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Clinico-morphological and Molecular Detection of Bovine Tropical Theileriosis in Cattle

Anil Kumar[1], Rajesh Kumar[2], Ramesh Tiwary[2], Bhoomika[3], G.D Singh[1], Ajit Kumar[4], Anil Gattani[5], Pallav Shekhar[6]

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ABSTRACT

Background: *Theileria* spp. were targeted for detection and differentiation from clinical samples using PCR in the current investigation and the diagnostic sensitivity of this method was evaluated in comparison to microscopic observations. The clinical characteristics of bovine tropical theileriosis as clinical markers were also analysed based on the PCR data.

Methods: On the basis of clinical evaluation and microscopic inspection of blood smears, 200 blood samples from cattle suspected of having theileriosis were examined. To confirm the disease, the PCR method was employed by using 18S rRNA and *Cyto* b1 gene to confirm *Theileria* spp. and *Theileria annulata* respectively in clinical samples.

Result: The results showed that microscopic examination detected 65/200 (32.50%), whereas, the PCR assay identified 120/200 (60.00%) for *Theileria* spp. Of the total 200 samples studied, 77 (38.50.3%) were positive for *T. annulata* specific PCR. Based on PCR confirmation of *T. annulata* infection in cattle, the predominant clinical signs were pyrexia, ticks' infestations, anorexia, enlargement of lymph node, pale mucous membrane, lacrimation, coughing, drop in milk production and emaciation. The other clinical signs were observed as icterus, nasal discharge, salivation, melena and exophthalmia.

Key words: Clinical signs, Microscopic examination, PCR, *Theileria* spp. *Theileria annulata*.

INTRODUCTION

Bovine theileriosis is a widespread tick-borne disease of cattle in tropical and subtropical regions brought on by intracellular hemoparasites of the genus Theileria (Bursakov and Kovalchuk, 2019). *Thileria annulata* (*T. annulata*) widely considered to be more pathogenic causing Tropical Theileriosis and *Theileria orientalis* generally considered to cause Benign Theileriosis are the two main species found in India, that infect bovines and transmitted by *Hyalomma anatolicum anatolicum* and *Haemophyssalis bispinosa* respectively (Aparna *et al.,* 2011).

Theileriosis is mostly diagnosed in the field based on clinical symptoms and a tick infestation on the diseased animals (Qayyum *et al.,* 2010) and conventional laboratory techniques. The "Gold Standard" diagnostic test among conventional diagnostic methods is still the direct microscopic identification of theileria in blood smears and/or lymph node fluid smears. However, these tests are robust, have low sensitivity and are completely dependent on trained laboratory technicians. Giemsa stained thin blood smears are usually sufficient for detecting acute infections, as evidenced by *Theileria* schizonts in lymph node smears and piroplasms alone or in conjunction with schizonts in blood smears, but may not be in sub-clinical and carrier infections due to very low parasitemia (Salih *et al.*, 2015). On blood slides, it is challenging to distinguish the morphology of Theileria species' piroplasms (Almeria *et al.,* 2001) especially due to mixed infections. Deoxyribonucleic acid (DNA)-DNA hybridization and the polymerase chain reaction (PCR) have combined to produce very sensitive and specific molecular diagnostic techniques for the detection and characterization of the organisms that cause theileriosis (Collins *et al.,* 2002). *Theileria annulata* can be distinguished from other non-pathogenic *Theileria* species using PCR, which is thought to be a very sensitive and specific diagnostic method for pathogen identification and differentiation.

Aiming to identify and distinguish *Theileria* spp. from clinical samples using PCR assays, this study's diagnostic

[1]Department of Veterinary Clinical Complex, Bihar Veterinary College, Bihar Animal Sciences University, Patna-800 014, Bihar, India.
[2]Department of Veterinary Surgery and Radiology, Bihar Veterinary College, Bihar Animal Sciences University, Patna-800 014, Bihar, India.
[3]Department of Veterinary Public health, Bihar Veterinary College, Bihar Animal Sciences University Patna-800 014, Bihar, India.
[4]Department of Veterinary Parasitology, Bihar Veterinary College, Bihar Animal Sciences University Patna-800 014, Bihar, India.
[5]Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry, Jabalpur-482 001, Madhya Pradesh, India.
[6]Department of Veterinary Medicine, Bihar Veterinary College, Bihar Animal Sciences University, Patna-800 014, Bihar, India.

Corresponding Author: Anil Kumar, Department of Veterinary Clinical Complex, Bihar Veterinary College, Bihar Animal Sciences University, Patna-800 014, Bihar, India.
Email: dranilvet95@gmail.com

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sensitivity was compared to microscopic observations in light of the limitations of conventional diagnostic approaches. Based on PCR results, the clinical signs of bovine tropical theileriosis as clinical markers were also evaluated.

MATERIALS AND METHODS

Selection of animals

The present study was held in the Department of Veterinary Medicine, BVC, BASU, Patna during March 2020 to February 2022. A total of 200 cattle of age ranging 3 months-6 years, either sex and irrespective of breed were selected based on the clinical signs suggestive of theileriosis like high fever of 104-107°F, swelling of parotid lymph nodes, pre-scapular and pre-femoral lymph nodes, pale mucous membrane, suspended rumination, bilateral nasal discharge, lacrimation and or which have the problem of tick infestation *etc*.

Collection of blood samples

The blood samples @ 3 ml from each suspected cases were collected aseptically from jugular vein and transferred in EDTA coated vial for preparation of thin blood smears and DNA extraction.

Microscopic examination of blood smears

Blood smears were stained by Giemsa's staining technique. The smears were examined microscopically for the presence of *Theileria* piroplasms in erythrocytes and Koch's blue bodies in lymphocytes. The presence of even a single piroplasm was considered positive for *Theileria* spp. Before considering it negative, at least 50 microscopic fields per slide were observed. The morphological characteristics of *Theileria* were identified according to key described by Soulsby (1982).

DNA isolation and PCR Amplification

Genomic DNA from each blood sample was isolated by using the Wizard® Genomic DNA Purification Kit (Promega, USA), as per the manufacturer's instructions and stored at - 20°C till future use. The different sets of primers used in the study were custom synthesized from Eurofins Genomics Pvt. Ltd. (India) (Table 1) for the amplification of targeted genes of *Theileria* spp. both on genus and species-specific grounds. The PCR was performed as per the method described previously (Cao *et al.,* 2013) with slight modification briefly, 25 µl reaction mixture containing 12.5 µl of MAX PCR Master Mix (2×Premix) (Takara), 2.0 µl (10 pmol/ µl) of each primers, 2 ìl of the DNA template and nuclease free water (Thermo Scientific, USA) to make total volume up to 25 µl. Positive control and negative control have been included in each run. The cycling condition for *Theileria* genus was, Initial denaturation at 95°C for 5 minutes and 35 cycles of denaturation; 1 min. at 94°C, annealing; 1 min. at 60°C and extension 90 seconds at 72°C and final extension for10 minutes at 72°C. The cycling condition for *T. annulata* was initial denaturation at 95°C for 2 minutes and 35 cycles of: denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The amplified PCR product was checked by electrophoresis on 1.5% agarose gel stained with ethidium bromide in horizontal electrophoresis apparatus (Thermo Scientific, China) and viewed in gel documentation system (VILBER).

Data analysis

Chi square test was performed to test the association between blood smear examination and PCR.

RESULTS AND DISCUSSION

The results of present investigation showed that microscopic examination detected 65/200 (32.50%), whereas, the PCR assay identified 120/200 (60.00%) for theileria spp. Of the total 200 samples studied, 77 (38.50.3%) were positive for *T. annulata* specific PCR (Table 2). Under microscopic examination, low to high numbers of polymorphous theileria parasites were observed mostly as annular or round form and some were detected as in oval, coma or dot form inside the red blood cells of the stained blood smears (Fig 1) and also in few cases Koch's blue bodies inside lymphocytes were observed (Fig 2).

Table 1: *Theileria* genus specific, species specific and self-designed primers.

Pathogens	Primername		Oligonucleotide sequences (5′-3′)	Product size (bp)	References
Theileria spp.	18SrRNA	F	GAAACGGCTACCACATCT	778	Cao *et al.* (2013)
		R	AGTTTCCCCGTGTTGAGT		
T. annulata	Cytob1	F	ACT TTG GCC GTA ATG TTA AAC	312	Bilgiç *et al.* (2013)
		R	CTC TGG ACC AAC TGT TTGG		

Table 2: Animals positive for the presence of *Theileria* sp. by microscopic examination (ME) of blood smears and by PCR for *Theileria* spp. and *Theileria annulata*.

Technique	Animal screened	Positive for theileriosis	Percent positive (%)
Microscopic examination of *Theileria* spp.	200	65	32.50
Theileria spp. by PCR	200	120	60.00
T. annulata by PCR	200	77	38.50
Theileria spp. other than *T. annulata*	200	43	21.50

The results of present study are in agreement with those of Kumar *et al*. (2022). *Theileria* genus specific *18S rRNA* gene was used (Fig 3), because it has been shown to be an effective marker for investigation in *Theileria* spp. (Cao *et al.,* 2013). PCR assay targeting *cytob1* gene was used to amplify the single and specific 312 bp fragment to diagnose the tropical theileriosis (Fig 4). Bilgic *et al*. (2010) reported that cytochrome b gene is highly sensitive in detection of *T. annulata* infections in cattle and *cytob1* gene is highly specific for the detection of *T. annulata* parasites at the level of low parasitaemia, especially in carrier cattle and can also discriminate *T. annulata* from non-pathogenic *Theileria* species and other haemoparasites (Bilgic *et al*., 2013). This demonstrates the effectiveness of the PCR technique for theileriosis confirmation and supports the use of genus- and species-specific primers in our research.

Previous research has conclusively demonstrated that, when used to diagnose *Babesia* species and *T. annulata*, PCR-based methods are more sensitive than other diagnostic techniques (Kundave *et al.,* 2017).

In the present study, 65 (32.50%) and 120 (60%) samples were found positive for *Theileria* spp. by microscopic examination and PCR, respectively. Chi-square statistical analysis revealed a significant (p<0.05) difference in detection sensitivity when PCR assay was compared with microscopic examination. The PCR included those 55 samples which were found negative by Giemsa's staining (Table 3). Considering microscopic blood smear examination as the gold standard method, the sensitivity of PCR was found to be 100% in clinically suspected animals. The present study findings are in agreement with Charaya *et al.* (2016), Rajkumar *et al*. (2020) and Ullah *et al.,* (2021) who reported a significantly higher sensitivity of PCR technique in detection of *Theileria* spp. as compared to microscopic blood smear examination and the technique also allowed for specific discrimination between pathogenic and non-pathogenic theilerias which cannot be accomplished by traditional diagnosis by microscopic observation (Almeria *et al.,* 2001).

Due to artefacts, incorrect staining, inexperience, loss of the piroplasmic form as a result of hemolysis and inadequate sensitivity, the microscopic examination of blood smears revealed false negative results (Chauhan *et al.,* 2015) and lack of discrimination of other morphologically related parasites if mixed infections (Ullah *et al.,* 2021). This makes it quite evident that PCR assays are better to microscopy examinations.

Out of 200 clinically examined animals in our study, 77 (38.50%) animals tested positive for *T. annulata* on PCR assays and displayed clinical indications of disease

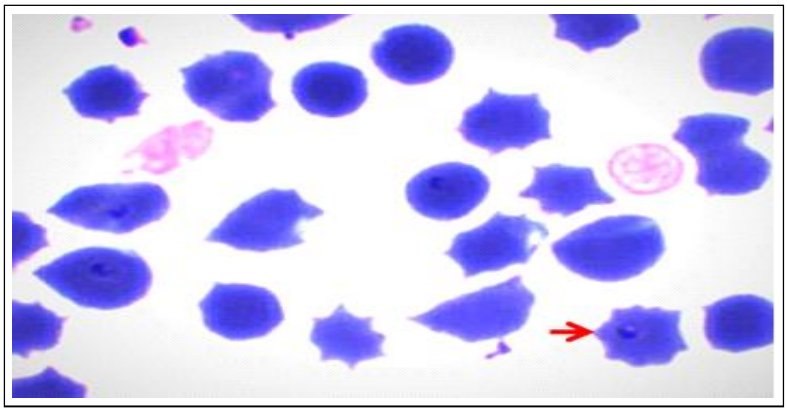

Fig 1: (GSTBS × 100) showing signet ring shaped piroplasms of *Theileria* spp.

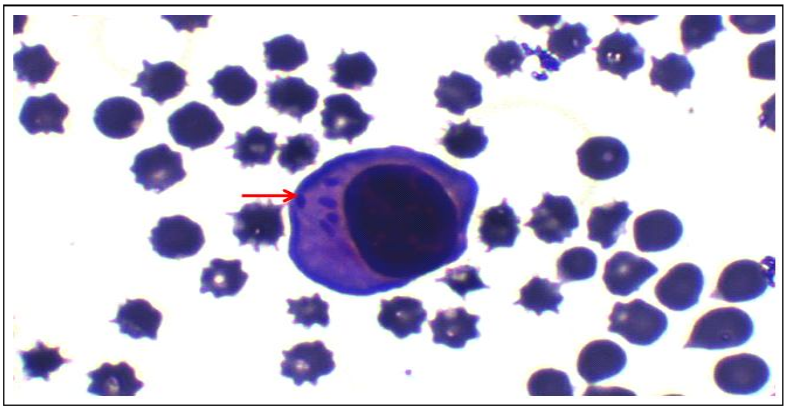

Fig 2: Photomicrograph (GSTBS × 100) showing Koch's blue bodies of *Theileria* spp. in lymphocytes.

(Table 4). The predominant clinical signs in cattle suffering from tropical theileriosis were pyrexia (100%), ticks infestations (92.20%), Anorexia (80.51%), enlargement of lymph node (75.32%), pale mucous membrane (70.12%), lacrimation (68.83%), coughing (59.74%), drop in milk production (49.35%) and emaciation (41.55%).

The other clinical signs were observed as icterus (24.67%), nasal discharge (23.40%), salivation (22.10%), melena (15.58%) and exophthalmia (11.68%). Clinical indicators of T. annulata infection include anaemia, wasting, jaundice and enlargement of superficial lymph nodes (Ma *et al*., 2020). In the present study, 92.20 percent of theileriosis affected animals had tick infestation which is comparable to the results obtained by Khawale *et al*. (2020), who had observed 92.53 per cent of theileriosis affected animals with tick infestation, which are a major risk factor for the spread of theileriosis (Khattak *et al.,* 2012). Variation in clinical signs shown by the animals in the present study might be attributed to various housing and management practices along with the degree of infection. In the early stages of the disease, due to increase in microschizont proliferation inside lymphocytes and inflammatory reactions in the infected lymph nodes, lymphoid hyperplasia superficial lymph nodes is observed (Al-Emarah *et al.,* 2012).

In the present study, hyperthermia ranged from 103°F to 105°F was observed in cattle affected with bovine tropical theileriosis. The diverse nature of clinical symptoms of Theleriosis are due to high levels of inflammatory cytokines (TNF-α, IL-1 and IL-6), produced by infected mononuclear cells (Col and Uslu, 2006). The clinical signs like anorexia, emaciation and melena in the present study might be due to the increased cytokines. Overproduction of TNF-α and lymphocyte infiltration are thought to be contributing factors for ophthalmopathy in calf theileriosis (Shanker *et al.,* 2013).

A significant loss in milk production and milk composition has been reported because of Theileriosis (Memon *et al.,* 2017; Perera *et al.,* 2014).

Pale mucus membranes indicator of anaemia, might be due to removal of the parasitized erythrocytes by reticulo-endothelial system (Farooq *et al.* 2019), persistent blood loss due to permanent blood sucking ticks (Durrani *et al.,* 2008), reduced erythrogenesis due to TNF-α (Boulter and Hall, 2000) or due to erythrophagocytosis (Modi *et al.,* 2015).

The respiratory symptoms like nasal discharge, respiratory discomfort and cough, could be attributed to advanced cases of severe pulmonary edema caused by released vasoactive substances from collapsing alveolar cells (Abdel-Hamied *et al.,* 2020).

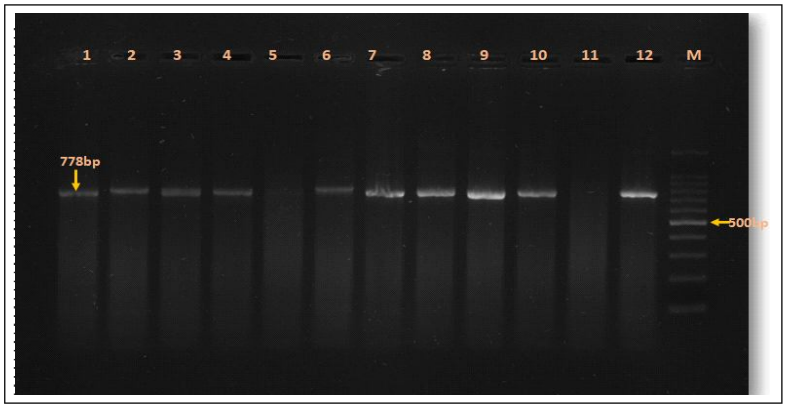

Fig 3: Showing the gel electrophoresis image of *18srRNA* amplification of *Theileria* genus, where Lane 5 negative sample; Lane 11 negative control; Lane 12 positive control; other lane positive samples; lane M: 100 bp ladder.

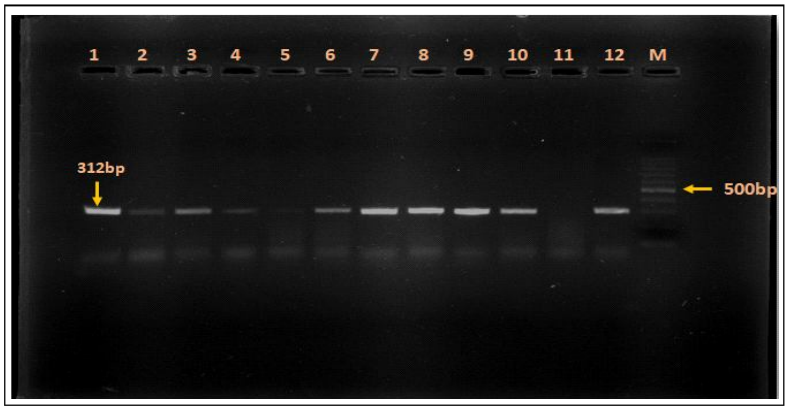

Fig 4: Showing the gel electrophoresis image of *Cytob1* gene amplification of *T. annulata*, where Lane 1-10 positive sample; Lane 11 negative control; Lane 12 positive control; lane M: 100 bp ladder.

Table 3: 2×2 Contingency table for comparison of blood smear and PCR.

PCR	Blood smear examination		Total
	Positive	Negative	
Positive	65[a]	55[b]	120
Negative	0[c]	80[d]	80
Total	65	135	200

a= True positive, b= False positive, c= False negative, d= True negative.
Sensitivity of PCR= a/a+c × 100= 100% at CI 95% (0.9442 to 100.00).
Specificity of PCR= d/b+d × 100= 59.26% at CI 95% (0.5083 to 0.6718).

Table 4: Clinical signs in cattle infected with *T. annulata*.

Clinical signs	No. of animals (N= 77)	Percentage (%)
Fever (103-105°F)	77	100
Ticks infestation	71	92.20
Swollen lymph nodes	58	75.32
Coughing	46	59.74
Nasal discharge	18	23.40
Lacrimation	53	68.83
Salivation	17	22.10
Anorexia	62	80.51
Drop in milk production	38	49.35
Pale mucous membranes	54	70.12
Icterus	19	24.67
Emaciation	32	41.55
Exophthalmia	09	11.68
Melena	12	15.58

Therefore, thorough inspection of diseased cattle based on clinical signs especially of swollen lymph nodes, pallor mucous membranes, pyrexia, coughing respiratory distress, lacrimation and exophthalmia, as major clinical markers might be used in clinical diagnosis of *T. annulata* infection in the field conditions.

CONCLUSION

PCR assay demonstrated higher sensitivity for diagnosing Bovine theileriosis than microscopy. *Theileria annulata* may be distinguished from non-pathogenic Theileria species and other hemoparasites using the 18S rRNA gene and Cyto b1 gene based PCR assay, which can also be used to monitor the effectiveness of pharmacological treatments. Despite the lack of diagnostics in the field, considering these clinical manifestations, particularly swollen lymph nodes, pallor of the mucous membranes, pyrexia, coughing, respiratory distress, lacrimation and exophthalmia as the major clinical markers could help in making a clinical diagnosis of theileriosis in cattle.

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Conflict of interest: None.

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