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PCR based unraveling of the cryptic epizootiology of bubaline theileriosis targeting the merozoite surface antigen gene

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ABSTRACT

Lower parasitemia often skips the conventional microscopic and serological techniques from detecting latent, cryptic and chronic carrier states of bovine tropical theileriosis infection in buffalo hosts. A PCR was laboratory standardized and later validated on 80 field samples of suspected bubaline (*Bubalis bubalis*) hosts. Oligonucleotide primers (TBR F/R), selected from the gene encoding the 30-kDa major *Theileria annulata* merozoite surface antigen were custom designed and used for PCR amplifications. The sensitivity and specificity of PCR in relation to blood smear was compared based on kappa value predictions. Keeping blood smear examination as a gold standard for detecting actual number of confirmed positive cases, PCR on blood was 100 % sensitive and 88.6 % specific. The described PCR-based assay provides a valuable tool to study the epidemiology of bovine tropical theileriosis (BTT) in buffaloes and some vital data regarding epidemiology of theileriosis in water buffaloes from semi arid parts of India was generated.

Key words: Blood smear examination, Bovine tropical theileriosis, PCR on blood

Bovine tropical theileriosis (BTT), a tick borne lymphoproliferative disease of ruminants, causes significant economic losses in large parts of Asia and Africa (Hassanpour *et al.* 2013). BTT accounts for an estimated annual global loss of US$ 800 million (Brown 1997) besides, US$ 384.3 million annually in India alone (Minjauw and McLeod 2003). *Theileria* piroplasms are difficult to find in stained blood smears and it is not possible to discriminate between different *Theileria* species based on morphology alone. Alongside, serological tests suffer from the limitation in sensitivity because of cross-reactivity with antibodies directed against other *Theileria* species (Burridge *et al.* 1974). The present study was designed with the objective of validating a PCR based assay for the accurate and sensitive diagnosis of cryptic cases of BTT in water buffaloes.

MATERIALS AND METHODS

Collection of blood samples: Blood samples (aliquot of 1 ml) from each animal, were collected in clean sterile vaccutainer, containing ethylene diamine tetra acetic acid (EDTA), from the jugular vein of earlier theileriosis confirmed water buffaloes (microscopic observation of blood smears) for isolation of positive controls for laboratory standardization of PCR assay. Later on, blood samples and lymph node aspirates from 85 suspected buffaloes were also collected for validation of the PCR assay.

DNA isolation from whole blood and lymph node aspirate: DNA was isolated using standard phenol chloroform method with minor modifications (Pruvot *et al.* 2010). Briefly, 100 microliter of blood was added into a 1.5 ml tube containing 500 µl of denaturing solution (guanidinium thiocyanate, 10%) and vortexed at high-speed for 5 min; 150 µl each of chloroform and phenol were added and vortexed at high speed for 5–10 min. The content was centrifuged at 13,000 rpm (15,493×g) for 5 min and the supernatant (upper phase) was collected. Again 150 µl each of chloroform and phenol were added to the supernatant, vortexed for 5–10 min and centrifuged at 13,000 rpm for 5 min. After collection of supernatant (about 400 µl), 1 ml of absolute ethanol was added and left the sample to precipitate at –20 °C overnight. DNA was pelleted by centrifugation at 13,000 rpm for 10 min, the pellet was washed with 75% alcohol twice. The pellet was finally air dried before re-suspension into 50 µl of TE buffer (Tris, EDTA). Thus, the final prepared sample was concentrated 2:1 compared to the initial blood sample.

Primers selection and polymerase chain reaction (PCR): Primers Tams F/R were custom synthesized using sequence published elsewhere (d' Oliveira *et al.* 1995). This pair of

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Table 1. Primer sequences along with thermal cycling profile

Primer name	Primer sequence	Reference
Tams F	(5/)- GTAACCTTTAAAAACG -(3/)	d'Oliveira *et al.* 1995
Tams R	(5/)- GTTACGAACATGGGTT-(3/)	

	Thermal cycling profile				
	Initial denaturation	Denaturation	Hybridization	Extension	Termination
PCR	94°C, 60 sec	94°C, 45 sec	55°C, 60 sec × 30 cycles	72°C, 60 sec	72°C, 300 sec

Table 2. Diagnostic performance of blood PCR in respect to blood microscopy for diagnosing bubaline theileriosis in suspected buffalo samples (85)

Test	Blood smear			Sensitivity (95% CI)	Specificity (95% CI)	Kappa value
PCR	Positive	Negative	Total			
Positive	26	0	26	100%	88.6%	Kappa=0.757 The strength of agreement is considered to be 'good'
Negative	8	49	57			
Total	34	51	85			

primers was used for the synthesis of the primary PCR amplification product of the gene encoding the 30-kDa major *T. annulata* merozoite surface antigen. Details for primers design including position of nucleotides, nucleotide sequences, and expected PCR products are shown in Table 1.The PCR reaction was set up into 25 µl volume containing 12.5 µl PCR Master Mix (0.05/ 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 (Tams F/R), 2 µl of the DNA template and total volume was made up to 25 µl using nuclease free water. The details of thermal cycling profile is given in Table 1. The PCR amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel.

Sensitivity and specificity assay: The sensitivity of the present assay was determined firstly by spectrophotometric estimation of purified DNA concentration (1020 µg/ml). In order to check the specificity of these primers, PCR was performed with DNA extracted from the blood of buffaloes parasitologically positive (microscopic observation of blood smears and confirmed by PCR) for *Trypanosoma evansi* and *Babesia* sp. using standard primers (Desquesnes *et al.* 2001, Singh *et al.* 2007).

Statistical analysis: The PCR results on blood were compared with that of blood smear examination. The sensitivity and specificity were calculated and compared with that of blood smear examination, as a gold standard, using online software (http://graphpad.com/quickcalcs/kappa1.cfm). The kappa value, hence calculated, was compared and results were formulated (Table 2).

RESULTS AND DISCUSSION

The PCR-based assay afforded sensitive and specific detection of theileriosis in all naturally infected buffaloes. The PCR produced a 721 bp PCR product from all the positive samples which is highly specific for *T. annulata*

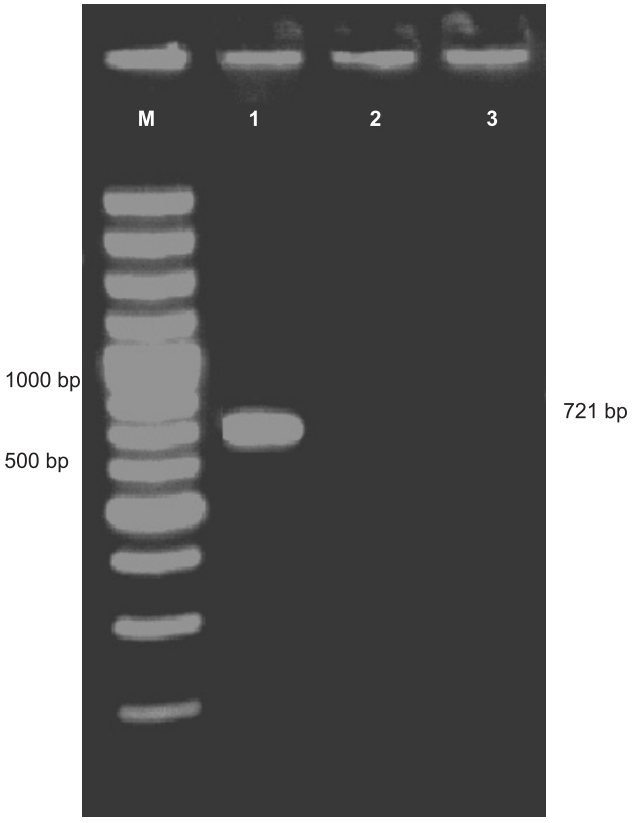

Fig. 1. Agarose gel electrophoresis depicting the desirable amplicons; Lane M, molecular weight marker 100 bp; lane 1, desired amplicon from positive sample; lane 2,3, negative sample.

(Fig. 1). The DNA from other blood parasites failed to produce any of the amplification products accounting for its high specificity.

When compared with blood smear examination, PCR on blood detected 26 cases positive in comparison to blood smear examination, which detected 18 positive cases. Keeping blood smear examination as a gold standard for detecting actual number of confirmed positive cases, PCR on blood was 100 % sensitive and 88.6 % specific based on their kappa value predictions.

Under the field conditions, the animals having subclinical infection are the major source of infection to the ticks. The subclinical infection cannot be easily diagnosed on blood smear examination whereas PCR detects very minute infection of *T. annulata* and can be used as an excellent tool for the diagnosis of BTT in carrier buffaloes. Discrimination of *T. annulata* from nonpathogenic *Theileria* species and other hemoparasites that may occur simultaneously in the same carrier animal is feasible using PCR (d'Oliveira *et al.* 1995). In addition, PCR can be used to determine whether animals translocated from regions where theileriosis is endemic, are carriers of *T. annulata* or not. The much higher sensitivity of the PCR also facilitates monitoring of animals after vaccination with attenuated macroschizont-infected cell cultures.

Selection of target primer molecule is very vital for the optimum and accurate diagnosis using PCR. The primers used in the present study were derived from the gene encoding the 30-kDa major *T. annulata* merozoite surface antigen (d'Oliveira *et al*. 1995). These primers are considered to be the most ideal primers for the specific detection of *T. annulata* and they did not give any cross reaction either with other *Theileria* species (*T. parva*, *T. mutans*, *T. sergenti*, *T. buffeli*, *T. velifera* and *T. taurotragi*) and/or with other similar haemoprotozoans (*Anaplasma centrale*, *A. marginale*, *Babesia bovis* and *B. bigemina*) (d'Oliveira *et al.* 1995). Moreover, the detection limit of these primers is very high. The lowest detection limit of the PCR is reportedly 2 to 3 parasites/ml of infected blood, which corresponds with a parasitemia of 0.000048% (d'Oliveira *et al.* 1995).

In conclusion, the described PCR assay provides a simple, rapid, sensitive and specific method for detection of theileriosis in naturally infected latent carrier states in buffaloes as well as in other species also and can be recommended for inclusion in survey and control programmes. The detection limit of these primers along with the technique used for confirmation could be very detrimental in diagnosis of cryptic and latent carriers where parasite concentration is very much lower to be detected by conventional serological and microscopic techniques.

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