



A comparative Study between Blood Smear, Whole Blood PCR and FTA Card PCR for Diagnosis of *Theileria annulata* and *Theileria orientalis* in Cattle

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ABSTRACT

Bovine theileriosis, a tick borne haemoprotozoan disease, caused by, *Theileria annulata* and *Theileria orientalis* and responsible for heavy economical losses in dairy industry. Diagnosis of theileriosis is mainly based on clinical signs and conventional techniques. Polymerase chain reaction using whole blood is a modern method of diagnosis of theileriosis as it can detect infection even in low parasitemia but only concern is that its very time consuming and chances of loss of DNA is more. Hence, to overcome this, the present study reports the diagnosis of *T. annulata* and *T. orientalis* by comparing Whole blood PCR and FTA card PCR whereas a comparison between blood smear examination, whole blood PCR and FTA card PCR was done to determine the sensitivity of the techniques. The study was conducted on 50 blood samples of cattle having typical clinical signs and blood smear after staining with Giemsa's stain were examined for presence of *Theileria spp.* in R.B.C. The primer sequences were used as per Kolte et al and Kakati *et al.* The assay employs primers specific for the gene encoding the 30-kDa major merozoite surface antigen of *T. annulata* and Major merozoite surface protein gene of *T. orientalis*. Out of total 50 blood samples, 16 were positive for *Theileria sp.* by blood smear examination, 19 were positive for *T. annulata* by Whole blood PCR and FTA card PCR whereas for *T. orientalis*, 26 were positive by whole blood and 27 samples by FTA card PCR. So the diagnosis of theileriosis by using FTA card PCR is found to be sensitive and less time consuming method as compared to Whole blood PCR.

Keywords: Cattle, theileriosis, diagnosis, whole Blood PCR and FTA Card PCR

Livestock sector plays most important role in India's rural population by contributing 16% to the income of small farm households and an average of 14% for all rural households. The two-third population of rural community is dependent on livestock for their livelihood. Livestock has played tremendous role in providing employment to 8.8% population of our country. Livestock sector contributes 4.11% to GDP and major contribution has been found towards agriculture with the total gross domestic product of 25.6%. Demand of food derived from animals is on rise that has led to the introduction of new breeds and innovative technologies like cross breeding among dairy cattle and buffaloes to enhance the produce and product development. Due to cross-breeding, the population of dairy animals and milk yield has increased but the disease tolerance has been decreased and cross-bred animals

have been found more prone to various infectious and haemoprotozoan diseases like Theileriosis and Babesiosis.

Theileriosis is a tick borne haemoprotozoan disease of ruminants caused by parasite belonging to the genus *Theileria* under the sub-order Piroplasmorina (Demessie and Derso, 2015). Theileriosis infection in cattle is characterized by clinical signs like pyrexia, enlargement of lymph node, reduction in milk yield, emaciation, nasal and ocular discharges and dyspnea in some cases (Soulsby, 2012). In India, cross breeding programs to increase the milk production, bovine tropical theileriosis

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has emerged as one of the most important and most economical tick borne disease of bovines with the huge loss of \$800 million per annum in India (Devendra, 1995) and huge money is spend by the government to control the disease with the approximate estimate of \$384.3 million per annum (Kumar *et al.*, 2018).

Diagnosis of theileriosis is mainly based on clinical signs and conventional techniques like microscopic examination of stained blood smears or lymph node biopsy smears. But this technique is often associated with wrong diagnosing the carrier animals, low sensitivity and other technical problems (Shayan and Rahbari, 2005). Serological tests like ELISA and IFAT has limitations of cross reactivity and show false positive and false negative results against other *Theileria* sp.

In order to find the best method of diagnosis, the present study was designed for the comparison between Blood smear, Whole blood PCR and FTA card PCR for diagnosis of *Theileria annulata* and *Theileria orientalis* in cattle.

MATERIALS AND METHODS

Animals

The study was conducted on 50 cattle with the history of any of the following symptoms, which includes; high temperature (102-105°F), swollen lymph node, reduced milk yield, emaciation, depression, anorexia, diarrhoea, petechial haemorrhages in eye.

Blood sampling and blood smear

Blood samples were collected individually in 2 ml of EDTA coated vials for DNA isolation, labelled and brought to the lab. A drop of fresh blood collected from jugular vein was taken on FTA card (Himedia) and were transported to lab in polythene zip lock bags as per the instructions given by manufacturer. Blood smears were prepared from the blood from jugular vein, marked and processed in laboratory by using Giemsa’s stain.

Isolation of genomic DNA

The DNA was isolated from whole blood and FTA card by using Favorgen Blood Genomic DNA Extraction

Mini Kit and Himedia Insta DNA kit, respectively. The concentration of DNA was quantified by using Nano-drop spectrophotometer. The samples with ratio of O.D. 260/280 of 1.8 and above were selected for PCR whereas samples below the ratio 1.8 conc. were again subjected to DNA isolation.

Polymerase chain reaction (PCR)

The assay employs primers specific (Table 1) for the gene encoding the 30-kDa major merozoite surface antigen of *Theileria annulata* (Kolte *et al.*, 2017) and Major merozoite surface protein gene of *Theileria orientalis* (Kakati *et al.*, 2015).

The PCR reaction was set up into 25µl reaction containing 1µl of each Forward and Reverse primers, 12.5µl Mater Mix (dNTPs, MgCl₂, Dye and Taq DNA Polymerase), 9.5µl of nuclease free water and 1µl of DNA template. The reaction condition for Tams1 was initial denaturation at 95.0°C for 5 minutes followed by 30 cycles each consisting of individual cycle with 95.0°C (30 seconds), 55.0°C (30 seconds), 72.0°C (1 min 10 seconds) with the final extension at 72.0°C (1 cycle) for 5 minutes. The reaction condition for MPSP was initial denaturation at 98.0°C for 10 seconds followed by 35 cycles each consisting of individual cycle with 98.0°C (10 seconds), 55.0°C for 45 seconds, 72.0°C (30 seconds) with the final extension of 72.0°C for 2 minutes. The PCR amplicons were then subjected to 0.8% of agarose gel electrophoresis and gel was examined under Gel documentation System (Gel Pro Analyser).

Table 1: Primers of different genes of theileria

Gene	Primer	Product size (Bp)	Reference
Tams 1	F- 5’ TACTTGAAGCTTCCATGT TGCCAGGACCAC 3’	846	Kolte <i>et al.</i> , 2017
	R- 5’ ATCTTGCTCGAGAAGGAAG TAAAGGACTGATGA 3’		
MPSP	F- 5’ CTTGCCTAGGATACTT CCT 3’	776	Kakati <i>et al.</i> , 2015
	R- 5’ ACGGCAAGTGGTGAGAA CT 3’		

RESULTS AND DISCUSSION

Out of total 50 blood samples, 16 (32%) were found positive for *Theileria sp.* by blood smear examination (Fig. 1 and 2) whereas for *Theileria annulata*, PCR found 19 (38%) were found positive by both whole blood PCR and FTA card PCR while for *Theileria orientalis*, 26 (52%) were positive by whole blood PCR and 27 (54%) by FTA card (Table 2).

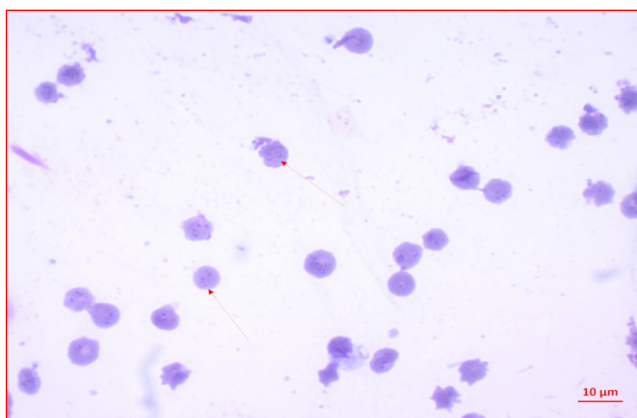


Fig. 1: Photomicrograph of ring form of *T. annulata* in erythrocytes

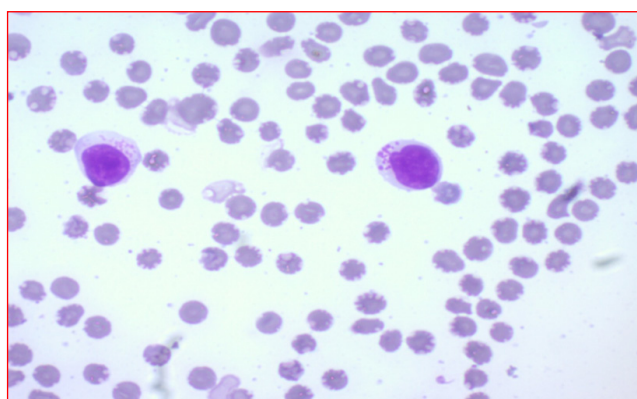


Fig. 2: Photomicrograph of blood smear showing Koch blue body in lymphocytes

Table 2: Comparison between Whole Blood PCR and FTA card PCR positive for *Theileria annulata* and *Theileria orientalis*

Sl. No.	Test	<i>T. annulata</i>	<i>T. orientalis</i>
1	Whole blood PCR	38%	52%
2	FTA card PCR	38%	54%

On comparison of all the methods, it was found that 16(32%) samples were positive by blood smear, 45 (90%) by whole blood PCR and 46 (92%) were found positive by FTA card PCR (Table 3). The present study is new and no comparison between blood smear, whole blood PCR and FTA card PCR has been studied yet.

Table 3: Percent prevalence of theilariosis by different tests in the cattle

Sl. No.	Test	Cattle
1	Blood smear	32%
2	Whole blood PCR	90%
3	FTA card PCR	92%

The study has found that PCR is recommended as best tool for diagnosis of *Theileria annulata* and *Theileria orientalis* due to its efficacy to detect infection in chronic and carrier stages and DNA isolation by using FTA card is highly recommended technique over whole blood for diagnosis of theileriosis as it can detect infection from a drop of blood, less laborious, less amount of blood is required and quick results are seen. The present study is in partial agreement with Muhaanguzi *et al.* (2014).

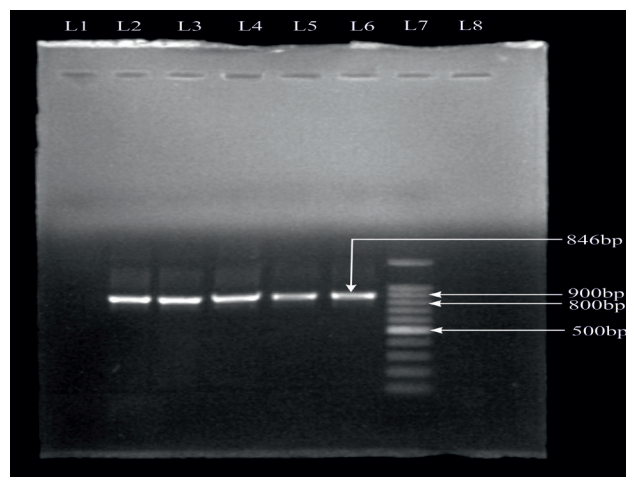


Fig. 3: Agarose gel electrophoresis of amplified *T. annulata* DNA

L1: Negative control, L2: Positive control, L3-L4: FTA card DNA, L5-L6: Whole blood DNA, L7: 100 bp DNA ladder

They used Whatman FTA cards to study the epidemiology of *T. parva* (5.3%) in Eastern Uganda. Similarly, Hassan *et al.* (2018) used FTA cards to determine the prevalence of

Theileria spp. in Pakistan. Several workers like Kolte *et al.* (2017), Paliwal *et al.* (2019), Kundave *et al.* (2015) have performed PCR using whole blood and also reported that PCR was most sensitive tool for diagnosis of theileriosis. Similar observations have been recorded earlier by various workers (Khatoon *et al.*, 2015; Sahoo *et al.*, 2017).

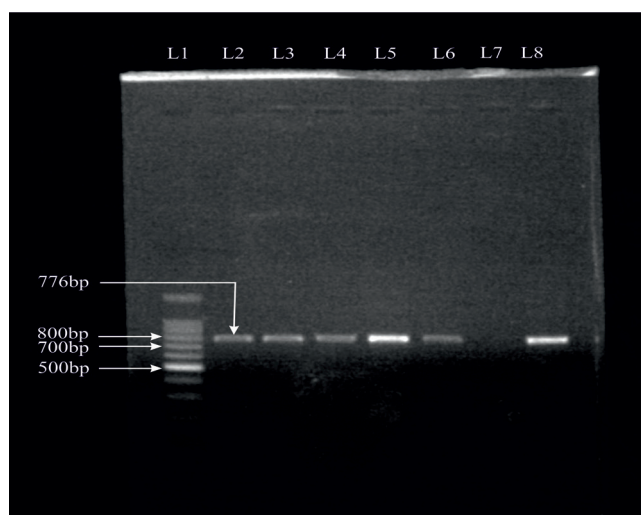


Fig. 4: Agarose gel electrophoresis of amplified *T. orientalis* DNA

L1: DNA ladder 100 bp, L2-L4: FTA card DNA, L5-L6: Whole blood DNA, L7: Negative control, L8: Positive control

The primers specific for the gene i.e. Tams1 and MPSP were used to detect *Theileria annulata* and *Theileria orientalis* with the amplicon size of 846bp for *Theileria annulata* (Fig. 3) and 776bp for *Theileria orientalis* (Fig. 4) was detected in blood samples. Similar primers were also used by Kolte et al and Kakati et al for diagnosis of *Theileria annulata* and *Theileria orientalis*, respectively.

CONCLUSION

PCR is recommended as best tool for diagnosis of *Theileria annulata* and *Theileria orientalis* due to its efficacy to detect infection in chronic and carrier stages. The DNA isolation by using FTA card is highly recommended technique over whole blood for diagnosis of theileriosis as it can detect infection from a drop of blood, less laborious, less amount of blood is required, chances of DNA loss is less and quick results. PCR using FTA card DNA showed higher sensitivity as compared to PCR using whole blood DNA and blood smear examination.

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