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Comparative Evaluation of Microscopy and PCR Assay for Detection of *Theileria annulata* Infection in Ruminants

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ABSTRACT

Bovine tropical theileriosis, a tick borne haemoprotozoan disease, is one of the major threats to the health and production of cattle in the tropics and sub tropics. Diagnosis of the disease mainly relies upon clinical signs and microscopic examination of blood and lymphnode aspirate smears, which suffers with low diagnostic sensitivity, especially in carrier animals. Hence, the microscopy (gold standard test) and a highly sensitive polymerase chain reaction assay (PCR) were compared in terms of sensitivity, in the prsesnt study. For this, a total of 250 blood samples (cattle-215 and buffaloe-35) suspected for theileriosis were collected and initially screened by microscopic examination of Giemsa stained thin blood smear followed by Tams1 gene based PCR assay. A total of 55 (22.0%) and 95 (38.0%) samples were found positive for *Theileria annulata* infection by microscopy and PCR assay, respectively. In order to analyze the detection limit of the given PCR assay, the amplified product was cloned in pTZ57R/T cloning vector with DH5α (*E. coli*) as host cells. The recombinant plasmid was isolated from the bacterial cells and concentration of the same was measured and then, a 10 fold serial dilution of the same was used as template in PCR assay. Present study concludes that Tams1 based PCR has very high level of sensitivity (with respect to microscopy) and detection limit of the test is 10^{-7}ng/µl parasite DNA.

Keywords: Theileriosis, microscopy, polymerase chain reaction, diagnostic sensitivity

Bovine tropical theileriosis, caused by intracellular blood protozoan *Theileria annulata*, is considered to be a life threatening disease of cross bred cattle in many parts of the world including India (Uilenberg, 1982). In India, the parasite is transmitted by *Hyalomma anatolicum anatolicum* ticks with an annual loss of US$ 384.3 million to dairy sector (Minjauw and Mc Leod, 2003). Theileriosis has been reported from different parts of India by several workers (Kohli *et al*., 2014; Velusamy *et al*., 2014; Bhatnagar *et al*., 2015; Naik *et al*., 2016). Conventionally, the disease is being diagnosed by microscopic examination of thin blood smear (gold standard) from the infected animals but sensitivity of this technique is extremely low as it often fails to detect carrier animals, due to low parasitemia (Friedhoff and Bose, 1994). The available data on prevalence of infection in animals, based on microscopy alone may not be representing the true picture of *T. annulata* infection in a geographical area and thus use of molecular tests seems to be a viable option. Tams1 is a merozoite surface protein of *T. annulata,* with molecular mass of 30 to 32 kDa (Katzer *et al*., 2002). Reports are available on the use of Tams1 target based PCR for sensitive and specific amplification of *T. annulata* DNA from blood of cattle and ticks (D'Oliveira *et al*., 1995; Gubbels *et al*., 2000; Kirvar *et al*., 2000). In the present study, microscopy and Tams1 based PCR were compared for their diagnostic sensitivity for detection of *T. annulata* infection in suspected large ruminants.

MATERIALS AND METHODS

Sample collection

During a period of one year (December, 2015 to November,

2016), a total of 250 blood samples (215 cattle and 35 buffaloes) were collected from animals of Bareilly district of Uttar Pradesh; presented to Veterinary Hospitals/Clinics with the complaint of symptoms like pyrexia, anorexia, lymphnode enlargement, pale mucous membrane, drop in milk production, tick infestation, bilateral nasal discharge and lacrimation suggestive of theileriosis. From each animal, 2 ml fresh blood was taken from jugular vein and thin blood smear was prepared immediately. Part of remaining blood was utilized for DNA isolation, for use in polymerase chain reaction (PCR assay).

Blood smear examination

Thin blood smears were fixed with absolute methanol for one minute, air dried and stained with Giemsa stain (1:20) as per standard method (Soulsby, 1982) for detection of blood parasites. The percentage of parasitemia was determined by counting the infected RBCs in the proportion of total RBCs in Giemsa's stained blood smears under light microscope and at least, 15 to 20 microscopic fields were counted. Following formula was followed for determination of % of parasitaemia:

$$\% \text{ Parasitaemia} = \frac{\text{Total no.of infected RBC}}{\text{Total no.of RBC's}} \times 100$$

Isolation of DNA from blood samples

Genomic DNA was extracted from 200µl of the whole blood using DNA extraction kit (DNeasy blood kit, Qiagen) following the manufacturer's protocols. Concentration of isolated DNA was measured using spectrophotometer and purity was checked in agarose gel electrophoresis (1.5 % gel). Aliquots of extracted DNA were stored at -20 ºC until further use.

Polymerase chain reaction

Self-designed primers (forward 5'-CCG TTA ATG CTG CAA ATG AGG AGG) and (reverse 5'-GAG GCG AAG ACT GCA AGG GGA G) targeting 751 bp amplicon of the major merozoite surface antigen (Tams 1 gene) of *T. annulata* was custom synthesized based on the sequence from GenBank with Acession no. AF214840.1. PCR reaction was performed in 25 µl total volume containing 20-30 ng of template DNA, 2.5 µl of 10X PCR Green buffer (Puregene), 0.5 µl of 10 mM dNTP, 0.5 µl of each forward and reverse primer (20 pmol/µl), 0.2 µl Dream *Taq* DNA polymerase and nuclease free water to make the volume 25 µl. The cycling conditions used for PCR amplification of target gene were: ininial denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 45 sec denaturation, 58ºC for 45 sec anaeling, 72ºC for 45 sec elongation with a final extension at 72ºC for 10 min. A negative control (nuclease free water), and a positive control (DNA isolated from blood of a known *T. annulata* infected cattle) was included for every series of amplification. PCR amplified products were analyzed by agarose gel electrophoresis (120 V/208 mA) in the 1.5% agarose gel following ethidium bromide staining (0.5µg/ml). The results were recorded using gel documentation system (Syngene).

Cloning and sequencing for confirmation of target gene

PCR amplified product of the known *T. annulata* positive sample was gel purified and subjected to cloning using the pTZ57 R/T cloning vector and DH5α (*E. coli*) as host cell following the standard protocol. The recombinant clones were confirmed by colony PCR and plasmid PCR and after that recombinant plasmid DNA was purified from the overnight grown bacterial culture using GeneJET plasmid Miniprep kit (Thermo Scientific).

Thereafter, the concentration of plasmid DNA was quantified by Nanodrop® and the purified plasmid was used to determine analytical sensitivity/detection limit of the Tams1 gene based PCR assay. A stab culture of positive clone harbouring the desired fragment of Tams1 gene was custom DNA sequenced and analyzed using DNASTAR and Basic Local Alignment search tool (BLAST, NCBI).

Analytical sensitivity of Tams1 based assay

The cloned plasmid (pTZ57R/T-Tams1) containing the target region of Tams1 gene of *T. annulata* was diluted serially in proportion of 10-fold (100 ng/µl to 10 fg/µl) with nuclease free water and 2 µl of the cloned plasmid DNA from each dilution was used as template for the detection of threshold limit of PCR assay. The cloning vector pTZ57R/T was used as negative control. To calculate the detection limit of PCR assay in serially diluted plasmid DNA in the sense of number of molecules, the following formula was used.

$$n = \frac{N \times Q}{660B \times 10^9}$$

Where, n: copy number of plasmid/µl, N: avagrado number, Q: quantity of recombinant plasmid in ng/µl, B: size of the recombinant plasmid (plasmid plus insert) in bp, 660: molecular wt. of one base pair.

Specificity of Tams1 based assay

The total genomic DNA samples extracted from whole blood infected with other common haemoparasites of cattle viz. *Babesia bigemina* and *Anaplasma marginale* were used as templates for the PCR.

Data analysis

Results of both microscopy and PCR assay were analyzed, and incidence of infection was determined accordingly (Table 1). The sensitivity and specificity of PCR assay and microscopy were determined by formula used by Noaman (2014).

RESULTS AND DISCUSSION

Microscopic examination of 250 Giemsa stained blood smears revealed the presence of *Theileria annulata* piroplasm (Fig. 1) in 55 samples (51 cattle, 4 buffaloes) with the overall prevalence of 22.0%. The minimum and maximum parasitemia in the blood samples were found to be 0.03% to 12% by microscopy.

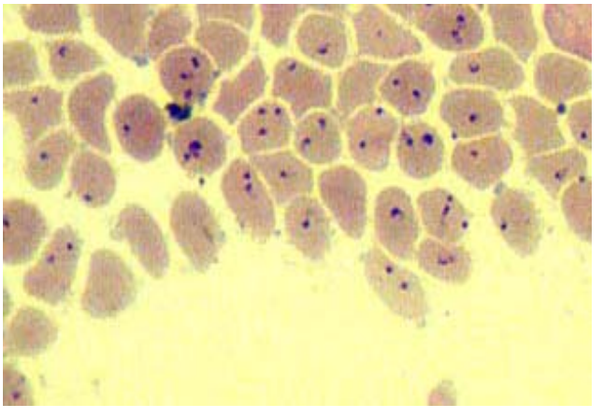

Fig. 1: *Theileria annulata* infected RBCs of cattle

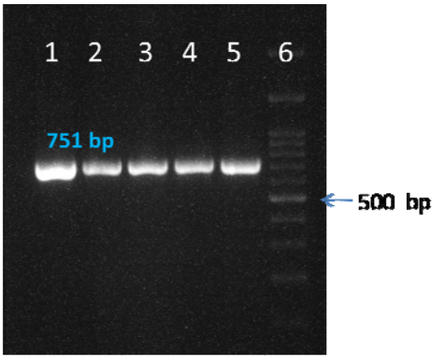

Fig. 2: Agarose gel electrophoresis of PCR amplified *T. annulata* DNA

Lane 1: positive control (positive DNA), Lane 2-5: field samples, Lane 6: 100bp DNA ladder

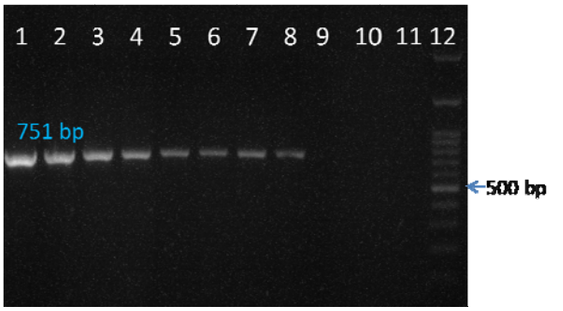

Fig. 3: Sensitivity of Tams1 gene based PCR assay

Lane 1: positive control, lanes 2-11: ten fold serial dilution of *T. annulata* plasmid DNA ranging from 100 ng/µl to 10^{-10} ng/µl, lane 12: 100 bp DNA ladder

However, gene specific PCR amplification (751 bp) (Fig. 2) was recorded in 95 samples (90 cattle, 5 buffaloes) with the overall prevalence of 38.0%. In the study, Tams1 gene specific PCR could detect *T. annulata* infection in 40 animals in addition to 55 detected positive by microscopy. Analysis of the PCR products revealed positivity up to 10^{-7} dilution of recombinant plasmid (Fig. 3). The dilution of 10^{-7} corresponded to plasmid amount of 0.01 picogram. The detection limit of Tams1 based PCR was found to be 2.5 parasites/ml of blood by using the formula described above. Further, nucleotide sequence generated from the recombinant plasmid (Provisional Acc. No. MF346028) confirms presence of 751 continuous base pairs in the

cloned product. PCR amplification was not recorded in other parasitic DNA, showing high specificity of the test. The observation made in the study partially corroborate with the finding of D'oliveira *et al*. (1995), who reported lower detection limit of PCR to be as 2-3 parasites per µl of infected blood, which corresponds with parasitaemia of 0.000048%. Kirvar *et al*. (2000) reported the sensitivity of PCR detection to be as one parasite per milliliter of blood, which was three times accurate than microscopic examination of 200 fields. In the present study, blood smear examination of 50 microscopic fields showed 58 % sensitivity and 100 % specificity in comparison to 100 % sensitivity and specificity of Tams1 based PCR assay (Table 1).

Table 1: Sensitivity and specificity of microscopy with respect to Tams1 based PCR

Method	No. of samples examined	No. of positive detected	Sensitivity[a]	Specificity[b]
PCR	250	95	100	100
Microscopy (50 fields)	250	55	58	100

[a]Calculated as follows: [Number of true positive / (number of true positive + number of false negative)] ×100

[b]Calculated as follows: [Number of true negative / (number of true negative + number of false positive)] ×100 (Noaman, 2014)

Microscopy, despite being less expensive, quick and easy to perform, has much lower sensitivity and requires expertise as well. Due to this limitation only, PCR has been highly recommended by investigators for the detection of *Theileria* infections (Shayan and Rahbari, 2005; Bilgic *et al*., 2013). Molecular and microscopic techniques for detection of *T. annulata* infection in cattle have been compared by Chauhan *et al*. (2015) in which 32.69% animals were found positive for theileriosis by microscopy and 46.15% by PCR assay. Charaya *et al.* (2016) also compared PCR assay with microscopy for detection of theileriosis in crossbred cattle population and recorded 62.06% *Theileria* sp. infection by PCR and only 34.48% by microscopy. Tams1 based PCR assay was carried by several workers throughout the country (Kundave *et al*., 2014., Sudan et al *et al*., 2015; Chauhan *et al*., 2015; Padhiyar *et al*., 2016).

Of late, incidence of *T. annulata* infection is increasing, and it is being recorded from almost every state of the country. Though, infection in particular area is maintained in animals at a low level but under stress conditions like environmental extremes, pregnancy, nutritional and transportation stress, infection may flare up and pose threat to the health and survival of susceptible animals. Hence, for the timely and sensitive detection of *T. annulata* infection in animals, Tams1 gene based PCR assay could be used as adjunct test along with microscopic examination. This will be helpful in devising more effective preventive and control strategies against theileriosis.

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