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Short communication

Babesia bigemina infection in yak (*Poephagus grunniens* L.): Molecular detection and characterization

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ABSTRACT

Yaks contribute significantly in the Himalayan high land economy. Specific information on prevalence of babesiosis in yaks is lacking. A fast and reliable PCR assay targeting *Babesia bigemina* small subunit ribosomal RNA sequence (SS rRNA) was laboratory standardized for molecular detection of *B. bigemina* in yaks. Restriction digestion of the PCR amplified 675 bp target sequence with *Vsp* I confirmed the prevalent species of *Babesia* as *B. bigemina*. Nucleotide sequencing and phylogenetic analysis of PCR amplified 675 bp SS rRNA sequence revealed a close genetic relationship with other bovine isolates of *B. bigemina*. A PCR based survey involving 94 blood samples of yak from the National Research Centre on Yak, Dirang, Arunachal Pradesh detected infection in 5.32% of yak blood samples, which was significantly higher in comparison to microscope based detection of infection in 2.13% blood smears. This is the first report on sensitive PCR based detection of *B. bigemina* infection in yaks and PCR-RFLP and nucleotide sequence analysis based molecular characterization of the *B. bigemina* isolated from yaks.

1. Introduction

Yaks (*Poephagus grunniens* L.) are reared under transhumance system with vertical migration in high altitudes of Himalayan range. Presently, there are around 71,000 yaks in India which are spread across the Ladakh region of Jammu and Kashmir, West Kameng and Tawang districts of Arunachal Pradesh, North and East districts of Sikkim and Lahul, Spiti and Kinaur districts of Himachal Pradesh (Ramesha et al., 2006). The yak contributes significantly to the high land economy and nutritional security of the tribal population living in the difficult terrains of Himalayas. During summer the animals are moved to higher altitude of more than 4500 m above mean sea level (msl) and brought back during winter. The sharing of common grazing lands with cattle during winter (October–April) predisposes them to several vector born infections including babesiosis. Though the tick vector for *Babesia bigemina*, *Rhipicephalus* (*Boophilus*) *microplus*, is abundant at Arunachal Pradesh, information on occurrence of babesiosis in yaks is scanty. *Babesia* infection in yaks has been reported from China and Nepal (Yu et al., 1989; Graves et al., 1975), however, the infection was not identified at species level. So far as prevalence of *Babesia* infection in yak is concerned, no convincing report is available worldwide (Uilenberg, 2006). The present study was, therefore,

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undertaken to assess the prevalence of *Babesia* infection in yaks, being maintained at the Institute farm of National Research Center on Yak, Dirang using a sensitive PCR targeting the small-subunit rRNA (SS rRNA) gene of *B. bigemina*. Further, identity of the infection associated species of *Babesia* was confirmed by sequence analysis as well as by studying its restriction profile of the amplified fragment.

2. Materials and methods

2.1. Yaks

A total of 94 adult yaks of either sex, maintained at the Institute farm, National Research Centre on Yak, Dirang were randomly selected for the study.

2.2. Extraction of genomic DNA of Babesia sp.

About 20 ml of blood was collected aseptically in heparinized tubes by puncturing the jugular vein from a clinically infected adult female yak (no. 218) with clinical signs of high fever, anorexia and haemoglobinuria. Thin blood smears were prepared on spot for microscopical examination.

Leukocyte-free, piroplasm-rich erythrocytes were isolated (Ray et al., 1998) for extraction of the genomic DNA using QIAamp DNA Blood mini kit (Qiagen, Germany).

2.3. Oligonucleotides primers

A pair of PCR primers, described earlier by Ananyutthawongese et al. (1999), specific for a 675 bp fragment of *Babesia bigemina* small subunit ribosomal RNA (SS rRNA) gene between nucleotide positions 814 and 1488, was custom synthesized and used in the present study. The nucleotide sequence of the forward and the reverse primers were Bg3F – 5′-TAG TTG TAT TTC AGC CTC GCG – 3′ and Bg4R – 5′-AAC ATC CAA GCA GCT AGT TAG-3′, respectively. A specific inclusion of nucleotide 'G' instead of 'H' was made at position 17 in the reverse primer.

2.4. Polymerase chain reaction

The PCR assay was laboratory standardized in 25 μl reaction volume containing 10 ng of genomic DNA, 10 pmol of each primer (Bg3F and Bg4R), 200 μmol of each dNTP and 1.0 U *Taq* DNA polymerase (MBI Fermentas Life Sciences, Lithuania) in 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% w/v gelatin. The final volume was made to 25 μl with nuclease-free water (MBI Fermentas Life Sciences, Lithuania). The reactions were performed on a thermocycler (Gene Amp PCR System – 2700, Perkin Elmer, USA) with a preheated lid. The cycling conditions were standardized as an initial denaturation of strands for 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing of primers at 50 °C for 45 s and extension of strands at 72 °C for 45 s. A final extension of the synthesized strands was given at 72 °C for 10 min. The PCR amplified product was electrophoressed on an ethidium bromide stained 1.5% agarose gel and visualized in a transilluminator under UV light.

2.5. RE analysis of PCR product

The PCR amplified product was digested with *Vsp* I at 37 °C for 3 h. The digested product was electrophoressed in an ethidium bromide stained 1.5% agarose gel and visualized on a transilluminator under UV light.

2.6. Cloning and nucleotide sequencing of PCR amplified product

The 675 bp PCR amplified product was eluted from the agarose gel using gel extraction kit (Qiagen, Germany). The purified SS rRNA amplicon was cloned in pTZ57R/T cloning vector (Fermentas, USA) following standard protocol. Competent *Escherichia coli* (DH5α) cells were transformed with the recombinant plasmid construct and plated on Luria–Bertani agar medium containing ampicillin (50 μg ml^{-1}), X-gal (80 μg ml^{-1}) and IPTG (50 μmol). The recombinant clones were selected initially by blue–white colony screening method. A white colony was picked up and sub-cultured for 8 h in LB medium for extraction of recombinant plasmid DNA. The plasmid DNA was extracted using mini-prep plasmid DNA isolation kit (Fermentas, USA) and the insert was released by restriction digestion of the plasmid with *Xba* I and *Pst* I.

The white colonies were further tested by colony PCR to confirm the presence of specific insert. One such confirmed positive clone was selected and custom sequenced for nucleotides from the Department of Biochemistry, South Campus, Delhi University, New Delhi. Sequence information was submitted for accession number in primary bioinformatics web servers.

2.7. Sequence alignment and phylogenetic analysis

The sequence analysis and similarity searches were performed with the basic local alignment search tool after converting to FASTA format on the Internet (website: http://www.ncbi.nlm.nih.gov) using default matrix. Phylogenies were constructed by neighbor-joining using Kimura 2-parameter model using homogeneous pattern among lineages and tested by bootstrap with 1000 replicates, using MEGA version 4.0 (Tamura et al., 2007). Pair wise distance of *Babesia* sp. infective for cattle from GenBank and Indian yak isolate of *Babesia* sp. was calculated using Kimura 2-parameter model using homogeneous pattern among lineages and tested by bootstrap with 1000 replicates, using MEGA version 4.0 (Tamura et al., 2007).

2.8. Specificity of PCR assay

The specificity of the primers used in the present study was cross checked using genomic DNA from other common haemoparasites of bovids, *viz*. *Theileria annulata*, *Trypanosoma evansi* and *Anaplasma marginale* (available at IVRI, Izatnagar, UP) and yak genomic DNA isolated from