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Comparative Performance and Evaluation of Two Molecular Assays and Conventional Detection of *Theileria annulata* in Bovines

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ABSTRACT

Background: *Theileria annulata* is predominant and of utmost economical importance tick borne pathogen of bovines in the region which is routinely diagnosed based on the microscopic examination of Romanowsky stained thin blood smears. Present study was intended to evaluate and analyze the detection efficacy of a commercial polymerase chain reaction kit based assay in comparison to conventional PCR assay and classical microscopy for detection of *T. annulata* from blood samples of bovines from Punjab state.

Methods: In this comparative study 360 bovine blood samples from various districts of agro-climatic zones in Punjab were first screened for *T. annulata* by Giemsa-stained thin blood smear (GSTBS) examination. The same panel of blood samples was tested for *T. annulata* by a commercial PCR kit Bovi-TheiDX *Theileria annulata* (Genext Genomics) (PCR1) and established conventional PCR assay targeting merozoite piroplasm surface antigen (*Tams1*) gene of *T. annulata* (PCR2).

Result: Out of 360 samples screened, positivity of *T. annulata* by GSTBS was found to be 12.5% (45/360) with a sensitivity of 37.20% and specificity of 96.00% when compared with commercial kit (PCR1), the difference was statistically significant (p<.0001). The detection prevalence by PCR1 29.70% (107/360) and PCR2 assay 33.90% (122/360) showed significant (p<.0001) difference. The conventional PCR targeting the *Tams1* gene (PCR2) was found to be more sensitive (84.6%) (p<0.001) than PCR1 (69.7%).

Key words: Bovines, Bovi-TheiDX PCR, Conventional Polymerase Chain Reaction, Prevalence, *Theileria annulata.*

INTRODUCTION

Bovine tropical theileriosis (BTT), caused by *Theileria annulata,* an apicomplexan haemo-protozoan parasite, transmitted by *Hyalomma anatolicum anatolicum* in the Indo-Asian continent, is a major tick borne ailment of economic significance (Salih *et al.,* 2015). The infection threatens 39 million crossbred cattle in India (Kolte *et al*., 2017) and causes an annual monetary loss escalating up to US$ 384.3 million (Minjauw and McLeod, 2003), but this figure may be underestimated because sub-clinical infections are not incorporated (Gharbi *et al*., 2011). As in other haemoprotozoa, indigenous and treated animals may serve as a nidus for the spread of infection and further play a salient role in the shift of the life cycle between susceptible host and tick vector (Diallo *et al.,* 2018*;* Larcombe *et al*., 2019). Usually, these carrier animals have no any obscure symptoms and may acquire very low parasitaemia, unable to be detected by the gold standard microscopy method for recognition of intraerythrocytic piroplasms and schizont stage of *Theileria* spp. parasites (Sahoo *et al*., 2017).

Series of serological tests are employed for the detection of antibodies for surveillances purpose lack the specificity and not able to distinguish between the pre and post exposure to infection (Kaur *et al.,* 2016; Sudan *et al*., 2017). The molecular revolution saw an explosion of diagnostic assays (Criado-Fornelio, 2007), that target specific genes and species and are able to detect the existence of direct pathogen's DNA (Mans *et al*., 2015; Bal *et al.,* 2014). A number of genes of different species of *Theileria* have been partially explored include major surface protein (msp), ribosomal rRNA (18S rRNA), heat shock protein (hsp), merozoite piroplasm surface antigen (Tams) and lactate dehydrogenase. The polymorphism is more in *Tams1* gene and variety of sequences of this gene were published and employed in the molecular diagnosis of *Theileria* species (Santos *et al.,* 2013; Kundave *et al*., 2018; Ganguly *et al*., 2020; Selim *et al*., 2021). The core objective of the present work was to evaluate and compare the

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detection efficacy of two nucleic acid based assays (commercial Kit based PCR and conventional PCR assay targeting *Tams1* gene) for *T. annulata* corresponding to conventional microscopic (GSTBS) diagnostic assay in bovines from Punjab state.

MATERIALS AND METHODS

Sample collection

A total of 360 blood samples for analyses were collected from various districts of five agro-climatic zones of Punjab, through field visits and GADVASU Outpatient Department (OPD), for the period from October 2020 to August 2021. The samples were processed at departmental laboratory of Veterinary Parasitology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The ethical permission was granted by the Institutional Animal Ethical Committee of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (GADVASU/2020/IAEC/53/15). Prior consent was obtained from the owners for the collection of blood of their livestock animals. Proper precautionary measures were undertaken during the sample collection from the animals.

Microscopic examination

Two thin blood smears were prepared within 6 hours after the blood sample collection and subsequently fixed in methanol and then stained with Giemsa stain (Kelly, 1979). The smears were observed under 100X of the microscope for the presence of *Theileria* piroplasms in erythrocytes and Koch's blue bodies in lymphocytes.

PCR1 assay: (Bovi-TheiDX PCR assay)

A commercial kit, Bovi-TheiDX *Theileria annulata* (Genext Genomics Pvt. Ltd) was employed in PCR1 assay. In PCR 1 assay (Bovi-TheiDX), extraction of the DNA from blood samples were performed as per the recommended protocol. Reaction mixture (20 µL) included 3.2 µL of PCR buffer; 0.8 µL Bovi-TheiDX Primer mix; 1.6 µL GNG Taq polymerase, 13.4 µL of nuclease-free water, 1 µL of genomic DNA mixed as prescribed by manufacturer's protocol. PCR conditions for assay were initial denaturation step of 95°C for 5 min followed by 30 cycles of denaturation (95°C for 1min), annealing (57°C for 40 s), extension (72°C for 2 m) and final extension at 72°C for 10 min.

PCR 2 (*Tams1* PCR assay)

Conventional PCR targeting the *Tams1* gene of *T. annulata* was standardized and employed as per the conditions of Ganguly *et al*. (2020). For PCR2 assay genomic DNA from all the samples was extracted by using QIAGEN™ blood and tissue isolation kit as per the manufacturer's protocol and was stored at -20°C, till further use.

The primers targeting *Tams1* gene of *T. annulata* were as TAF5′ -TGAGTTAACTGTCGCGGATG-3′ and TAR 5′ -TGGGCAGGGTGAAGATTAAG-3′ (Ganguly *et al*., 2020). The PCR reaction mixture (25 µL) included 12.5 µL of master mix (GeneDireX OnePCR™), primers forward (TAF) and reverse primer (TAR) (10 pmol) 1 µL each, DNA template (2 µL) and 8.5 µL of nuclease-free water (NFW). The reaction was amplified in Thermal Cycler (Applied Biosystems,Veriti™ 96-Well) with amplification condition assay as initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, 53°C for 30 s and 72°C for 45 s, with a final extension at 72°C for 10 min. The negative or non-template control was run together with the samples to ensure the cross contamination and the quality of the results. The amplified PCR products were separated on 2% agarose gel stained with 5 µL of ethidium bromide by gel electrophoreses assembly (Bio-Rad Electrophoresis system) and visualized under gel documentation sysytem (Syngene, UK) for 420 bp in PCR1 (Fig 2) and for 156 bp in PCR2 assay (Fig 3) of *Theileria annulata.*

Statistical analysis

The results of the GSTBS, PCR1 (Bovi-TheiDX PCR assay) and PCR2 (*Tams*1 gene PCR assay) assays were statistically compared by chi-square test using the SAS software. The sensitivity and specificity of the GSTBS, PCR1 and PCR assays were analyzed by AUC (Area under the curve) and ROC (Receiver Operating Characteristics) curve using the SAS software.

RESULTS AND DISCUSSION

The microscopic examination of Giesma stained thin blood smears (GSTBS) smears revealed pleomorphic piroplasms of round, oval, elongate, ring and anaplasmoid forms in the erythrocytes and schizonts in lymphocytes (Koch's blue body) of *T. annulata* (Fig 1a 1b; Soulsby, 2006) in 45 out of 360 animals examined with an overall positivity of 12.5% (Table 1). Similarly, earlier reports from the Punjab province based on the microscopy showed positivity of bovine theileriosis in range of 9.23-14.65% (Tuli *et al*., 2015). However, varied prevalence from different states of the country including Tamil Nadu (13.0-55.27%) (Velusamy *et al.,* 2014; Reetha *et al.,* 2012), Uttarakhand (27.2%) (Kohli *et al.,* 2014), Gujarat (7.08-82.94%) (Vahora *et al.,* 2012; Maharana *et al.,* 2016) and in West Bengal (22.9%) (Debbarma *et al.,* 2018) has been reported earlier. The

Table 1: Comparative evaluation of microscopic examination along with commercial kit based PCR assay and convention PCR assay for detection of *Theileria annulata* in bovine samples.

Samples examined	Microscopic examination	PCR1 (Bovi-TheiDX PCR assay)	PCR 2 (*Tams1* gene PCR assay)
Total number of samples	360	360	360
Positive	45	107	122
Prevalence	12.5%	29.7%	33.9%
Sensitivity	32.7%	69.7%	79.4%
Specificity	96.0%	92.0%	85.4%
Level of significance	<.0001	<.0001	<.0001

variation in prevalence is attributed to the biotic and abiotic factors responsible for the tick propagation and the managemental conditions adopted by the farmers (Singh *et al.,* 2000). The detection prevalence by PCR1 29.7% (107/360) and PCR2 assay 33.9% (122/360) showed significant (p<.0001) difference. The prevalence by both PCR assays was higher than the microscopy (12.5%.), indicating that the conventional GSTBS technique not able to detect the carrier or latent cases of theileriosis with less than 1% parasitaemia and moreover unable to differentiate the different species of *Theileria* that are morphologically homogeneous (Friedhoff and Bose, 1994; Maharana *et al*., 2016). A peculiar feature of bovine theileriosis is the persistent carrier stage of infection that acts as a source of infection for the whole herd. Moreover, such animals often skip microscopic detection due to lower parasitemia and hence, detection by PCR remains an important alternative. The sensitivity of molecular technologies in comparison to conventional parasitological methods have been elucidated by various authors (Tuli *et al*., 2015; Maharana *et al*., 2016; Sudan *et al*., 2017; Acharya *et al.,* 2017).

The sensitivity and specificity of PCR1 was found to be 69.7 and 92.0 per cent in relation to PCR2 (Table 1). The comparison between two assays showed the conventional PCR targeting the *Tams1* gene PCR2 was found to be more sensitive (84.6%) (p<0.001) than PCR1 (69.7%) (Table 1). Generally the efficacy of any PCR protocol depends greatly upon the choice of primer preferred. The merozoite surface antigen (*Tams1*) is highly specific for *T. annulata* and it does not show cross reactivity with other species of *Theileria* including *T. parva, T. mutans, T. sergenti* and *T. buffali* (d'Oliveira *et al.,* 1995). The molecular diagnosis of *T. annulata* targeting *Tams1* gene was widely exploited throughout the globe including India (Tuli *et al*., 2015; Kundave *et al*., 2018; Ganguly *et al*., 2020; Selim *et al*., 2021). The limitation of PCR Bovi-TheiDX PCR assay that gene was not mentioned and is laborious. Additionally about 400 µL of blood is required for the DNA extraction in the PCR1 assay.

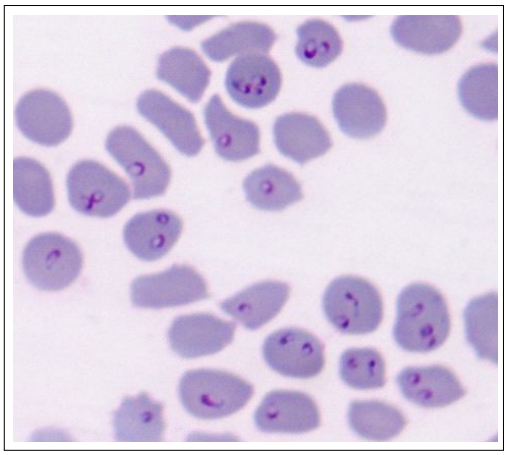

Fig 1a: Photomicrograph of Giemsa stained thin blood smear revealing pleomorphic Theileria annulata piroplasms in the erythrocytes (100X).

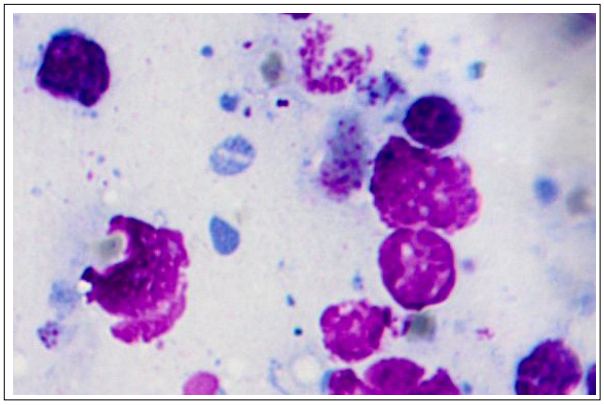

Fig 1b: Photomicrograph of Giemsa stained thin blood smear showing schizonts (Koch's blue body) of *Theileria annulata* (100X).

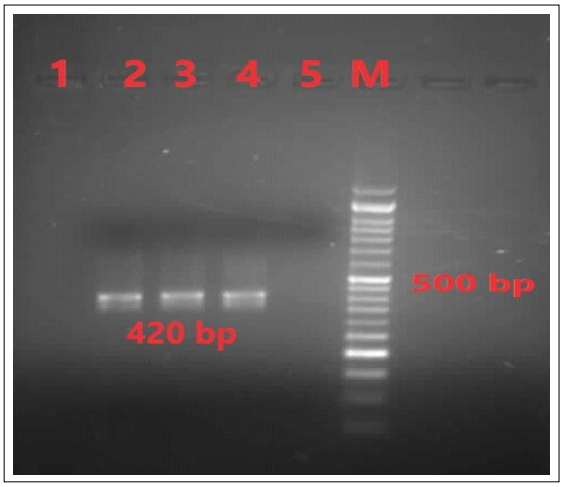

Fig 2: Amplified DNA product of 482 bp by Commercial kit (Bovi-TheiDX).
Lane 1-4: Test samples; Lane 5-: Negative control; M -: Marker 50 bp.

Fig 3: *Tams1* PCR showing amplified DNA product of 156 bp.
Lane-1-4, 6-15: Field samples; Lane 5: negative control; Lane M: 100bp marker
Lane10: Positive control.

CONCLUSION

To conclude, conventional PCR2 assay is a better tool than the commercial kit PCR1 assay and the conventional parasitological GSTBS technique. Further the choice of diagnostic method should be evaluated in accordance with the reality of sensitivity, reproducibility, time consumption and reliability.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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