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Genetic characterization and phylogenetic relationships based on 18S rRNA and ITS1 region of small form of canine *Babesia* spp. from India

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ABSTRACT

Canine babesiosis is a vector borne disease caused by intra-erythrocytic apicomplexan parasites *Babesia canis* (large form) and *Babesia gibsoni* (small form), throughout the globe. Apart from few sporadic reports on the occurrence of *B. gibsoni* infection in dogs, no attempt has been made to characterize *Babesia* spp. of dogs in India. Fifteen canine blood samples, positive for small form of *Babesia*, collected from northern to eastern parts of India, were used for amplification of 18S rRNA gene (~1665 bp) of *Babesia* sp. and partial ITS1 region (~254 bp) of *B. gibsoni* Asian genotype. Cloning and sequencing of the amplified products of each sample was performed separately. Based on sequences and phylogenetic analysis of 18S rRNA and ITS1 sequences, 13 were considered to be *B. gibsoni*. These thirteen isolates shared high sequence identity with each other and with *B. gibsoni* Asian genotype. The other two isolates could not be assigned to any particular species because of the difference(s) in 18S rRNA sequence with *B. gibsoni* and closer identity with *Babesia occultans* and *Babesia orientalis*. In the phylogenetic tree, all the isolates of *B. gibsoni* Asian genotype formed a separate major clade named as *Babesia* spp. *sensu stricto* clade with high bootstrap support. The two unnamed *Babesia* sp. (Malbazar and Ludhiana isolates) clustered close together with *B. orientalis*, *Babesia* sp. (Kashi 1 isolate) and *B. occultans* of bovines. It can be inferred from this study that 18S rRNA gene and ITS1 region are highly conserved among 13 *B. gibsoni* isolates from India. It is the maiden attempt of genetic characterization by sequencing of 18S rRNA gene and ITS1 region of *B. gibsoni* from India and is also the first record on the occurrence of an unknown *Babesia* sp. of dogs from south and south-east Asia.

1. Introduction

Canine babesiosis is a life threatening disease caused by intra-erythrocytic apicomplexan parasites of the genus *Babesia*. It is one of the most important vector-borne diseases of dogs caused by *Babesia canis* and *Babesia gibsoni* throughout the globe (Bourdoiseau, 2006; Kjemtrup and Conrad, 2006). *B. canis* is the large form (4–5 μm), while *B. gibsoni* is a small pleomorphic organism (1–2.5 μm) and appears most commonly as ring form. It is found in almost all parts of Asia, Europe, Africa, America and Australia (Conrad et al., 1991; Casapulla et al., 1998; Macintire et al., 2002). Acute infections are typically associated with remittent fever, progressive anemia, lethargy, thrombocytopenia, haemoglobinuria, marked splenomegaly and hepatomegaly (Wozniak et al., 1997; Goo et al., 2008). Chronic infections are more common and infected dogs remain as carriers without any overt clinical signs (Conrad et al., 1991).

Traditionally, all small canine piroplasms are recognized as *B. gibsoni* and it is assumed that no other small *Babesia* species infect dogs. This hypothesis was proven wrong by characterization of small piroplasms of dogs from Spain and California (Kjemtrup et al., 2000; Zahler et al., 2000a). Genetic characterization and phylogenetic analysis of the 18S rRNA gene of canine small piroplasms from Asia, the Midwestern United States, California, Africa and Spain conclusively proved that there are three genotypically distinct small *Babesia* of canines (Kjemtrup et al., 2000), *B. gibsoni sensu stricto* from Asia and the Midwestern United States (Zahler et al., 2000b), *Babesia microti*-type from Spain, named as *Theileria annae* (Zahler et al., 2000a; Garcia, 2006) and the Californian isolate, known as *Babesia conradae* (Kjemtrup and Conrad, 2006). Few other small piroplasms, viz. *Theileria equi*, *Theileria annulata* and an unnamed *Theileria* sp. were also reported in canines from Spain and South Africa (Criado-Fornelio et al., 2003c, 2006; Matjila et al., 2008).

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The 18S rRNA gene is widely used to establish phylogenetic relationships as well as to differentiate the genotypes or subspecies of canine *Babesia* (Kjemtrup et al., 2000; Uilenberg et al., 1989). Small canine piroplasms have been classified into three distinct clades based on characterization of 18S rRNA gene; *B. gibsoni* has been categorized under *Babesia sensu stricto* clade, *B. conradae* has been kept in Western clade and the ancestral *B. microti*-type isolate has been assigned to the *B. microti* clade (Zahler et al., 2000a,b; Kjemtrup et al., 2000; Kjemtrup and Conrad, 2006). In addition, ITS1 region often demonstrates marked divergence among species and even among strains of the same species. Intraspecific variation within the ITS1 region has already been reported in isolates of three sub species of *B. canis* and *Eimeria* sp. (Barta et al., 1998; Zahler et al., 1998). Despite difference in pathogenicity and vector specificity, three sub species of *B. canis* are not genetically different in their 18S rRNA sequences but differ in ITS1 region indicating the utility of this region for differentiation at subspecies level (Zahler et al., 1998). As for *B. gibsoni* Asian genotype, only one study has been carried out using partial sequence of ITS1 region for phylogenetic placements of different isolates and to study the intraspecific genetic variability between isolates (Bostrom et al., 2008).

As the clinical picture, choice of drug for treatment as well as epidemiology and control strategies of the disease varies depending upon the genotype of the parasite in question, it is imperative to ascertain the species, sub-species/genotype involved in canine babesiosis. However, the epidemiology and clinical importance of *B. gibsoni* infections in India are not well understood. Apart from few sporadic reports on the occurrence of *B. gibsoni* infection in dogs on the basis of microscopic examination of blood smears, no information is available on genetic characterization of *Babesia* spp. of dogs in India (Mitra et al., 1987; Sundar et al., 2004; Varshney et al., 2009). Therefore, in this study, sequence and phylogenetic analyses of both ITS1 region and 18S rRNA gene were carried out for genotyping of small form of canine *Babesia* in India.

2. Materials and methods

2.1. Parasite and isolation of genomic DNA

Genomic DNA was isolated from 300 μl of EDTA anticoagulated whole blood of 15 dogs of 4 north Indian states (4 from district Ludhiana of Punjab state, 1each from district Bareilly and Sidharthnagar of Uttar Pradesh state and 1 from district Dehradoon of Uttarakhand state), 1 east Indian state, West Bengal (4 from district Kolkata, 1 from Siliguri of district Darjeeling and 1 from Malbazar of district Jalpaiguri) and 1 north-east Indian state, Assam (2 isolates), using Genomic DNA Mini Kit (IBI Scientific, USA) following manufacturer's protocol and stored at −20 °C for future use. The Giemsa-stained blood smears of all these dogs were microscopically positive for small form of canine *Babesia* sp.

2.2. PCR amplification and purification

The universal *Babesia* genus specific published primer set, B18S-F and B18S-R was used in this study to amplify the partial sequence of 18S rRNA gene (1665 bp) with minor modifications (Ikadai et al., 2004). The PCR reaction was carried out in 25 μl of 1X PCR green buffer (Thermo Scientific) containing 0.2 μl of Dream *Taq* DNA polymerase, 10 pmol of each primer and 0.2 mM concentration of each deoxynucleotide triphosphate, with 4 μl of template DNA. Amplification was performed using a S1000 thermal cycler (Bio-Rad, USA) under following conditions: initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles (94 °C for 30 s, 55 °C for 2 min and 72 °C for 2 min) and a final extension step at 72 °C for 15 min. For amplification of the partial ITS1 region (254 bp) from *B. gibsoni* Asian genotype, the forward oligonucleotide primer, Bg ITS1-F (5′-ACATTGAAACTTGTCGAGCTGCG-3′) and reverse primer, Bg ITS1-R (5′-AGATCCCGCACCCAGCCAC-3′) were designed using reference sequences from GenBank (accession nos. EU084672, EU084673, EF185062, EF185063, EF185064 and EF185065) and PCR was carried out in 25 μl reaction mixture comprised of 1X PCR green buffer (Thermo Scientific) containing 0.2 μl of Dream *Taq* DNA polymerase, 10 pmol of each primer and 0.5 μl of 10X dNTP with 4 μl of extracted DNA as template. The following cycling conditions were standardized for amplification of the ITS1 region: initial denaturation at 95 °C for 3 min, followed by 34 cycles (95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min) and a final extension step at 72 °C for 15 min. A negative control without template DNA was included in each PCR reaction.

The amplified PCR product was checked by electrophoresis on 1.5% low melting agarose gel and purified using MinElute® Gel Extraction kit (Qiagen, GmbH, Hilden, Germany) following manufacturer's protocol.

2.3. Cloning and sequencing

For cloning of 18S rRNA gene and ITS1 region, the purified PCR products from each sample were separately ligated into pTZ57R/T TA cloning vector (Thermo Scientific, USA) and incubated at 4 °C for overnight. The plasmid DNA constructs were transformed into competent DH5α *Escherichia coli* cells using InsTAclone™ PCR cloning kit (Thermo Scientific, USA) in accordance with manufacturer's protocol. The transformed cells were then plated immediately on pre-warmed LB agar plates supplemented with ampicillin (50 μg/ml), X-gal (30 μg/ml) and IPTG (0.5 mM/ml for the development of blue and white colonies. The positive clones were confirmed by colony PCR using gene specific primers and restriction enzyme digestion of extracted plasmid with *Bam*HI and *Hind*III. The stab cultures of two positive clones per sample harboring the desired gene were custom nucleotide sequenced from the Department of Biochemistry, Delhi University, South Campus, India. The fragments were sequenced at least twice to reduce possibility of sequencing artifacts.

2.4. Sequence analysis

All the newly generated sequences of both 18S rRNA gene and ITS1 region of small form *Babesia* spp. isolates were compared with each other and with published sequences in the nucleotide database in GenBank by BLAST program of the National Center for Biotechnology Information (NCBI: http://blas.ncbi.nlm.nih.gov) and Megalign in DNASTAR in order to analyze sequence variations.

2.5. Phylogenetic analyses

Phylogenetic analyses of *B. gibsoni* or other small piroplasm isolates collected from different geographical regions of India along with other small as well as large form of *Babesia* spp. from different hosts were done independently with the sequences of the 18S rRNA gene and ITS1 region by using the MEGA5, version 5.2 software (Tamura et al., 2011).

A total of thirty-five 18S rRNA gene sequences (1617–1665 bp) including 15 newly generated sequences and 27 ITS1 sequences (254 bp) including 13 newly generated sequences were used in the analyses. Twenty 18S and fourteen ITS1 sequences of various species were obtained from nucleotide database in GenBank with their accession number as well as host and region of origin (Table 1). All the available sequences of 18S rRNA gene of *T. annae* in GenBank database were too small in size (avg. 500 bp) in comparison to the newly generated sequences (1665 bp) and thus *T. annae* was not included in the present study.