Indian Journal of Animal Sciences **90** (7): 1006–1008, July 2020/Short communication

Tams 1 based PCR assay for detection of theileriosis in indigenous calves from semi-arid regions of India

KAPIL KUMAR GUPTA, MUKESH KUMAR SRIVASTAVA, VIKRANT SUDAN* and DAYA SHANKER

UP Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura, Uttar Pradesh 281 001 India

Received: 18 July 2017; Accepted: 7 August 2017

Keywords: BTT, Indigenous cattle calves, PCR on blood, Tams 1

Bovine Tropical Theileriosis (BTT), a tick borne lympho-proliferative disease of ruminants, caused by apicomplex parasite *Theileria annulata* inflicts significant impact on health and productivity of dairy animals (Pandey *et al.* 2017). In India, BTT accounts for loss of US$ 384.3 million annually (Minjauw and McLeod 2003). Calf theileriosis causes heavy mortality along with specific clinical manifestations (Singh *et al.* 2014) including exopthalmia (Sudan *et al.* 2012, Singh *et al.* 2015). *Theileria* piroplasms are difficult to find in stained blood smears especially in cases of chronic carriers (Jaiswal *et al.* 2016). Therefore, molecular detection based on DNA probes remains an important tool for sensitive diagnosis of chronic carrier states. The present study was designed with the objective of standardization and validation of a PCR assay for sensitive and specific detection of theileriosis in calves.

Collection of blood samples: Blood samples (1 ml aliquot from each animal) were collected in clean sterile vaccutainer, containing ethylene diamine tetra acetic acid (EDTA), from the jugular veins of earlier theileriosis confirmed cattle (microscopic observation of blood smears) for isolation of positive controls and laboratory standardization of PCR assay. Later on, blood samples were collected from 71 cattle calves from various *goshalas* of Mathura-Vrindavan area alongside those which were brought to TVCC, DUVASU, Mathura. Peripheral blood smears were also made using standard procedures.

DNA isolation, primers selection and polymerase chain reaction (PCR): DNA was isolated using standard blood isolation kit (Puregene) following manufacturer's protocol. Primers TAMS F/R were custom synthesized using sequence published elsewhere (Sudan *et al.* 2016). This pair of 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 profiles are given in Table 1. The PCR amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel.

Sensitivity and specificity assay: Serial dilutions of the positive DNA was performed in order to obtain regular scale of dilution from 1/100 to 1/200,000. In order to check the specificity of these primers, PCR on *Trypanosoma evansi* and *Babesia* sp. using standard primers was also performed (Parashar *et al.* 2015, Sudan *et al.* 2017).

Statistical analysis: The results of the PCR on blood were compared with that of blood smear examination. The sensitivity and specificity were calculated and compared with that of blood smear examination (kept as gold standard) using online software (http://graphpad.com/quickcalcs/kappa1.cfm).

The PCR produced a 721 bp PCR product from all the positive samples which is highly specific for *T.annulata*. The serial dilution results revealed a very good response. 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 12 positive cases in comparison to blood smear examination, which detected 8 positive cases. (Table 2). Keeping blood smear examination as a gold standard for detecting actual number of confirmed positive cases, PCR on blood was found to be 100% sensitive and 93.65% 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 routine 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. Discrimination of *T. annulata* from non-pathogenic *Theileria* species and other hemoparasites that may occur simultaneously in the same carrier animal is feasible using PCR (d'Oliveira *et al.* 1995).

*Corresponding author e-mail: viks.sudan@gmail.com

Table 1. Primer sequences along with thermal cycling profile

	Primer name	Primer sequence	Reference
	TAMS F	(5')- GTAACCTTTAAAAACG -(3')	Sudan *et al.* 2016
	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	72°C, 60 sec	72°C, 300 sec
			× 30 cycles		

The much higher sensitivity of the PCR also facilitates monitoring of animals after vaccination with attenuated macroschizont-infected 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 (Sudan *et al.* 2016). 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 had been reported to be 2–3 parasites per ml of infected blood, which corresponds with a parasitemia of 0.000048% (d'Oliveira *et al.* 1995).

Table 2. Comparison of blood in respect to blood PCR for diagnosing calf (n=71) based on kappa value prediction

Blood	Blood microscopy		Kappa value
PCR	+ (%)	– (%)	
12	8.0	4.0	Kappa= 0.769
–59	0.0	59.0	SE of kappa = 0.109
Total	8.0	63.0	95% confidence interval: From 0.555 to 0.983
(%)	–11.3	–88.7	The strength of agreement is considered to be 'good'.

*Kappa value > 0.81, almost perfect agreement; 0.61–0.80, substantial agreement; 0.41–0.60, moderate agreement; 0.21–0.40, fair agreement; 0.01–0.20, slight agreement and 0.00, poor agreement.

In conclusion, the described PCR assay provides a simple, rapid, sensitive and specific method for detection of theileriosis in naturally infected calves and can be recommended for inclusion in survey and control programmes.

SUMMARY

Lower parasitemia often skips the conventional microscopic and serological techniques from detecting latent, cryptic and/or chronic carrier states of bovine tropical theileriosis (BTT). Hence the molecular detection of the parasitic DNA remains a highly authenticated tool. Oligonucleotide primers (TBR F/R) were custom designed and used for PCR amplification of *T. annulata*. The sensitivity and specificity of PCR was compared with blood microscopy based on kappa value predictions. A total of 8 samples were found positive by blood smear examination whereas PCR detected 12 infections. Blood smear examination was kept as a gold standard for detecting actual number of confirmed positive cases, for being 100% sensitive along with 93.65% specific, respectively, in detecting calf theileriosis. The described PCR-based assay provides a valuable tool to study the epidemiology of BTT in calves and some vital data regarding epidemiology of theileriosis in calves from semi-arid parts of India has been generated. Such a record for screening of calves for theileriosis is missing from Indian context.

ACKNOWLEDGEMENT

The authors are highly thankful to the Vice Chancellor, DUVASU for the facilities provided.

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