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Molecular characterization and sequence phylogenetic analysis of *Babesia bigemina* cattle isolate from Mathura based on 18S ribosomal DNA gene

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Babesiosis is an economically important disease in milch animals accounting for significant mortality and morbidity rates alongside decrease in milk production. A loss of about 51 litre milk in a month from a cross-bred cow has been estimated due to *Babesia bigemina* infection (Laha *et al*. 2012a). The infection causes high rise of temperature, haemoglobinurea, anaemia, anorexia and diarrhoea in cattle (Laha *et al*. 2012b). The pathogenesis of the disease varies with the various isolates found across the world. The study of literature on phylogenetic relation of *B. bigemina* of cattle isolates revealed that scanty study on cattle has been done in India. The present communication deals with molecular characterization of 18s ribosomal subunit of *B. bigemina* Mathura isolate, alongside its phylogenetic relationship with other *B. bigemina* isolates from India and across the world.

Sample collection and DNA isolation from blood: Blood samples (1 ml aliquot) were collected in clean sterile vacutainer, coated with EDTA anticoagulant, from the jugular vein of earlier confirmed cross-bred cow (through microscopic observation of blood smears). DNA was isolated from blood using standard phenol chloroform method with minor modifications (Sudan *et al.* 2015). Briefly, 200 µl of blood was added into a 1.5 ml tube containing 500 µl of denaturing solution (guanidinium thiocyanate) and vortexed at high speed for 5 min. Total volumes of 150 µl of chloroform and 150 µl of phenol were added and vortexed at high speed for 5–10 min. The microtube was then centrifuged at 15,493g for 5 min, and the supernatant was collected. Another 150 µl of chloroform and 150 µl of phenol were added to the supernatant, vortexed for 5–10 min, and centrifuged at 15,493g for 5 min. A volume of 400 µl of supernatant was collected, and 1 ml of absolute ethanol was added into a 1.5 ml tube, and the sample was left to precipitate at -20°C overnight. After 10 min of centrifugation at 15,493g, the pellet was washed with 75% alcohol twice. The pellet was finally air dried before resuspension into 50 µl of TE buffer (Tris, EDTA). Thus, the final prepared sample was concentrated 2:1 compared to the initial blood sample.

Primer selection and PCR amplification: Oligonucleotide primers targeting the 18s ribosomal RNA (18s Babes F/R) were self synthesized using the available sequences in the pubmed (Accession No. KF606863). The PCR reactions were set up into 25 µl volume containing 12.5 µl PCR Master Mix (0.05U/µl Taq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 1.5 µl of each primer (18s Babes F/R; 20 pmol of each primer) and 5 µl of the extracted DNA template. The total volume of the PCR mix was made up to 25 µl using nuclease-free water. Details for primer design including position of nucleotides, nucleotide sequences, expected PCR products and thermal cycling profile are shown in Table 1. The amplified amplicon was analyzed by agarose gel electrophoresis in 1.5% agarose gel.

Molecular cloning and characterization of 18s ribosomal gene of B. bigemina*:* The amplified product of 18s ribosomal gene was purified using Gel Purification Kit (Puregene) following manufacturer's protocol. Thereafter, competent *Escherichia coli* DH5α cells (Puregene) were prepared following the standard calcium chloride treatment method (Sambrook *et al.* 1989). Ligation reaction for cloning of amplified 18s ribosomal gene product into TA cloning vector (InsTA, Fermentas) as well as transformation of DH5α cells was carried out as per manufacturer's protocol. The positive clones were identified by blue-white colony screening method. Further confirmation was done by colony PCR following standard protocol (Sambrook *et al.* 1989). The colony PCR amplified products were visualized in the ethidium bromide incorporated agarose gel following agarose electrophoresis. A subculture of positive clone harboring the desired gene was subcultured into 100 ml of LB broth (Himedia) for overnight growth and thereafter plasmid was isolated using Plasmid Purification Kit (Banglore Genei) following manufacturer's protocol. Thereafter the plasmids were sent for custom

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Table 1. Primer sequence, PCR conditions as well as expected amplicon size

Primer		Primer sequence		Amplicon size
18s Bab F		5' CCCAATCCTGACACAGGGAG3'		446 bp
18s Bab R		5' GCAAATGCTTTCGCAGTGGT3'		
		Thermocyclic profile		
Initial denaturation	Denaturation	Hybridization	Extension	Termination
95°C, 300 sec	94°C, 45 sec	57°C, 60 sec	72°C, 60 sec	72°C, 600 sec
		× 35 cycles		

sequencing. The sequence information received was analyzed using DNASTAR and GeneTool softwares. Based on the sequence, the structure of 18s ribosomal gene was modulated using online softwares.

PCR amplification, molecular cloning and molecular characterization of the 18s ribosomal gene of B. bigemina*:* 18s ribosomal gene of *B. bigemina* gene was amplified from the genomic DNA of infected crossbred cattle using the specific forward and reverse primers. The amplicons were resolved as a single band of 446 bp (Fig. 1A). It was further purified for ligation in TA cloning vector. The selection of positive colonies was performed by colony PCR using the specific primers. The results of colony PCR (Fig. 1B) were checked by agarose gel electrophoresis.

Data analysis: BLAST analysis confirmed the sequence was from 18S ribosomal RNA gene of *B. bigemina*. The sequence was submitted to GenBank database under the accession number KY038944. Analyses revealed that this sequence had 100% identity with two sequences from North Eastern part of India, viz. KF606863 and KF606866, respectively (Laha *et al.* 2015) while with other two isolates from same region it showed slightly lesser homologies, viz. KF606864 and KF606865, respectively (Laha *et al.* 2015). Besides, it had 99.1–99.9 and 99.0–99.7% nucleotide identity with *B. bigemina* from Argentina and Kenya, respectively, thereby attributing close phylogenetic relation with isolates from Argentina and Kenya (Fig. 2). Distant relationship was observed with f cognate gene sequences from China.

The conserved 18S rRNA gene sequence have been used

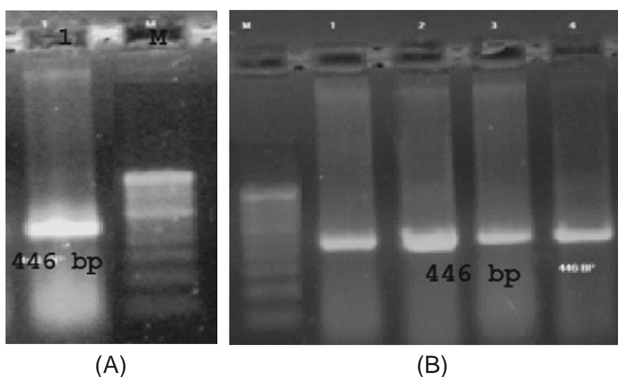

Fig. 1. PCR amplification of 18S ribosomal DNA of *B. bigemina* from blood of cattle (A); and colony PCR of recombinant clones (B).

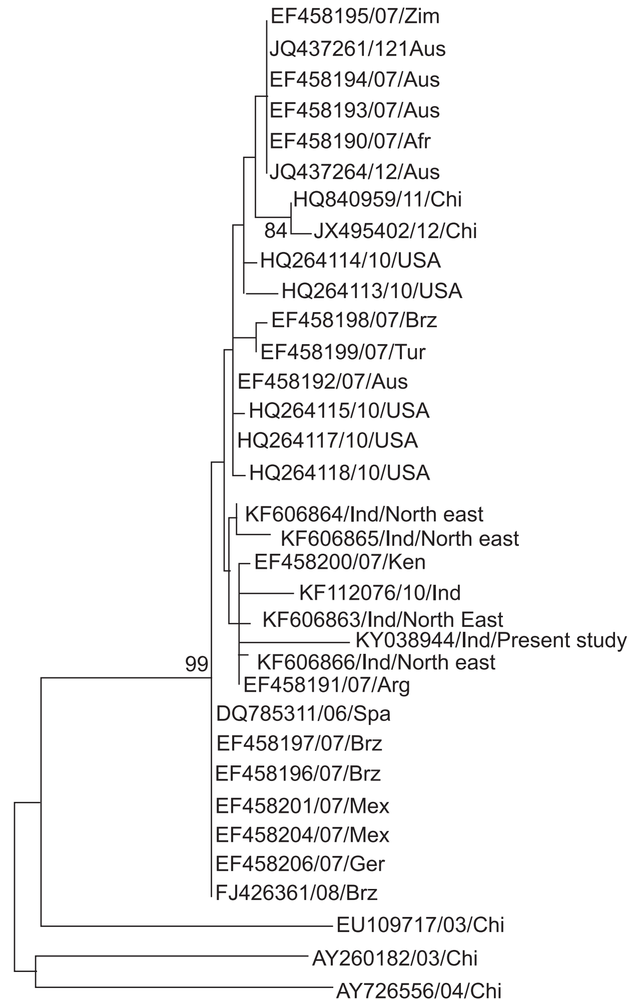

Fig. 2. Maximum likelihood tree depicting phylogenetic relationship of *B. bigemina* organisms at 18S rRNA gene sequence level. Brz, Brazil; Chi, China; Ger, Germany; Mex, Mexico; Spa, Spain; Ken, Kenya; Arg, Argentina; Ind, India; Aus, Australia; Tur, Turkey; Afr, Africa; and Zim, Zimbabwe.

earlier for specific identification and classification of several species of piroplasms (Yin *et al.* 2004). In the present study, the 18S ribosomal RNA gene sequences of Mathura isolate of *B. bigemina* isolates had cent percent homology with two sequences from North Eastern part of India (Laha *et al.* 2015) alongside 99.1–99.9% homology with other two sequences from the same region and 99.0–99.7% nucleotide identities with isolates from Argentina and Kenya, respectively. Thus, a close genetic relatedness was observed

between *B. bigemina* isolates from this region of India with Argentina and Kenya. Isolates of *B. bigemina* from yak in Arunachal Pradesh has been shown to have a close relation with isolates from China, Mexico, Australia and Zimbabwe (Saravanan *et al*. 2013), although the target sequence for estimating the phylogenetic relation was different. The present study on phylogenetic relation of *B. bigemina* of cattle isolate of semi arid India with other isolates throughout the world may be considered as significant from phylogenetic point of view. Thus, the present isolate was more close to Argentina and Kenya isolates than the isolates from China, Mexico, Australia and Zimbabwe.

SUMMARY

The present communication deals with the molecular characterization and phylogenetic relationship of 18S ribosomal DNA of *B. bigemina* from cattle of Mathura with other isolates across the globe. The 446 bp product PCR amplified and was cloned into TA vector. Upon sequencing, the nucleotide homology was established and phylogenetic relationship was calculated with other *B. bigemina* isolates from India and across the world. The present isolate was more close to Argentina and Kenya isolates than the isolates from China, Mexico, Australia and Zimbabwe. The finding is important from the molecular phylogeny point of view.

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