Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http://ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Case Report

Molecular Characterization of *Theileria annulata* by *HSP70* Gene of India and Management of Its Infection in Cattle: A Case Report

***Mritunjay Kumar [1], Apurba Debbarma [2], Joyabrata Roy [3], Debasish Behera [4], Hmar Lalrinkima [5], Pratik Bhowmik [6]**

1. *Department of Veterinary Medicine, Bihar Veterinary College, Patna, India*
2. *Department of Veterinary Parasitology, College of Veterinary Sciences & Animal Husbandry, West Tripura, India*
3. *Veterinary Clinical Complex, College of Veterinary Sciences & Animal Husbandry, West Tripura, India*
4. *Department of Veterinary Pathology, College of Veterinary Sciences & Animal Husbandry, West Tripura, India*
5. *Department of Veterinary Parasitology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl, Mizoram, India*
6. *Department of Veterinary Parasitology, College of Veterinary Science, Assam Agricultural University, Guwahati, India*

Received 15 Feb 2025
Accepted 15 Apr 2025

Keywords:
Anaemia;
Buparvaquone;
Phylogenetic inference;
Theileria annulata hsp70 gene

***Correspondence Email:**
mritunjay_medicine@rediffmail.com

Abstract

A 3-year-old cow which suffered from anorexia and high fever for the last 2 days was investigated at farmer door step. Detailed examination of cow showed high temperature (40.5 ^{0}C, pale conjunctival mucous membrane, increased size of pre-scapular lymph nodes and severe tick infestation. Examination of blood smear showed the presence of *Theileria* spp. Haematological values showed low value of hemoglobin (4 gm/dl), TEC (2.3x10^6/µl), PCV (14%) with mild leukocytosis (14.5x10^3/µl). The biochemical values were almost within the normal reference range except for AST (88U/L). PCR amplification of the *hsp70* gene was done from genomic DNA which was isolated from cattle blood and the results showed amplification of approx. 270 bp. In silico analysis of the generated DNA sequence confirm the species of the parasite as *T. annulata* and the phylogenetic inference of the generated sequence (MH178373) showed cladding with *Theileria* infecting domesticate bovines. This study proposed the use of heat-shock protein 70 (*hsp70*) gene of *Theileria* species for inter-species characterization and phylogeny. Treatment with intra-muscular single dose of Buparvaquone (Intas Pharmaceutical Pvt. Ltd., India; 2.50mg kg^{-1}along with intra-muscular antipyrectic drugs Meloxicam and Paracetamol combination (Intas Pharmaceutical Pvt. Ltd., India;15 mg kg^{-1} q24)for 2 days and oral preparation of haematinics (Intas Pharmaceutical Pvt. Ltd., India; 50 ml q24) for 10 days. This is the first report on molecular detection of *Theileria annulata* infection from cattle in Tripura, as per available scientific literature.

DOI: https://doi.org/10.18502/ijpa.v20i3.19627

Introduction

Bovine tropical theileriosis is a tick-borne intra-cellular protozoan disease caused by *Theileria annulata* (1) threatening about 250 million bovine livestock worldwide in developing countries (2). The prevalence of vector borne diseases is being reported in increasing trends in various parts of India that is the bottle-neck in livestock production especially in large ruminant farming (3,4). Upon transmission of the sporozoite by the bite of *Hyalomma anatolicum* tick, the parasite invades mononuclear leukocytes causing acute lymphoproliferative disease characterized by febrile condition, hyper-salivation and lymphnode enlargement. The disease progresses upon invasion of erythrocytes by piroplasmic stage of the parasite. The common clinical manifestations include general lymphadenopathy, fever, anorexia, anemia, jaundice, weight loss and eventually death, if untreated in susceptible animals (5).

The crossbred cattle of India are highly susceptible to *T. annulata* infection, a disease with significant economic loss that costs the country's livestock industry US$ 384.3 million annually (6). Hence, it is the need of hours to identify the vector borne parasite species at the earliest possible and administer prompt medication especially in an area where it is never reported earlier. Northeast India is rich in biodiversity and considered as one of the hot-spot biodiversity area in India, and therefore the vector-parasite transmission cycle is virtually unknown. The areas are bordering Myanmar in the east and Bangladesh in the west and thus the cross-boundary transmission of infectious agent is being reported (7). The area is not found with *H. anatolicum* tick (8) which is really alarming for the possible introduction of theileriosis in NEH region.

Thus, we aimed to report a prompt and successful treatment of theileriosis in cow and possibly de-tick the animal before it spread into a new area. In addition, study the phylogenetic inferences of *T. annulata* from India upon molecular confirmation in relation to other species of the parasite.

Case description

A 3-year-old cow in Radhakishore Nagar, West Tripura, Tripura, India was investigated at farmer's doorstep for anorexia and high fever (40.5 °C) for the past 2 days. The animal was having history of transportation from Bihar to Tripura, 3 days ago. Detail examination of the animal showed high temperature (40.5 °C), pale conjunctival mucous membrane, increased in the size of pre-scapular lymph nodes with abundance of tick infestation. Blood sample was collected from the jugular vein for various haemato-biochemical examinations on day 0 and day 10 of treatment. Blood smear was prepared from peripheral blood for microscopic examination of haemoprotozoa.

The hematological parameters *viz.*, Hb, TEC, TLC, DLC were estimated by using Vet haematology Analyser (Mindray 2800) and biochemical parameters *viz.*, serum creatinine, BUN, ALT and AST were estimated on day 0 and day 10 of post-treatment by using standard kits. Treatment of the affected animal was instituted with intra-muscular single dose of Buparvaquone (Intas Pharmaceutical Pvt. Ltd., India; 2.50 mg kg^{-1} along with intra-muscular anti-pyrectic drugs Meloxicam and Paracetamol combination (Intas Pharmaceutical Pvt. Ltd., India; 15 mg kg^{-1}q24) for 2 days and oral preparation of haematinics (Intas Pharmaceutical Pvt. Ltd., India; 50 ml q24) for 10 days. Tick infestation in cow was controlled by using pour on solution 1% Flumethrin (Bayer Pharmaceuticals Ltd., 1ml kg^{-1}). Simultaneously, genomic DNA was isolated from the whole blood by using HipurA Blood Genomic DNA Miniprep Purification Kit (Cataloque no: MB504-50PR) for molecular confirmation of the parasite species. PCR amplification was

done targeting heat-shock protein 70 (*hsp 70*) with oligonucleotide primers (Forward primer -TGTCAAGGAGGCCTCAAATT and reverse primer- TTTGACTTTGAATAGGGTGC) synthesize by GCC Biotech (INDIA) Pvt. Ltd., Kolkata. The following cyclical conditions were used for PCR amplification: initial denaturation at 95°C for 5 mins; cyclical denaturation at 95 °C for 30sec; annealing temperature at 56 °C for 30 sec for 33 cycles; extension at 72 °C for 30 sec and final extension, 72 °C for 5 min. The PCR product (5µl out of the 25 µl) was subjected to submarine electrophoresis in 1.5% agarose gel readded with ethidium bromide. The agarose gel was visualized in gel documentation system and the remaining portion of the PCR product was sent for custom sequencing. The DNA samples were sent to Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi for Sanger DNA sequencing both in forward and reverse directions.

The generated DNA sequence was deposited in NCBI database and analyzed upon comparison with *T. annulata* Ankara strain (XM_946872) by ClustalW in MEGA 11 software. To compare the evolutionary distance of *T. annulata* from other isolate and strain as well as among different species of *Theileria* such as *T. annulata* of Ankara strain (XM_946872), *T. annulata* of Turkey (AY271268), *T. parva* Muguga strain (XM_760227), *T. orientalis* (CP056068), *T. ovis* (AB248747), *T. cervi* (AB248748) and *T. equi* (AB248743), phylogenetic tree was constructed by using MEGA software version 11.0 (10) by using Maximum-Likelihood and Kimura-2-parameter. A tree was constructed keeping the bootstrap value at 1000 replicates after trimming the non-align sites and the p-distances is provided in supplementary material. During the construction of a tree, *Toxoplasma gondii* (AB019539) and *Babesia bigemina* (AB482178) *hsp70* sequence were used as an out-group for comparison.

Examination of blood smear showed the presence of *Theileria* spp. (Fig. 1) with annular shaped parasite within erythrocytes. Confirmation of the parasite species was done by amplification of the *hsp70* gene (Fig. 2) and subsequent DNA sequence analysis upon comparison with those DNA species available in the public data base.

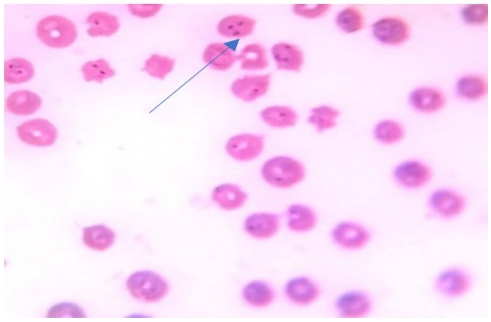

Fig. 1: Piroplasm of *T. annulata* in the arrows

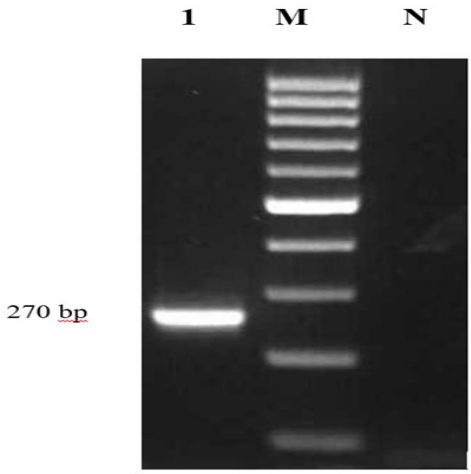

Fig. 2: Showing PCR amplification *Theileria annulata hsp70* gene

Lane 1 : Amplification of *hsp70* gene at 270 bp
Lane M: 100 bp ladder
Lane N: Negative control

There was transition of nucleotides at three positions at C>T (151), T>C (190) and C>T (214) and transversion at T>G (55), C>A (250) and C>G (260). Despite these point mutations of the nucleotides, alignment of the amino acids of our sequence with Ankara

strain (XM_946872) did not yield any amino acid change at the protein sequence. The DNA sequence generated in this study was deposited in NCBI data base and accession number was obtained as MH178373. The phylogenetic inferences drawn in this study showed *T. annulata* of India, Turkey and Ankara strain are homologous with high bootstrap value. In fact, other species of *Theileria* infects domestic bovines such as *T. parva* and *T. orientalis* fall nearer to each other (≥ 90) with high bootstrap value upon comparison with other *Theileria* species infecting equines, cervid and small ruminants signifying a homology within these species. In this study, *T. gondii* and *B. bigemina* was intended to keep as an out-group (Table 1), surprisingly other *Theileria* species *viz. T. equi, T. cervi* and *T. ovis* branch along with the intended out-group apart from *Theileria* infecting domestic bovines (Fig. 3).

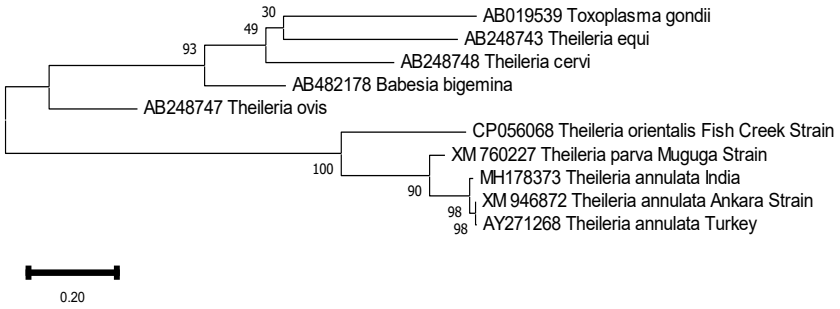

Fig. 3: Phylogenetic inferences of *T. annulata* cladding with *Theileria* infection of domesticated bovines from other *Theileria* species

Table 1: P-distance values of *T. annulata* from India cladding with *Theileria* infection of domesticated bovines from other *Theileria* species

AB019539_ Toxoplasma_gondii									
MH178373_Theileria_annulata_India	2.32885								
XM_946872_Theileria_annulata_Ankara_Strain	2.40420	0.02039							
AY271268_Theileria_annulata_Turkey	2.40706	0.02460	0.00399						
XM_760227_Theileria_parva_Muguga_Strain	2.75825	0.14184	0.14753	0.15329					
CP056068_Theileria_orientalis_Fish_Creek_Strain	2.42400	0.58330	0.58365	0.59654	0.51243				
AB482178_Babesia_bigemina	0.78007	1.79225	1.88932	1.94137	1.95301	1.61348			
AB248743_Theileria_equi	0.93629	3.26272	3.05572	2.96030	2.99685	4.60690	0.86165		
AB248748_Theileria_cervi	0.77646	2.57646	2.50842	2.58659	2.61953	1.61551	0.63165	0.74241	
AB248747_Theileria_ovis	0.90336	1.60499	1.60591	1.60654	1.46391	1.59486	0.77354	1.25640	1.04622

Investigation of haematological values showed low value of haemoglobin (4 gm/dl), TEC (2.3 X10^6/µl), PCV (18%) with mild leukocytosis (14.5 X10^3/µl) whereas serum creatinine (0.9 mg/dl), BUN (12 mg/dl) and ALT (40 U/L) were within normal reference range except for AST (88U/L) on day 0 of treatment. The animal showed gradual improvement from day 2nd of treatment and the parameters became within normal reference range on day 10th of treatment.

Discussion

This study calls for more intense and deeper study of *T. annulata*, *T. orientalis* and *T. parva* that are infecting domestic bovines with stronger DNA sequences or concatenated genes. Yet, this study proposed the prospect of the use of *Theileria hsp70* for studying the inter-species phylogenetic inferences as did in Babesia (11).In Tripura, the microscopic prevalence of *Theileria* spp. was recorded as 18.7% in cattle but the confirmation of parasite species was not carried out yet. The highest prevalence of theileriosis was seen during monsoon season reflecting conducive environment for tick population during this season in subtropical climate (12). Clinical signs and alternation in hematological profile of affected cow were consistent with earlier reports of lower values of hemoglobin, PCV and TEC suggestive of anemia (13).

Early detection of *T. annulata* infection and its prompt treatment with buparvaquone is 100% effective in eliminating the protozoan parasites from the blood and lymph nodes, results in improvement in clinical cases (7,14) as reported in this study. Due to latent infection of *T. annulata* upon clinical recovery of animal from clinical disease (15) and the identification of its intermediate host in NEH region (8), utmost care must be taken for rapid identification of the parasitic infection and prompt treatment is imperative for prevention of the disease outbreak in the area where the disease is not yet endemic. This study reflects the importance of quarantining and de-ticking of newly purchased animals prior to mingling them with existing stock of animals. At the same time, study of the identification and re-appraisal of the tick species in northeast India is warranted as the areas considered one of the biodiversity hotspots in India and internationally bordering porous borders with Bangladesh and Myanmar.

Conclusion

Transportation of cattle without screening for haemoprotozoan diseases from endemic region to disease free region may be a potential threat for the outbreak of diseases in susceptible animal. The detection of *T. annulata* in this study indicates a pre-requisite for further molecular investigation to identify the *Theileria* spp. from other parts of the Tripura and its neighboring states. This study calls for more intense and deeper study of *T. annulata*, *T. orientalis* and *T. parva* that are infecting domestic bovines with stronger DNA sequences or concatenated genes in Tripura.

Acknowledgements

Authors are thankful to Principal, College of Veterinary Sciences & A.H., R.K. Nagar for providing necessary facilities.

Conflict of interest

Authors declares no conflict of interest.

References

1. Parveen A, Alkhaibari AM, Asif M, et al. Molecular epidemiology of *Theileria annulata* in cattle from two districts in Punjab (Pakistan). Animals (Basel). 2021;11(12):3443.
2. El Damaty HM, Yousef SG, El-Balkemy FA, et al. Seroprevalence and risk factors of

tropical theileriosis in small holder asymptomatic large ruminants in Egypt. Front Vet Sci. 2022; 9: 1004378.
3. Ghosh S, Azhahianambi P, De La Fuente J. Control of ticks of ruminants, with special emphasis on livestock farming systems in India: present and future possibilities for integrated control-a review. Exp Appl Acarol. 2006;40(1): 49–66.
4. Jacob SS, Sengupta PP, Krishnamoorthy P, et al. Bovine babesiosis in India: Estimation of prevalence by systematic review and meta-analysis. Exp Parasitol. 2022; 239:108318.
5. Onizawa E, Jenkins C. Epidemiology, clinical signs, and risk factors associated with theileriosis in Australian cattle (2006-2022). Pathogens. 2024; 13(3): 253.
6. Minjauw B, McLeod, A. Tick-borne diseases and poverty. The impact of ticks and tick-borne diseases on the livelihood of small scale and marginal livestock owners in India and eastern and southern Africa. 2003; Research report, DFID Animal Health Programme, Centre for Tropical Veterinary Medicine, University of Edinburgh, U. K.
7. Kumar S, Mohmad A, Parthasarathi BC et al. Current epidemiological status of bovine theileriosis in Indian scenario. Ann Clin Cytol Pathol. 2018; 4(1): 1090.
8. Patra G, Ghosh S, PolleyS, et al. Molecular detection and genetic characterization of *Coxiella*-like endosymbionts in dogs and ticks infesting dogs in Northeast India. Exp Appl Acarol. 2022; 86(4):549-566.
9. Akbari J, Javanbakht J,Tavassouli M, et al. Molecular survey of *Theileria annulata* in cattle by PCR-RFLP method in Iran. J Bacteriol Parasitol. 2012;3(2):135.
10. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021; 38(7):3022–3027.
11. Yamasaki M, Inokuma H, Sugimoto C, et al. Comparison and phylogenetic analysis of the heat shock protein 70 gene of *Babesia* parasites from dogs. Vet Parasitol. 2007; 145(3-4):217-27.
12. Debbarma A, Pandit S, Jas R, et al. Prevalence of naturally occurring theileriosis in cattle of Tripura, India. Indian J Anim Health. 2023; 62(1): 107-113.
13. Arif S, Lahkar D, Gogoi S, et al. The molecular detection and therapeutic management of pathogenic *Theileria luwenshuni* infection in a goat: a case report. Iran J Parasitol. 2024;19(4):502-508.
14. Verma R, Das G, Kumar S, et al. Molecular investigation of bovine tropical theileriosis outbreak in an organized dairy cattle farm in Madhya Pradesh, India. Parasitol Res. 2023; 122: 2079–2089.
15. Abid K, Bukhari S, Asif M, et al. Molecular detection, and prevalence of *Theileria ovis* and *Anaplasma marginale* in sheep blood samples collected from district Layyah in Punjab Pakistan. Trop Anim Health Prod. 2021; 53:439.