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Research paper

Molecular detection and genetic diversity of *Babesia gibsoni* in dogs in India

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ABSTRACT

Babesia gibsoni is a tick borne intraerythrocytic protozoan parasite causing piroplasmosis in dogs and has been predominantly reported in Asian countries, including Japan, Korea, Taiwan, Malaysia, Bangladesh and India. The present communication is the first evidence on the genetic diversity of *B. gibsoni* of dogs in India. Blood samples were collected from 164 dogs in north and northeast states of India and 13 dogs (7.9%) were found positive for *B. gibsoni* infection by microscopic examination of blood smears. Molecular confirmation of these microscopic positive cases for *B. gibsoni* was carried out by 18S rRNA nested-PCR, followed by sequencing. Nested-PCR for the 18S rRNA gene was also carried out on microscopically *B. gibsoni* negative samples that detected a higher percentage of dogs (28.6%) infected with *B. gibsoni*. Genetic diversity in *B. gibsoni* in India was determined by studying *B. gibsoni* thrombospondin-related adhesive protein (*BgTRAP*) gene fragments (855 bp) in 19 isolates from four north and northeast states of India. Phylogenetic analysis of the *BgTRAP* gene revealed that *B. gibsoni* parasite in India and Bangladesh formed a distinct cluster away from other Asian *B. gibsoni* isolates available from Japan, Taiwan and Korea. In addition, tandem repeat analysis of the *BgTRAP* gene clearly showed considerable genetic variation among Indian isolates that was shared by *B. gibsoni* isolates of Bangladesh. These results suggested that *B. gibsoni* parasites in a different genetic clade are endemic in dogs in India and Bangladesh. Further studies are required for better understanding of the genetic diversity of *B. gibsoni* prevalent in India and in its neighbouring countries.

1. Introduction

Babesia gibsoni is a tick borne intraerythrocytic protozoan parasite causing piroplasmosis in dogs globally (Irwin, 2009). Several *Babesia* species/sub-species including *B. gibsoni*, *B. conradae*, *B. canis*, *B. vogeli*, *B. rossi* and *B. vulpes* have been characterized at molecular level. *B. gibsoni* is mainly distributed in Southeast Asia, including India, Bangladesh, Japan, Korea, Taiwan and Malaysia (Inokuma et al., 2004; Miyama et al., 2005; Lee et al., 2009, 2010; Mokhtar et al., 2013, Mandal et al., 2014; Terao et al., 2015). Genetic characterization of canine *B. gibsoni* parasites in some of the southeast Asian countries including Japan, Korea, Taiwan and Malaysia has been done earlier but characterization of *B. gibsoni* in India and Bangladesh was reported only recently (Mandal et al., 2014; Terao et al., 2015). The 18S rRNA gene is widely used to differentiate the species or sub-species of canine piroplasms and nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the 18S rRNA gene has been reported to differentiate *B. vogeli*, *B. canis* and *B. gibsoni* (Jefferies et al., 2007). But, sequence conservation of the 18S rRNA gene and the ITS1 region of *B. gibsoni* isolates was shown from geographically distant regions of the world, including Japan, Taiwan, Australia, USA and India, suggesting a clonal expansion of a single strain by direct dog-to-dog transmission (Bostrom et al., 2008; Irwin, 2009; Mandal et al., 2014). However, genetic diversity of the *B. gibsoni* thrombospondin related adhesive protein (*BgTRAP*) gene has been reported by several authors (Jia et al., 2009; Lee et al., 2010; Terao et al., 2015). TRAP is a type-I transmembrane protein that contains a von Willebrand Factor-like A (vWF-like A) domain, thrombospondin type I (TSR) domain and a short acidic cytoplasmic tail domain (CTD) (Morahan et al., 2009). The genetic diversity of the *BgTRAP* genes was found in regions between TSR and the CTD in Asian isolates (Jia et al., 2009; Lee et al., 2010; Terao et al., 2015). The thrombospondin related adhesive protein was first reported in *Plasmodium* (Trottein et al., 1995) and homologues were subsequently identified in other apicomplexan parasites including *Babesia* (Gaffar et al., 2004). The protein is implicated to function in the parasite's motility and invasion and is expressed in sporozoites and merozoites (Naitza et al., 1998; Morahan et al., 2009). *BgTRAP* gene has been used for studying genetic diversity of *B. gibsoni* parasites (Lee et al., 2010; Terao et al., 2015) and is the only protein available for identifying the genetic variants of *B. gibsoni* as per the present literature. This protein has also been characterized as a sero-diagnostic and vaccine candidate in *B. gibsoni* (Fukumoto et al., 2001; Zhou et al., 2006). Epidemiology and control strategies of the disease including its treatment vary depending upon the genotype of the parasite, thus making it imperative

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to ascertain the species, sub-species/genotype involved in canine babesiosis. However, epidemiology and clinical importance of *B. gibsoni* infections in dogs in India are not well understood. Apart from few sporadic reports on the occurrence of *B. gibsoni* infection in dogs based on microscopic examination of blood smears (Mitra et al., 1987; Sundar et al., 2004; Varshney et al., 2009; Abd Rani et al., 2011), there is a single report on the molecular characterization of *Babesia* spp. of dogs in India (Mandal et al., 2014). Therefore, in this study, an epidemiological survey of *B. gibsoni* infection in dogs was conducted in north and northeast states of India. For identification of the *Babesia* species at molecular level 18S rRNA gene of the parasite was amplified by nested-PCR and sequenced. However, genotyping and phylogenetic analysis of *B. gibsoni* prevalent in India were carried out by PCR amplification and sequencing of the *BgTRAP* gene fragments and by their comparison with other Asian *B. gibsoni* parasites. In addition, tandem repeat analysis of the *BgTRAP* gene fragments was performed to examine genetic variation between Indian isolates and isolates from other countries.

2. Materials and methods

2.1. Blood sample collection and microscopic identification of B. gibsoni

Peripheral blood samples were collected from a total of 164 pet dogs including both males and females of different breeds brought to the referral polyclinic of Indian Veterinary Research Institute, Izatnagar, U.P, India with complications of high fever, anaemia, thrombocytopenia and history of tick infestation. In addition to the dogs screened at the institute's veterinary polyclinic, blood was also collected from the suspected dogs from the states of Uttar Pradesh, Uttarakhand, West Bengal and Assam from January 2014–December 2015 (Fig. 1). Blood was collected from the saphenous vein of these 164 dogs into an EDTA-tube and thin blood smears were prepared and Giemsa stained. Parasites were identified as *B. gibsoni* by microscopic examination of these blood smears. The genomic DNA was extracted from the blood samples of both microscopically *B. gibsoni* positive and negative dogs ($n = 164$) using commercial genomic DNA isolation kit (Qiagen, GmbH, Germany) as per the manufacturer's instructions. The eluted DNA was stored at -20 °C until use.

2.2. PCR amplification of 18S rRNA gene and sequencing

Identification of *B. gibsoni* at molecular level was done by PCR amplification of 18S rRNA gene from the genomic DNA of 164 blood samples including 13 microscopic positive *B. gibsoni* samples. A set of forward and reverse primers (5′-TGGTTGATCCTGCCAGTA-3′ and 5′-CTTCTCCTTCCTTTAAGTGA-3′) was used to amplify a gene fragment of 1665 bp and subsequently nested-PCR was performed on this primary PCR product with internal primers 5′-ATAACCGTGCTAATTGTAGG-3′ and 5′-TGTTATTTCTTGTCACTACC-3′ (specific for *B. gibsoni*) to amplify a product of 308 bp (Jefferies et al., 2007). As template DNA, 1.0–2.0 μl of the eluted DNA for primary PCR and 1.0–2.0 μl of 1:10 diluted primary PCR products were used for nested-PCR, respectively. The primary PCR reaction was carried out in 25 μl volume with initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 2 min with 20 pM of each primer and a final extension at 72 °C for 7 min in thermal cycler (PTC-200, MJ Research, USA). *B. gibsoni* genomic DNA and no DNA template in the reaction vials were used as positive and negative controls, respectively for each PCR. The above reaction conditions were maintained for the nested-PCR except for the primer extension of 1 min. The PCR products were electrophoresed in 0.5% Tris-borate-EDTA agarose gels, stained with ethidium bromide and visualized under UV light. The 18S rDNA gene fragments of 17 isolates were cloned in $p^{TZ57R/T}$ cloning vector (InsTA Clone PCR cloning Kit, Thermo Fisher Scientific, USA) and custom sequenced at University of Delhi, South campus, New Delhi, India. Restriction enzyme analysis with *Hinf*I was carried out with 18S rRNA gene fragments of the remaining 30 samples.

2.3. Genetic characterization of thrombospondin related adhesive protein gene

Total RNA was isolated from two *B. gibsoni* isolates (*Bg*Ind-01 and *Bg*Ind-02), collected from Uttar Pradesh, by Trizol method (Invitrogen, Thermo Fisher Scientific, USA) and cDNA synthesized by reverse transcriptase-PCR using oligo-dT primer and M-MLV reverse transcriptase enzyme (MBI Fermentas, USA) following standard cDNA synthesis protocol. Thrombospondin related adhesive protein gene of the above two isolates was PCR amplified using cDNA as a template and TRAP gene specific primers 5′-ATGGCGAGGATGAAGGGTGTAA-3′ and 5′-TCAG GCCCACATGGCTTCATTG-3′. The PCR conditions were same as described for 18S rRNA gene except for annealing at 60 °C. The PCR products of the full length TRAP cDNA of these two isolates were agarose gel purified, cloned in a p^{DRIVE} TA cloning vector (Qiagen, GmbH, Germany) and custom sequenced at University of Delhi, South campus, New Delhi, India.

For genotyping and phylogenetic analysis of *B. gibsoni* parasites in India, the *BgTRAP* gene fragments of 19 isolates (*Bg*Ind-01 to *Bg*Ind-19) were also PCR amplified from the genomic DNA. Out of these 19 isolates PCR amplified for *BgTRAP* DNA, 17 were already confirmed as *B. gibsoni* with 18S rRNA based nested-PCR and sequencing. Primers were selected based on conserved sequences of 9 available *BgTRAP* genes in GenBank, including TWN2 (GU447229), TWN3 (GU447230), TWN4 (GU447231), TWN5 (GU447232) and TWN6 (JN247443) from Taiwan, Honshu1 (AB478341), Okinawa1 (AB478346) and Shikoku1 (AB478348) from Japan and Jeju1 (AB478343) from Korea. Forward 5′-ACCCAGCGAATACAATGCACCACA-3′ and reverse 5′-AGCCCTGCAA AACCGGCAAGTATG-3′ primers were designed to amplify 855 bp region with variable nucleotide repeats. The PCR reactions were carried out in 25 μl reaction mixture with initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min and final extension at 72 °C for 7 min. Each amplicon was purified using QIAquick Gel Extraction Kit (QIAGEN, GmbH, Germany), cloned in p^{DRIVE} cloning vector and sequenced. The sequence of each clone was generated in triplicate.

2.4. Phylogenetic analysis

A total of 47 and 4 available TRAP gene sequences of *B. gibsoni* and *B. bovis*, respectively were considered for the phylogenetic analysis. The 47 *B. gibsoni* isolates included 19 newly generated sequences (accession nos.: KT750254, KT750255 and KU517673–KU517689) in the study. Phylogenetic analysis was done using Mega 6.0 with the Neighbour Joining method and bootstrap values determined for 1000 replicates of the data sets (Tamura et al., 2013). The Jones–Taylor–Thornton (JTT) Model was found to be the substitution model of choice using Mega 6.0. *Neospora caninum* MIC2 homologue (AF061273) was used as outgroup to root the tree.

The basic genetic variability parameters were also calculated using Mega 6.0 by grouping all the isolates into three groups. Group 01 consisted of India and Bangladesh isolates, group 2 included all the South-east Asian isolates and group 3 constituted the outgroup which included *B. bovis* isolates and *Neospora caninum*. Simpson's index of diversity (Hunter and Gaston, 1988) was applied to measure the discriminatory power of *BgTRAP*, used as a molecular marker.

2.5. Tandem repeat identification

Tandem repeat DNA sequences in *BgTRAP* gene fragments were detected using DNA STAR and Gene Tool software.