat and with reports of humans being accidental hosts of *B. vogeli* in splenectomised patients with acute renal failure (Marsaudon et al., 1995) along with high conglomerations and anthrophilic tendency of *R. sanguineus* (Goddard, 1989) has also sparked 'One Health' concerns associated to it.

5. Conclusion

Canine babesiosis due to *B. gibsoni* is of significant veterinary importance in India. The disease is endemic as observed by its prevalence across the different agro-climatic zones of this country. During the study it was also inferred that this small piroplasm has established itself predominantly over *B. vogeli* and is on the rise among the canine population of this country. Moreover, clinical disease due to *B. gibsoni* in this country is being manifested by three different forms viz., asymptomatic or subclinical-, classical or uncomplicated- and atypical or complicated- form of disease. Furthermore, from diagnostic point of view, conventional microscopic examination and simple PCR based molecular detection methods for these apicomplexan parasites were found to be inadequate especially in chronically infected animals

manifesting asymptomatic infection. Although, conventional/simple PCR protocols are considered to be ideal methods for laboratory diagnosis of canine babesiosis but the present study argues that nested PCR instead of simple PCR be relied upon as a screening assay and also for epidemiological studies as statistical significant false-negative animals were observed among the latter results. These findings not only provide better understanding of the canine babesiosis but will also assist in planning of pragmatic preventive strategies against this potent threat especially in countries akin to India where the endemic scenario of the disease exists.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.prevetmed.2019.104696>.

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