Indian Journal of Animal Sciences **86** (1): 11–13, January 2016/Article
https://doi.org/10.56093/ijans.v86i1.54908

A cross-sectional study to unravel the cryptic epidemiology of bovine tropical theileriosis by comparing four traditional and molecular techniques in water buffaloes

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Received: 19 January 2015; Accepted: 29 July 2015

ABSTRACT

A comparison of 4 techniques, viz. blood smear examination, lymph node examination, PCR on blood and PCR on lymph node aspirate for the accurate and sensitive diagnosis of cryptic cases of BTT in water buffaloes, was made. These tests were laboratory standardized and later validated on 85 field samples of suspected bubaline hosts. 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. When blood PCR was kept as standard, the lymph node biopsy and lymph node PCR were 90 and 86.96% sensitive along with 77.33 and 90.32% specific, respectively, in detecting theileriosis from suspected samples. The described PCR-based assay provides a valuable tool to study the epidemiology of 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, Bubaline BTT, Lymph node examination, PCR on blood, PCR on lymph node aspirate

Bovine tropical theileriosis (BTT), a tick-borne lymphoproliferative disease of ruminants, causes significant economic losses in large parts of Asia and Africa (Woods *et al.* 2013). Serological tests suffer from the limitation in sensitivity because of cross-reactivity with antibodies directed against other *Theileria* species (Burridge *et al.* 1974). Hence, animals with a negative serological test can still infect ticks. Therefore, molecular detection based on nuclear acid probes remains an important tool for sensitive diagnosis. The present study was designed with the objective of comparing 4 commonly used parasitological techniques, viz. blood smear examination, lymph node examination, PCR on blood and PCR on lymph node for the accurate and sensitive diagnosis of cryptic cases of BTT in water buffaloes.

MATERIALS AND METHODS

Collection of blood samples: Blood samples (1ml aliquot from each animal) were collected in clean sterile vaccutainer, **c**ontaining ethylene diamine tetra acetic acid (EDTA), from the jugular veins 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. Alongside, peripheral blood and lymph node biopsy smears were also made using standard procedures (Soulsby 1982).

DNA isolation from whole blood and lymph node aspirate: DNA was isolated using standard phenol chloroform method with minor modifications (Sudan *et al.* 2014). Briefly, 100 microliters of blood/lymph node aspirate were added into a 1.5 ml tube containing denaturing solution (500 µl; guanidinium thiocyanate) and vortexed at high-speed for 5 min. Chloroform (150 µl) and phenol (150 µl) were added and vortexed at high speed for 5–10 min. The microtube was centrifuged at 13,000 rpm (15,493 g) for 5 min, and supernatant (upper phase) 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 13,000 rpm for 5 min. Supernatant (400 µl) was collected and added to 1 ml absolute ethanol into a 1.5 ml tube, and sample was left to precipitate at –20°C overnight. After 10 min of centrifugation at 13,000 rpm, 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

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Table 1. Primer sequences along with thermal cycling profile

Primer name	Primer sequence				Reference
TAMS F	(5′)- GTAACCTTTAAAAACG -(3′)				Sudan *et al.* (2015)
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, 30 sec

compared to the initial blood sample.

Primers selection and Polymerase chain reaction (PCR): Primers TAMS F/R were custom synthesized using sequence published elsewhere (Sudan *et al.* 2015). This pair of primers was used for the synthesis of primary PCR amplification product of the gene encoding the 30kDa major *T. annulata* merozoite surface antigen. Details for primers design including position of nucleotides, nucleotide sequences, and expected PCR products are given 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.

Statistical analysis: The results of the PCR on both blood and lymph node aspirate were compared with that of blood smear examination and lymph node biopsy 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.

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).

PCR on blood detected 26 cases positive in comparison to blood smear examination, which detected 18 positive cases. Likewise, PCR on lymph node aspirate detected 20 cases positive in comparison to lymph node biopsy examination which detected 9 positive cases (Table 2). 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. When blood PCR was kept as standard, the lymph node biopsy and lymph node PCR were 90 and 86.96% sensitive along with 77.33 and 90.32% specific, respectively in detecting theileriosis from suspected samples (Table 3).

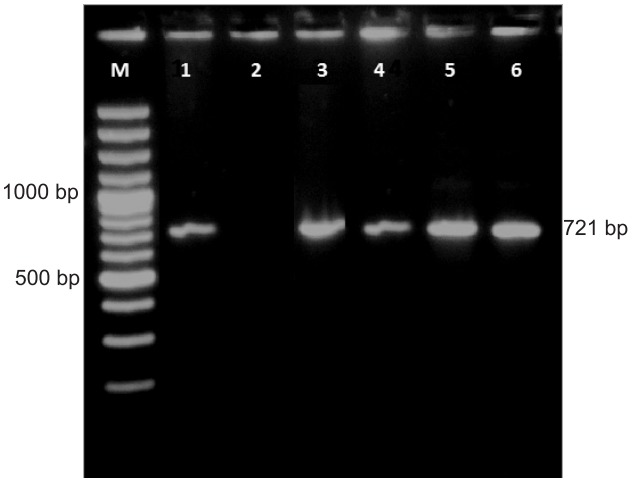

Fig. 1. Agarose electrophoresis gel depicting the desirable amplicons**.** Lane M, molecular weight marker; lane 1–4, lymph node PCR; lane 5–6, blood PCR.

Under the field conditions, the animals having subclinical infection are the major source of infection to the ticks (Sudan *et al.* 2015). 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

Table 2. Comparison of blood microscopy, lymph node biopsy and lymph node PCR in respect to blood PCR for diagnosing bubaline theileriosis in suspected buffalo samples (85)

BloodPCR	Blood microscopy		Lymph node biopsy		Lymph node PCR	
	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
+ (26)	18	8	9	17	20	6
- (59)	0	59	1	58	3	56
Total	18	67	10	75	23	62
(%)	(21.18)	(78.82)	(11.76)	(88.24)	(27.06)	(72.94)

Table 3. Diagnostic performance of blood PCR in respect to blood microscopy and subsequent performance of lymph node biopsy and lymph node PCR in respect to blood PCR for diagnosing bubaline theileriosis in suspected buffalo samples (85)

Assay	Sensitivity (%)	Specificity (%)	Kappa Value*
Blood PCR	100.0	88.06	0.757
Lymph node biopsy	90.0	77.33	0.398
Lymph node PCR	86.96	90.32	0.742

*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; 0.00, poor agreement.

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 that were translocated from theileriosis-endemic regions were 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 30kDa 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 had been reported to be 2 to 3 parasites/ml of infected blood, which corresponds with a parasitemia of 0.000048% (d'Oliveira *et al.* 1995).

With regards to comparison of various tests, PCR on blood was found to be superior than the other 3 techniques in respect to higher sensitivity and specificity values. This could be attributed to the fact that though lymph node involvement is seen in initial stages but as the disease progress the parasite shifts to blood and in severe cases leucopenia and lymph node enlargement (Soulsby 1982) occur, and chances of false positive increases as seen in the present study. So lymph node biopsies and PCR on lymph node aspirate may act as supportive facts in elucidating the conclusion about the presence of BTT in chronic or latent carrier stages but they should not be solely used as confirmative diagnostic tools because the parasite migrates from them in later stages.

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.

ACKNOWLEDGEMENT

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

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