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Development of a duplex PCR assay for simultaneous detection of *Babesia bigemina* and *Theileria annulata* infections in cattle

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ABSTRACT

Bovine babesiosis and theileriosis are fatal tick borne haemoparasites of vertebrates imposing serious constraints on health and productivity of livestock. Additionally, the recovered animals become persistent carriers and play a significant role in disease epidemiology. The present investigation describes the development and evaluation of duplex PCR assay for simultaneous detection of *Babesia bigemina* (*B. bigemina*) and *Theileria annulata* (*T. annulata*) in cattle. Following *in silico* analysis for candidate target genes representing each of the haemoparasites, an optimised duplex PCR assay was established using two sets of primers, ssurRNA and cytob1 for genomic DNA amplification of *B. bigemina* and *T. annulata* encoding product size of 689 and 312 bp, respectively. The results were compared with conventional microscopy and monoplex PCR assay. The sensitivity of each primer pair was checked using serial dilutions of parasite DNA, while specificity was determined by testing for amplification from DNA of different stocks of each pathogen. The duplex PCR detected each parasite species with the same level of sensitivity, irrespective of whether its DNA was amplified in isolation or with DNA mixture representing the other pathogens. Additionally, single and duplex PCRs could able to detect each species with equal sensitivity in serially diluted DNA representing mixtures of both the pathogen, and nonspecific amplification from non target species was not observed. The developed assay represents an economical, simple, sensitive, specific and reproducible diagnostic tool for simultaneous detection of tropical theileriosis and bovine babesiosis and boosting targeted selective control strategy in endemic areas.

Key words: *Babesia bigemina*, Cattle, Duplex PCR, Microscopy, *Theileria annulata*

Babesiosis and theileriosis are amongst the most economically important tick borne diseases encountered in India. In India, babesiosis caused by *Babesia bigemina* independently, accounts for annual losses to the tune of US$ 57.2 million (McLeod and Kristjanson 1999). Clinical signs of this disease are characterized by fever, anaemia, icterus and haemoglobinuria in infected animals (Sharma *et al*. 2013). Bovine tropical theileriosis (BTT) is caused by *Theileria annulata* alone claims for annual losses to the tune of US$ 800 million (Brown 1997) globally and US$ 384.3 million per annum in India alone (Minjauw and McLeod 2003). The disease is characterized by lymphadenopathy, splenomegaly, fever, anaemia, weakness and loss of body weight (Kumar *et al*. 2016, Tuli *et al*. 2015, Maharana *et al.* 2016a, b). Microscopy has the limitations of its inherent inability to detect the subclinical and carrier animals. Specificity of serological methods is limited by cross reactivity of antibodies between species and failure to differentiate between past and present infections (Sumbria *et al*. 2015). Nucleic acid based detection techniques like PCR assays allow diagnosis of parasite at levels far below the detection limit of the frequently used parasitological techniques (Bilgic *et al*. 2017, Kolte *et al*. 2017). Although conventional singleplex PCR designed to detect a single target efficiently, it is time consuming and expensive from epidemiological point of view. In contrast, duplex PCR allows for simultaneous amplification of two target loci and offers significant advantage over traditional singleplex PCR (Markoulatos *et al*. 2002). As both *B. bigemina* and *T. annulata* infections have more or less similar types of signs and arthropod vectors of these two diseases also exist together, the present communication describes the development and validation of a duplex PCR assay for simultaneous diagnosis of co-infections.

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MATERIALS AND METHODS

Sample collection: A total of 160 blood samples (2 ml

Table 1. Oligonucleotide primers used for standardization of Duplex PCR Assay

Name of haemoparasite	Primer target	Primer designation (Oligonucleotide) and sequence	Region amplified	Amplicon size	Annealing temperature	Reference
Babesia bigemina	ssurRNA	BB F: TAGTTGTATTTCAGCC TCGCG BB R: AACATCCAAGCAGCT AHTTAG	Small subunit ribosomal RNA sequence of *B. bigemina*	689 bp	50ºC	Ellis *et al*. (1992)
Theileria annulata	cytob1	TA F: ACT TTG GCC GTA ATG TTA AAC TA R: CTC TGG ACC AAC TGT TTG G	Cytochrome b1 gene of *T. annulata*	312 bp	50ºC	Bilgic *et al*. (2010)

from each animal) were randomly collected from jugular vein of suspected cattle in anticoagulant vial containing EDTA and kept on ice. This whole blood was used for smear preparation, DNA isolation and validation of PCR assay.

Microscopic examinations (ME): Thin and thick blood smear were prepared and subjected to Giemsa staining method following the standard protocol (Soulsby 1982). Each slide was examined for 75 different microscopic fields with a magnification of 100×. Giemsa stained lymphnode biopsy smears were also examined for detection of *T. annulata* macroschizonts (Soulsby 1982).

DNA extraction: DNA was isolated from blood/ lymphnode aspirate using standard phenol:chloroform extraction method as described by Sambrook and Russel (2001). The genomic DNA isolated from *B. bigemina* infected erythrocytes of clinically infected cattle (microscopically positive) was used as positive control. Similarly, DNA isolated from the clinically theileriosis (microscopically positive) confirmed cattle served as positive control.

Oligonucleotide primers for Duplex PCR: Oligonucleotide primers used for establishment of duplex PCR targeted the ssurRNA sequence of *B. bigemina* and cytochrome b gene of *T. annulata* (Table 1). Primer melting temperature (Tm), potential for self annealing was bioinformatically analysed using oligonucleotide analyser software 3.1 (https://eu.idtdna.com/calc/analyzer). Each set of primers was checked for specificity using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis in conjugation with NCBI database (https://www.ncbi.nlm.nih.gov/).

Single and duplex PCR amplification: The PCR reactions were set up into 25 µl volume containing 12.5 µl Top Taq® PCR Master Mix (QIAGEN, India), 1 µl of each primer (10 pmol each of BBF/R and TAF/R) and 1 µl of the extracted DNA template, and the total volume was made up to 25 µl using nuclease-free water. The PCR cycling conditions were set in automated thermal cycler (Applied Biosystem, USA) with the following programme: initial denaturation at 94°C for 3 min, 32 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1.2 min and the final extension at 72°C for 10 min. Additionally, monoplex PCRs with the same single primer set were also performed so as to compare the sensitivity of duplex PCR with single PCR as well as microscopy based thin blood smear examination. For each reaction, 10 µl of PCR amplicon was analyzed by agarose gel electrophoresis in 2% agarose gel containing 10 µg/ml ethidium bromide in Tris-acetate- EDTA (TAE) buffer at 50V for 1 h and visualized under UV light (Gel Doc^{TM} XR^{+}, BIORAD, USA).

Specificity of primers: PCR amplification was employed on each individual positive DNA sample (*B. bigemina* and *T. annulata*) as well as from the mixture of DNA samples derived from control positive samples (*B.bigemina* and *T. annulata*) of respective haemoprotozoan using their specific primers along with host leucocyte DNA to check cross reactivity, if any. DNA isolated from erythrocytes of cattle infected with *Anaplasma marginale* and *Trypanosoma evansi* was further used to ensure the specificity of these primers.

Sensitivity of single and duplex PCR: The sensitivities of single and duplex PCRs were determined by using equal concentration of purified DNA (300 ng determined by Eppendorf Biospectrometer®Kinetic, Germany) extracted from known positive *B. bigemina* and *T. annulata*. These purified parasitic DNA were used to produce two individual series of 10-fold dilutions using nuclease free water. In addition, equal quantities of DNA representing these two species were mixed and 10-fold serially diluted in nuclease free water to evaluate the sensitivities of the single and duplex PCR assays to amplify from samples containing mixed DNA templates (Table 2).

Table 2. Comparison of sensitivities of species specific oligonucleotide primer set in single and duplex PCR

Serial DNA dilution	Single PCR assay		Duplex PCR assay
	BBF/R	TAF/R	
B. bigemina alone	10^{-6}	-	10^{-5}
T. annulata alone	-	10^{-10}	10^{-8}
B. bigemina in mixture	10^{-5}	-	10^{-5}
T. annulata mixture	-	10^{-8}	10^{-8}

RESULTS AND DISCUSSION

Bovine babesiosis and tropical theileriosis are amongst the most abundant tick-borne diseases and exert their greatest impact in the tropical and subtropical regions.

Animals that have recovered from acute infection with *B. bigemina* and/or *T. annulata* remain persistently infected, act as carriers and play an important role in the transmission of the infection by ticks (Jonsson *et al*. 2008). Hence, it is the need of the hour to develop a laboratory based diagnostic tool capable of detecting the carrier animals. To overcome the shortcomings of monoplex PCR and to enhance the diagnostic efficiency, a variant termed duplex PCR has been described here (Sudan *et al*. 2015, Mitra *et al*. 2015, Sumbria *et al*. 2015).

PCR primers specificity: In the present study, the species-specific cytob1 primer set was used to amplify a 312 bp region of cytochrome b for *T. annulata* and ssurRNA sequence has been targeted for amplification of 689 bp specific for *B. bigemina*. BLAST analysis indicated that each oligonucleotide primer sequence was species-specific and did not possess additional local homology to the target sequence. Issues of loop formation, self-annealing and primer-dimer formation were overcome by optimising the duplex PCR conditions, including various cycling parameters, concentrations of primers, templates, $MgCl_2$ etc (Table 1).

rRNA sequences offer an alternate target for detecting parasites in a host even at very low parasitaemia, because it is the most abundant cellular macromolecule. This facilitates the development of sensitive diagnostic assays, in which rRNA based probes can be about 100 times more sensitive than probes based on repetitive DNA (Waters and McCutchan 1990). In the present study, ssurRNA sequence has been targeted for amplification of 689 bp specific for *B. bigemina*. This primer set has been shown to be highly sensitive and specific in detecting the parasite in latent carrier animals (Ellis *et al*. 1992). In the current study, the species-specific cytob1 primer set was used to amplify a 312 bp region of cytochrome b for *T. annulata*. This primer set has been shown previously to be capable of detecting the parasite in carrier animals in the field level with a high degree of sensitivity (Bilgic *et al*. 2010).

PCR amplification employed on each individual positive DNA sample using their specific primers led to the detection of expected fragments of size 689 bp (*B. bigemina*), 312 bp (*T. annulata*), respectively. Each set of the primers was specific for the respective parasite DNA, and amplification of non-target DNA samples did not lead to the production of spurious PCR products when specific primers for haemoprotozoa samples were interchanged.

Sensitivity of single and duplex PCR: The sensitivities of the single and duplex PCR assays were assessed using serially diluted DNA preparations. Starting with equal concentration of DNA from positive *B. bigemina* and *T. annulata* samples, 10-fold serial dilutions were generated for DNA of each species individually and for a combination representing both the species. As summarized in Table 2, the duplex PCR was able to detect *B. bigemina* and *T. annulata* at dilutions of 10^{-5} and 10^{-8}, with equivalent sensitivity for both single DNA and mixed DNA template dilutions. Agarose gel electrophoresis of duplex PCR assay

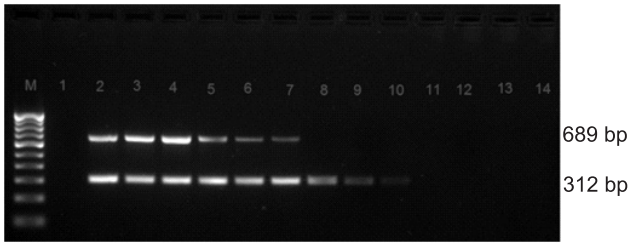

Fig. 1. Agarose gel electrophoresis of duplex PCR assay showing serial dilutions of a mixture of DNA from *B. bigemina* and *T. annulata*. Lane M, Step^{TM}Up 100 bp DNA Ladder (GeNei, India); lane 1, Known negative PCR control (water); lane 2, Undiluted mixed DNA sample; lanes 3–13, Ten-fold dilution series of mixed DNA sample ranging from 10^{-1} to 10^{-11}; lane 14, Uninfected bovine DNA.

exhibiting serial dilution of a mixture of DNA from *B. bigemina* and *T. annulata* is shown in Fig. 1. Apparently, there was no significant loss of sensitivity using the duplex PCR protocol compared to the single PCR assay since almost similar detection limits were observed when a single PCR was used to assay mixed DNA serial dilutions (Table 2). When the single PCR was used to amplify DNA dilutions representing each parasite species individually, the sensitivity of the assay improved to 10^{-6} and 10^{-10}, respectively (Table 2). This could be hugely qualified to the fact that the amount of template DNA present in the reaction mixture coupled with the competition for a limited amount of reagents between the primers affects the amplicon production quantitatively (Henegariu *et al*. 1997).Thus, in case of monoplex PCR with ample proportion of single target DNA template besides lack of competition between primers would yield larger amount of amplicon which would further enhance its sensitivity compared to duplex PCR. Yet, the duplex PCR assay was able to amplify target amplicons in mixed DNA templates with the same sensitivity as the single PCR (Bilgic *et al*. 2013, Sudan *et al.* 2015).

Relative efficacy of duplex PCR and compound microscopy based detection: A total of 160 blood samples were examined for infection with *B. bigemina*, *T. annulata* using microscopy, single and duplex PCR (Table 3). Out of 160 samples, 9 (5.62%) and 43 (26.87%) samples were positive for *B. bigemina* and *T. annulata* respectively by microscopy. Likewise, when compared with blood smear

Table 3. Single PCR, duplex PCR, and blood smear examination on suspected bovine blood samples

Name of test	Number of positive animals			Number of negative animals
	B. bigemina	*T. annulata*	*B. bigemina* + *T. annulata*	
Blood smear	9	43	3	108
Single PCR assay BBF/R	14	-	-	146
Single PCR assay TAF/R	-	58	-	102
Duplex PCR assay	12	52	8	96

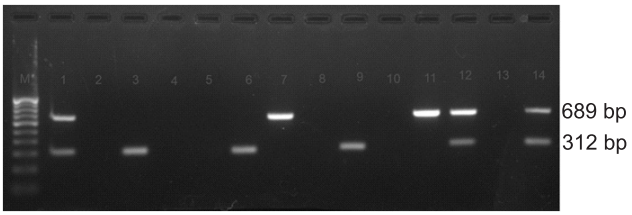

Fig. 2. Agarose gel electrophoresis of duplex PCR using DNA isolated from bovine field samples. Lane M, Step^{TM}Up 100 bp DNA Ladder (GeNei, India); lane1, Sample positive for *B. bigemina and T. annulata* (Known Positive Control); lane 2, Known negative PCR control (water); lanes 3, 6, 9, Sample positive for *T. annulata*; lanes 7, 11, Sample positive for *B. bigemina*; lanes 12, 14, Sample positive for both *B. bigemina* and *T. annulata*; lanes 4, 5, 8, 10, 13, Sample negative for both haemoparasite.

examination, single PCR on blood detected 14 (8.75%) and 58 (36.25%) positive cases of babesiosis and theileriosis respectively. When the samples were screened by duplex PCR, 12 (7.5%) and 52 (32.25%) number of animals had single infections with *B. bigemina* and *T. annulata*, respectively. Duplex PCR was able to detect 5% of mixed infections compared to 1.87% by microscopy (Fig. 2). These results defend the greater sensitivity of duplex PCR in detecting the subclinical and latent infections of both the haemoprotozoan (Sudan *et al*. 2015, Sumbria *et al*. 2015, Sumbria *et al*. 2016). The molecular prevalence of *T. annulata* was reasonably higher than that of *B. bigemina* because of abundance of tick vector population, i.e. *Hyalomma anatolicum* over *Boophilus microplus* in this geographical region. Earlier studies also suggested that *T. annulata* is the most prevalent and endemic hamoparasite in this region (Ganguly *et al*. 2017). It was also reported that *Hyalomma anatolicum*, transmitting agent of *T. annulata*, is a very common hard tick species abundant in the North India, including Haryana (Chillar *et al*. 2014). These reports justify our findings. The incidence of co-infection of both the haemoparasite was apparently less owing to different vectors responsible for their transmission (Sumbria *et al*. 2015).

In summary, the duplex PCR developed in the current study provides a simple, accurate diagnostic test that facilitates assessment of various risk factors associated with TBDs like identifying carriers of infection, detection of co-infections in the individuals etc. which will be very useful for establishment of more effective and selective control strategies.

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