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Comparison of microscopic and PCR technique for the detection of genus *Theileria* in large ruminants of Uttarakhand state

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Abstract
Bovine tropical theileriosis is a deadly tick borne disease of large ruminants caused by *Theileria annulata*. The aim of this study was to detect *T. annulata* in large ruminants of Uttarakhand state and comparison of microscopic and PCR technique. A total of 1006 blood samples of suspected animals for theileriosis across the Uttarakhand state. All the samples were initially screened for theileriosis by microscopic examination using Giemsa's staining technique and by doing Polymerase Chain Reaction (PCR) targeting 1098 bp fragment of *Tams1* gene. Total of 209(20.77%) and 283 (28.13%) of blood samples were found positive for theileriosis by microscopic and PCR technique respectively. The study showed that sensitivity and specificity of TBE was 73.85% and 100%, respectively while the same of PCR assay were 100%.

Keywords: Thin blood smear, Theileriosis, Tams 1 gene, large ruminants

Introduction
In India, theileriosis is one of the most economically important blood protozoan diseases of large ruminants that results in mortality and economic loss of amounting US$ 80 million approximately to livestock industry (Brown, 1997) [7]. It is caused by *Theileria annulata* an intracellular parasite of Apicomplexa group. The clinical symptom comprises of anorexia, enlarged superficial lymph nodes, fever, jaundice and anaemia and the disease become fatal when untreated. Since last decade theileriosis has become endemic and prevalent in the tarai region of Uttarakhand state resulting significant economic loss to the state. Therefore there is a need of early and precise diagnosis of theileriosis in carriers as well as in clinically diseased animals. The diagnosis of theileriosis is mainly based on clinical signs and was confirmed by microscopic examination using Giemsa stain. This conventional method have drawbacks like not able to identify piroplasmic stages of parasites in carrier animals and also require expertise in slide examination (Nourollahi *et al*., 2012) [13]. Serological methods like IFAT also show false negative or positive results during examination (Burridge *et al*., 1974) [6]. Therefore there is a need of that diagnostic method which has high specificity and sensitivity than conventional technique. In the present study, molecular technique (polymerase chain reaction) has been used for the diagnosis of *Theileria annulata* targeting 18SrRNA gene along with microscopic method.

Materials and Methods
Area of Study
Blood samples of cattle and buffaloes suspected for theileriosis infection were collected from different regions of Uttarakhand state from May, 2018 to June, 2021.

Examination of Blood Samples
Total of 1006 blood samples of bovines (754 of cattle and 252 of buffaloes) for a time of two years (May, 2018 to June, 2021) were collected from different regions of Uttarakhand state, which were presented to Veterinary Hospitals with the indications like anorexia, pyrexia, tick infestation, drop in milk production, nasal discharge, lacrimation and pale mucous membrane suggestive for theileriosis. Received blood samples from different areas were collected and blood smear was prepared instantly and rest part was stored at 4^0C for further examination.

Processing of blood samples

Giemsa Staining and Microscopic Examination

Thin smears of blood were made on grease free microscopic glass slides. Air dried blood smears were fixed for one min with absolute methanol and air dried followed by staining with Giemsa stain (1: 20) for 40 min as described by Soulsby (1982) [18]. After staining, the slides were washed in running water and air dried. The air dried smears were observed under oil immersion objective (magnification of 100 X). Piroplasms stage were searched in RBC's (50 fields x 400 RBC per field) (Aktas *et al*., 2006) [1].

Extraction of DNA from blood

The extraction of DNA from blood was done as per the protocol of Sambrook *et al*. (1989) [14] with slight modifications. Initially, the blood sample stored at -20^{0}C was thawed to room temperature. Blood sample (300µl) along with lysis buffer 700 µl (5M NaCl, 1M Tris-Cl, 10% SDS, 0.5 M EDTA), 2µl of RNase A and 5µl Proteinase K (Genei, Banglore, 20mg/ml) were taken in a 2ml capacity micro centrifuge tube and vortexed followed by overnight incubation at overnight at 56 ^{0}C in shaker incubator (New Brunswick Eppendorf, Germany).

Tris saturated phenol was added in same volume (1ml) to the lysed mixture and vortexed followed by centrifugation for 15 minutes. Supernatant was poured to a new sterile micro centrifuge tube and same amount of Phenol: Chloroform: Iso-amylalcohol (25: 24: 1) was added and followed by vortex and centrifugation. Upper aqueous part and Chloroform: Iso-amyalcohol (24:1) were vortexed and centrifuged. The upper aqueous part and double quantity of chilled isopropyl alcohol were centrifuged for 1 minute. A DNA pellet was seen in the micro centrifuge tube which was washed two times with chilled molecular grade ethanol (70%). The DNA pellet was kept at 37^{0}C for few minutes for air dry. To re-suspend the DNA, nuclease free water (NFW) (50µl) was added. The resuspended DNA was stored at-20^{0}C till further use.

Amplification of 18S rRNA gene of *Theileria* sp.

Amplification of 18S rRNA gene of *Theileria* sp. was done using oligonucleotide primer sequences: forward primer 5'-AGTTTCTGACCTATCAG-3' and reverse primer 5'-TTGCCTTAAACTTCCTTG-3' which amplifies 1098bp fragment of *Tams1* gene (Allsopp *et al*. 1993) [3]. In PCR tubes the volume reaction of 25µl have 1µl of DNA template, 12.5µl of PCR Master Mix (Genei, Bangalore), 1 µl of each forward and reverse primer and rest nuclease free water. The PCR-assay was conducted in an automated EP- Gradient S thermocycler (Eppendorf, Germany) programmed with following conditions: initial denaturation, annealing and final extension. Amplified PCR products were examined by agarose gel electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5µg/ml). The photograph and size of the gene was documented using a gel documentation system (Alpha Innotech, USA). The remaining PCR product was stored at -20^{0}C till further use.

Specificity and sensitivity of microscopic examination

The specificity and sensitivity of microscopic method was evaluated in respect to molecular (PCR assay) using following method used by Noaman, (2014) [12]

$$\text{Specificity (\%)} = \frac{\text{(Giemsa's staining and PCR negative)}}{\text{PCR negative}} \text{ x } 100$$

$$\text{Sensitivity (\%)} = \frac{\text{(Giemsa's staining and PCR positive)}}{\text{PCR positive}} \text{ x } 100$$

Results and Discussion

Giemsa stained thin blood smear examination of 1006 blood samples showed the presence of *Theileria* sp. piroplasm in 209 samples (buffalo, cattle) with overall prevalence of 20.77% as shown in Figure-1. While, PCR amplification of 18SrRNA gene was found in 283 blood samples (cattle, buffalo) with the overall prevalence of 28.13%. Results obtained from each method of examination of samples were compared to find out the sensitivity of detection methods.

PCR method was found more sensitive than thin blood smear examination as shown in Table - 1. PCR amplification of partial 18S rRNA *Tams1*gene was *Theileria* genus specific at 1098bp as shown in Figure-2.

Comparison of molecular assays and conventional methods to detect theileriosis has been attempted by several workers the world over (Durrani and Kamal, 2008; Shahnawaz *et al*., 2011) [9, 15] while in India such type of comparative studies are very few (Kundave *et al*., 2013; Singla *et al*., 2013; Arun Raj, 2014 [11, 17, 4]. In India, blood examination is the most common and convenient method for detection of *Theileria* sp. However, in the present study it was found that TBE has low sensitivity in detecting *Theileria* sp. in blood samples. On the other hand, in the present study it was found that PCR method was more sensitive and accurate in diagnosing *Theileria* sp. organism up to the species level. These findings are in accordance with the studies of Almeria *et al*. 2001 [2] and Azizi*et al*. 2004 [5]. In the present study, PCR assay was found 1.35 times more precise in diagnosis of theileriosis infection as compared to TBE while Arun Raj, 2014 [4] found PCR assay 1.77 times more accurate than TBE. Similarly, Kohli *et al*. 2014 [10] used AS-PCR assay for amplification of partial 18S rRNA gene and detected 16 more samples than TBE and found PCR 1.19 times more sensitive than TBE. Aktas *et al*. (2006) [1] found PCR 2.78 times more accurate than TBE.

Table 1: Sensitivity and specificity of TBE in respect to PCR assay for detection of *Theileria* sp. in blood samples

Method/Technique	NE	NP	Sensitivity (%)	Specificity (%)
TBE	1006	209	73.85	100
PCR	1006	283	100	100

NE: Number of samples examined
NP: Number of samples positive

The present study revealed that 209 and 283 blood samples of large ruminants of Uttarakhand state were found positive for theileriosis by TBE and PCR assay, respectively. On comparison of sensitivity and specificity of TBE and PCR in the present study, it was found that sensitivity and specificity of TBE was 73.85% and 100%, respectively while the same of PCR assay were 100% as shown in Table - 1. The study was in accordance with the findings of Chauhan *et al*. (2015) [8] who found the sensitivity as 70.83% and 100% for TBE and PCR assay and specificity as 100% for both TBE and PCR assay, while Noaman, 2014 [12] found sensitivity as 57% and 100% for TBE and PCR and specificity as 99% and 100% for TBE and PCR, respectively in TBE and PCR. In India, thin blood smear examination is the most conventional method for the diagnosis of theileriosis infection in blood samples. However, PCR is also now being routinely used for the diagnosis of *Theileria* sp. infections, mainly to detect carrier animals (Shayan and Rahbari, 2005) [16].

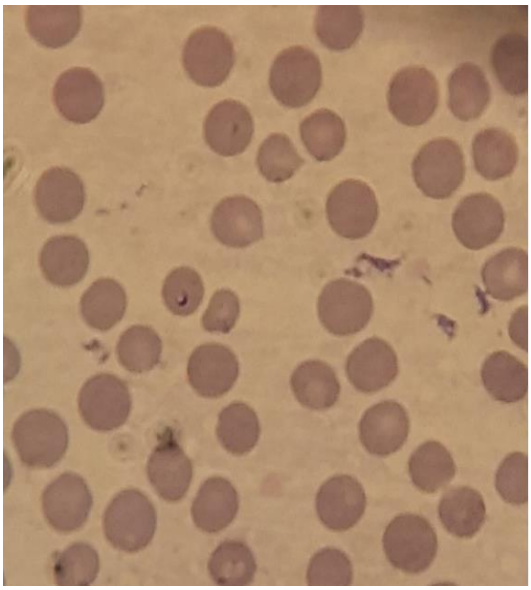

Fig 1: Piroplasmic stage of *Theileria* sp. in RBC after Giemsa staining

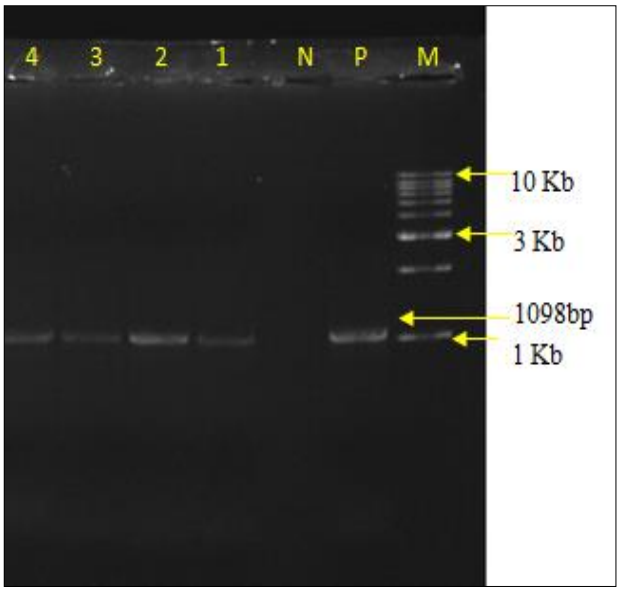

Fig 2: Agarose gel electrophoresis showing 1098bp PCR product. M: 1Kb mol. wt. DNA marker, Lane P: Positive sample, Lane N: Negative sample, Lane 1-4: Positive samples showing amplicon of 1098bp.

Conclusion of the study

The present study concluded that the molecular assay (PCR) is more sensitive than thin blood smear examination for the detection of theileriosis. Therefore, molecular technique should be preferred for screening the samples suspected for theileriosis as it detects infection in carriers as well as in clinically diseased animals.

References

1. Aktas M, Altay K, Dumanly N.A molecular survey of bovine Theileria parasites among apparently healthy cattle with a note on the distribution of ticks in eastern Turkey. Vet. Parasitol. 2006;138:179-85.
2. Almeria S, Castella J, Ferrer D, Ortuno A, Estrada-Pena A, Gutierrez JF. Bovine piroplasms in Minorca (Balearic Islands Spain): A comparison of PCR based and light microscopy detection. Vet. Parasitol. 2001;99:249-259.
3. Allsopp BA, Baylis HA, Allsopp MTEP, Cavalier-Smith T, Bishop RP, Carrington DM, *et al*. Discrimination between six species of Theileria using oligonucleotide probes which detect small subunit ribosomal RNA sequences. J Parasitol. 1993;107:157-165.
4. ArunRaj MR. Studies on molecular epidemiology of Theileria annulata and its genetic characterization based on 18S rRNA and Tams 1 gene.Thesis, Master in Veterinary Science. G.B.Pant University of Agriculture & Technology, Pantnagar; c2014. p. 104.
5. Azizi H, Shiran B, Dehkordi AF, Salih DA, Boguslawski K, Beyer D, *et al*. Detection of Theileria annulata by PCR and its comparison with smear method in native carrier cows. Biotech. 2004;7:574-577.
6. Burridge MJ, Brown CGD, Kimber CD. Theileria annulata: Cross reactions between a cell culture schizont antigen and antigens of East Africa Theileria Species in the indirect fluroscent antibody test. Experimental Parasitology. 1974;35:374-380.
7. Brown CGD. Dynamics and impact of tick-borne diseases of cattle. Tropical Animal Health Production. 1997;29:1S-3.
8. Chauhan HC, Patel AG, Bhagat AG, Patel MV, Patel SI, Raval SH, *et al*. Comparison of molecular and microscopic technique for detection of Theileria annulata from the field cases of cattle. Vet. World. 2015;8(11):1370-1374.
9. Durrani AZ, Kamal N. Identification of ticks and detection of blood protozoa in Friesian cattle by polymerase chain reaction test and estimation of blood parameters in district Kasur, Pakistan. Trop. Anim. Hlth. Prod. 2008;40:441-447.
10. Kohli S, Atheya UK, Srivastava SK, Banerjee PS, Garg R. Outbreak of theileriosis and anaplasmosis in herd of lolstein crossbredcows of Dehradun district of Uttaranchal, India: A himalayan region. Int. J. Livest. Prod. 2014;5(1):182-185
11. Kundave VR, Patel PV, Hasnani JJ, Patel AK, Joshi CG. Detection of theileriosis in cattle and buffaloes by polymerase chain reaction. J Vet. Parasitol. 2013;27(2):92-95.
12. Noaman V. Comparison of molecular and microscopic technique for detection of Theileria spp. in carrier cattle. J Parasit. Dis. 2014;38(1) 64-67.
13. Nourollahi-Fard SR, Khalili M, Ghalekkhani N. Detection of Theileria annulata in blood samples of native cattle by PCR and smear method in Southeast of Iran. J Parasitol. Dis. 2012;39(2):249-252.
14. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Vol. 2.2nd Ed. New York, Cold Spring Harbor Laboratory Press; c1989. p. 7.26-7.45.
15. Shahnawaz S, Ali M, Aslam MA, Fatima R, Chaudhry ZI, Hassan MU, *et al*. A study on the prevalence of a tick transmitted pathogen, Theileria annulata and hematological profile of cattle from Southern Punjab, Pakistan. Parasitol. Res. 2011;109:1155-1160.
16. Shayan P, Rahbari S. Simultaneous differentiation between Theileria sp. and Babesia sp. on stained blood smears using PCR. Parsitol. Res. 2005;97(4):281-286.
17. Singla LD, Sharma A, Sharma P, Kaur P. Management of multiple haematozoan infection at farm in Ludhiana.Ind. Vet. J. 2013;90(11):11-14.
18. Soulsby EJL. Helminths, Arthropods and Protozoa of Domesticated Animals. 7th Edition, Bailiere Tindall, Elsevier, London; c1982. p. 788.