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Comparative detection efficacy of primers targeting *SpeI-AvaI* restriction fragment and small subunit ribosomal RNA gene of *Babesia bigemina*

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ABSTRACT

The objective of the study is to evaluate the comparative detection efficacy of primers targeting *SpeI-AvaI* restriction fragment and small subunit ribosomal RNA (SSU rRNA) gene of *Babesia bigemina* by employing conventional polymerase chain reaction (PCR) on 783 animals (296 cattle and 487 buffaloes) of low lying (bet) area of Punjab. The detection rate of *SpeI-AvaI* and SSU rRNA PCR assays was 3.96% (31/783), and 6.64% (52/783), respectively. Among cattle and buffaloes, prevalence of *B. bigemina* was higher (P<0.01) in cattle by both the primers. The sensitivity and specificity of SSU rRNA PCR as compared to *SpeI-AvaI* restriction fragment PCR was 100% and 97.2%, respectively. The blast analysis of the nucleotides of the sequenced amplicons of Ludhiana isolates of *SpeI-AvaI* and SSU rRNA PCR assay of *B. bigemina* showed 83 and 100% similarity with available sequence in Genbank. The analysis of evolutionary divergence revealed that range of divergence was lying between 0.000 to 0.011 between SSU rRNA sequence with the other sequences of *B. bigemina* as well as *Babesia* species. To conclude, the primers targeting SSU rRNA gene are a better tool for amplification of the *B. bigemina*.

Keywords: *Babesia bigemina*, PCR, *SpeI-AvaI* restriction fragment, SSU rRNA gene

Babesia bigemina, an apicomplexan haemoprotozoa is the major causative agent of bovine babesiosis in the tropical and subtropical countries worldwide (Kaur *et al.* 2016). In India, the estimated economic impact of babesiosis in bovines is 57.2 million US dollars per annum (Narladkar 2018). Globally, 1.2 billion cattle population at the risk for *Babesia* species infection, however the true status in buffaloes has not been estimated (Terkawi *et al.* 2011). The direct pathogenic effect of *Babesia* infection is due to lysis of RBC by emerging parasites besides the other mechanisms including clumping of RBC, renal dysfunction, and release of vasoactive substances that contribute to the signs of fever, anorexia, haemglobinuria, and death in untreated cases (Bal *et al.* 2016, Sharma *et al*. 2016, Kaur *et al.* 2021).

Acute cases with predominant clinical signs are routinely diagnosed by microscopic examination of blood or organ smears stained with Giemsa (Bose *et al.* 1995), but the major inadequacy of this technique makes it obscure to detect the latent infection with low parasitaemia and the carrier animals (Terkawi *et al.* 2011a). On the other hand, abundant immunodiagnostic tests are employed for the detection of antigen or antibody of *Babesia* species in livestock animals with the constraint of less specificity, cross reactivity among different species, and incapability to distinguish between the prior exposure and existing infection (Alvarez *et al.* 2019). The above mentioned limitations of conventional microscopy and immunological tests have alerted the researchers towards the investigation of gene amplification methods by employing PCR with high sensitivity and specificity. Since the first description of PCR targeting the restriction fragment of *B. bigemina* (Figuorea *et al.* 1992), numerous published data available on the application of the PCR for detection of *B. bigemina* in host and tick vector worldwide targeting the diverse genes; ribosomal DNA (rDNA) (Chaudhry *et al.* 2010, Kumar *et al.* 2022), apical membrane antigen (AMA-1) (Sivakumar *et al.* 2012, Ganguly *et al.* 2017), putative aspartic proteinase babesipsin genes (Martins *et al.* 2008), rhoptry-associated protein 1 (rap-1) (Mtshali and Mtshali 2013) and putative methyltransferase gene (Bohaliga *et al.* 2018). Among the different partial or completely studied genes, the rDNA genes possess the better PCR diagnostic efficacy as of invariably multicopy and hence increase the sensitivity over assays using single copy sequences (Martins *et al.* 2008). Looking at the research gaps, the present study focused on to evaluate the efficacy of two PCR by utilizing the published primers targeting the *SpeI-AvaI* restriction fragment and SSU rRNA gene of *B. bigemina.*

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MATERIALS AND METHODS

Samples collection: Blood sample (5 ml) from jugular vein of 783 dairy animals (296 cattle and 487 buffaloes) of different age groups from bet area (low lying areas adjoining the water bodies) of Punjab were collected aseptically in ethylene diamine tetracetic acid (EDTA) coated vials and stored at –20°C for extraction of DNA.

DNA template preparation: Genomic DNA extraction from the whole blood was as per the given protocol of HiMedia HiPurA Blood Genomic DNA MiniPrep Purification Spin Kit (HiMedia Laboratories, India). The DNA extracted from a three day old neonatal calf blood served as negative template. The already available DNA samples of *Trypanosoma evansi*, *Theilerila annulata* and *Anaplasma marginale* in laboratory were used to check the specificity of primers. The blood sample of cattle positive for *B. bigemina* by Giemsa thin blood smear was used as positive DNA template. The purity and amount of extracted DNA was calculated at the ratio of OD260 to OD280 and OD260, respectively.

PCR assay optimization: Primers sequence and amplification conditions for *SpeI-AvaI* and SSU rRNA PCR is given in the Table 1. Optimization of *SpeI-AvaI* PCR condition with minor modifications was done according to Oliveira-Sequeira *et al.* (2005). PCR reaction mixture (25µL) contained 2.5µL of 10X Hotstart PCR Buffer, 1.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM (each) deoxynucleoside triphosphate (dNTPs), 2.5 U of Maxima Hotstart *Taq* DNA polymerase (Fermantas), 1 µL primer each (10 pmol), 13µL of nuclease free water (NFW) and 5 µL of template DNA. The amplification was done in thermocycler (Applied Biosystems, USA).

The SSU rRNA PCR assay reaction volume (25 µL) contained 12.5µL of KAPA 2G ™ Fast HotStart ReadyMix (2X containing KAPA 2G fast hot start DNA polymerase, KAPA 2G fast hot start PCR buffer, 0.2 mM dNTP each, 1.5mM $MgCl_2$), 1.5 µL of 10 pmol Bg3/Bg4 primers, 4.5 µL of NFW and 5 µL of DNA template. The reaction was carried out in thermocycler (Eppendrof, Germany). Amplified PCR products were visualized under UV transilluminator after electrophoresis in 1% agarose gel to detect *SpeI-AvaI* (278 bp) and SSU rRNA (689 bp) gene.

Analysis of nucleotide sequence: Amplified PCR products were sequenced from Xcelris Genomics, Ahmedabad, India. The obtained SSU rRNA and *SpeI-AvaI* sequences were subjected to Basic Local Alignment Search Tool (BLASTn) (Altschul *et al.* 1990). The 94 homologous sequences of SSU rRNA PCR showing E-value less than 10^{-5} were downloaded in FASTA format. Multiple alignments of the sequences using Clustal Omega were made (Thompson *et al.* 1994, Sievers *et al.* 2011). Phylogenetic and molecular evolutionary investigation using MEGA version 6 was carried out (Tamura *et al.* 2013). The phylogenetic tree was constructed with 1000 bootstrap resampling, using maximum likelihood method. The evolutionary divergence between sequences was estimated using the maximum composite likelihood model (Tamura *et al.* 2004).

Statistical analysis: Chi-square test was employed to access the association of *B. bigemina* detcted by *SpeI-AvaI* restriction fragment and SSU rRNA gene among cattle and buffaloes using SPSS 16.0 software. To evaluate the agreement between *SpeI-AvaI* restriction fragment and SSU rRNA gene based PCR assay Kappa value test was employed using Win Episcope 2.0 software.

RESULTS AND DISCUSSION

Specificity of the primers: The *SpeI-AvaI* and SSU rRNA PCR assays amplified 278 bp and 689 bp, respectively specific to *B. bigemina*, while non target DNA samples

Table 1. Details of the primers, conditions of *SpeI-AvaI* and SSU rRNA PCR assays

PCR	Primer sequence	PCR conditions	References
SpeI-AvaI	IA:5'CATCTAATTTCTCTC CATACCCCTCC3'	95°C for 5 min 95° C for 1 min 57°C for 1 min 73° C for 1.5 min } 35 cycles 73°C for 10 min	Figueroa *et al.* (1992)
	IB:5'CCTCGGCTTCAA CTCTGATGCCAAAG3'		
SSU rRNA	Bg3:TAGTTGTATTTCAGCCTCGCG3'	95°C for 5 min 95° C for 1 min 57°C for 1 min 73° C for 1.5 min } 35 cycles 73°C for 10 min	Ellis *et al.* (1992)
	Bg4:AACATCCAAGCAGCTAHTTTAG3'		

Table 2. Species wise analysis of *Babesia bigemina* by *SpeI-AvaI* and SSU rRNA PCR

Species	Total samples examined	*SpeI-AvaI* PCR		SSU rRNA PCR	
		Positive	Percentage	Positive	Percentage
Cattle	296	26	8.78	43	14.52
Buffaloes	487	5	1.02	9	1.84
Total	783	31	3.96	52	6.64
χ^2		29.134*		47.443*	

* Indicates difference at 1% level of significance.

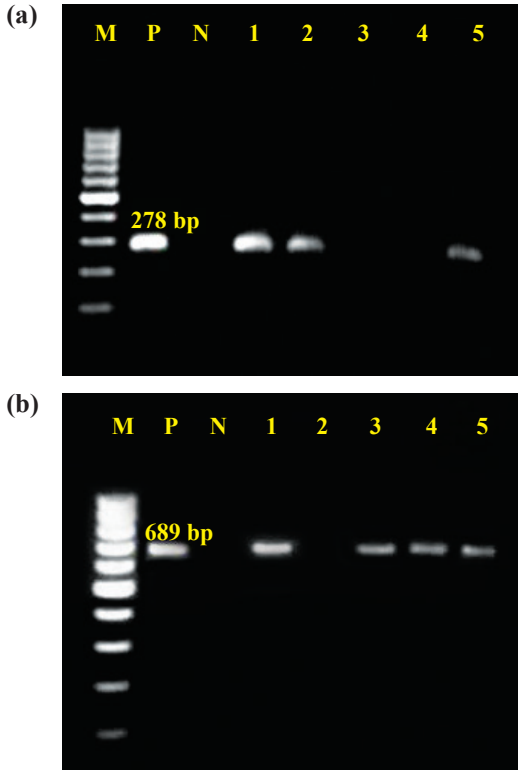

Fig. 1. **(a)** Agarose gel (1.5%) electrophoresis showing amplified DNA (278bp) for *SpeI-AvaI* restriction fragment *B. bigemina* in field samples. M= marker 100 bp; P: Positive control DNA; N: Non template control DNA; 1-5 Tested field samples DNA. **(b)** Agarose gel (1.5%) electrophoresis showing amplified DNA (689 bp) of SSU rRNA gene of *B. bigemina*. M: marker 100 bp; P: Positive control DNA; N: Negative control DNA; 1-5 tested field samples DNA.

of other haemoparasite of bovines did not showed any amplification (Supplementary Fig. 1 a, b). Application of *SpeI-AvaI* and SSU rRNA PCR protocols on 783 field samples (Fig. 1 a, b) revealed a detection of 3.96% (31/783), and 6.64% (52/783), respectively for *B. bigemina* (Table 2). The incidence of *B. bigemina* was significantly (P<0.01) higher in cattle than buffaloes by both *SpeI-AvaI* PCR and SSU rRNA PCR assays (Table 2). The detection rate in cattle was high by SSU rRNA (14.52%) as compared to *SpeI-AvaI* PCR assay (8.78%). Both the PCR assays showed that cattle are more susceptible to babesiosis than buffaloes and it is in conformity with Chaudhri *et al.* (2013) and Kaur *et al.* (2021). The SSU rRNA PCR assay detected all 31 samples positive by *SpeI-AvaI* PCR assay. The sensitivity and specificity of SSU rRNA PCR was 100% and 97.2%, respectively (Table 3). There was substantial agreement (Kappa value: 0.734) between the *SpeI-AvaI* PCR and SSU rRNA PCR used for evaluating the prevalence of *B. bigemina*. The lower detection rate by *SpeI-AvaI* PCR assay may be due to low level of DNA in 21 cases. Concordance to this finding, no amplification in 117 samples of cattle from Mozambique with primary PCR targeting the *SpeI-AvaI* restriction fragment observed and that explained by most probably due to low concentration of parasitic DNA (Martins *et al.* 2008).

The nucleotide sequences of *SpeI-AvaI* PCR amplicon of Punjab isolates (Accession number AB922127) showed 83% identity to *B. bigemina* sequence of GenBank (Accession number S45366) (Supplementary Fig. 2). In conformity to this finding, Portuguese isolates of *B. bigemina* revealed 82% identity with the published *SpeI-AvaI B. bigemina* restriction fragment (Figueroa *et al.* 1992). The scrutiny of the literature showed that *SpeI-AvaI* PCR is based on the unspecified DNA part of the parasites (Lew and Jorgensen 2005, Guerrero *et al.* 2007, Sivakumar *et al*. 2012).

The phylogenetic lineage of the SSU rRNA sequenced amplicon of Ludhiana, India (Accession number AB922126) was 100% with Accession numbers EF458206, X59605 and EF458199 of the NCBI nucleotide database. The multiple sequence alignment clearly demonstrated the conserved nature of SSU rRNA gene except for a few mismatches among the input sequences. The phylogenetic tree clustered the *B. bigemina* sequences together in one node (along with *Babesia* sp., *B. crassa*), while the other node harbored the *B. divergens* and *B. odocoilei* sequences (Supplementary Fig. 3). This result indicates that SSU rRNA sequence of *B. bigemina* is conserved among *Babesia*. The analysis of evolutionary divergence also revealed that range of divergence was lying between 0.000 to 0.011 between SSU rRNA sequence obtained in the present study and the other sequences of *B. bigemina* as well as *Babesia* sp. (China). However, the estimate of evolutionary divergence was higher between the sequence of the present study and those of *Babesia* sp., *B. divergens* and *B. odocoilei* (from 0.015 to 0.017). The primers used in SSU rRNA PCR assay were specific for *B. bigemina*, not for other related species of *Babesia*. The limitation of the present study is that the sensitivity of the primers could not be ascertained due to non availability of the cultured DNA template of *B. bigemina*.

The multiple sequence alignment of the amplicon of SSU rRNA with the sequences from a variety of *Babesia* species revealed that the primer binding sites were specific for *B. bigemina* and did not show the match with other species. The forward primer used in SSU rRNA PCR

Table 3. Sensitivity and specificity of SSU rRNA with *SpeI-AvaI* PCR assay of *B. bigemina*

SpeI-AvaI PCR				Total	Sensitivity (%)	Specificity (%)
SSU rRNA PCR		Positive	Negative			
	Positive	31	21	52	100	97.2
	Negative	0	731	731		
Total		31	752	783		

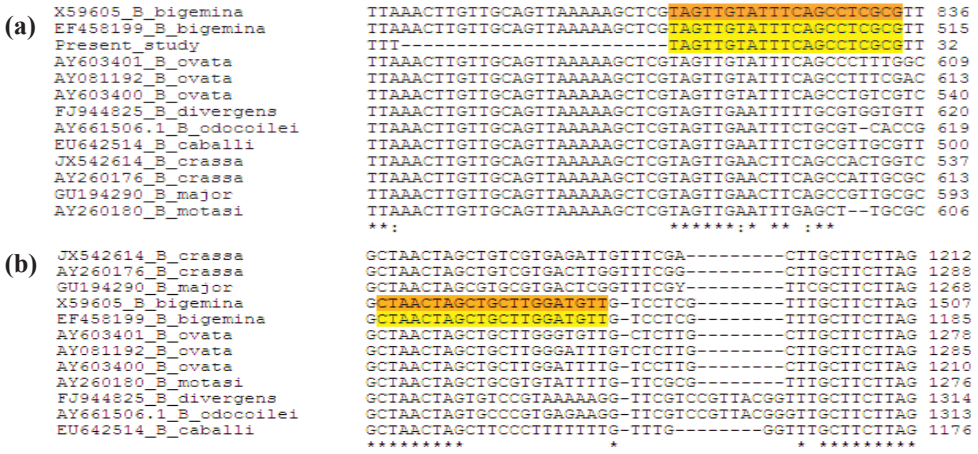

Fig. 2 (a) The multiple sequence alignments of various geographical isolates of *B. bigemina* showing the forward primer binding sites of SSU rRNA gene used in the present study. (b) The multiple sequence alignments of various geographical isolates of *B. bigemina* showing the reversed primer binding sites of SSU rRNA gene without the sequence of the present study due to mismatch one nucleotide

assay was present only in the sequences of *B. bigemina* (Fig. 2a). The reverse primer in present study sequence could not align due to mismatch of one nucleotide in SSU rRNA sequence (Fig. 2b).

The application of SSU rRNA gene has been explored for the diagnosis of bovine babesiosis in a number of published reports worldwide (Linhares *et al.* 2002, Durrani and Kamal 2008, Shams *et al.* 2013, Ganguly *et al.* 2017, Kumar *et al.* 2022) may be due to the availability of DNA sequences in molecular databases (Criado *et al.* 2006) or being multicopy gene that increases the sensitivity over the assays having single copy sequences. Martins *et al.* (2008) developed a novel hot start PCR targeting babesipsin, the putative gene of an undescribed aspartic protease present in *B. bovis* and *B. bigemina* of cattle from Mozambique. The comparison of semi-nested hot start PCR targeting the babesipsin with the nPCR targeting *SpeI-AvaI* restriction fragment gene of *B. bigemina* (Figueroa *et al.* 1993) showed that babesipsin semi-nested hot start PCR was more sensitive. Martins *et al. (*2008) indicated that the sensitivity of hot start PCR can be further increased if primer targeting the 18s rRNA a multicopy gene, is used instead of the apparent single copy babesipsin gene. Present study also accomplished that SSU rRNA PCR assay is more sensitive than *SpeI-AvaI* PCR assay, and being a housekeeping gene, could be better tool for diagnosis of the *B. bigemina* infections in bovines.

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