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Research Article

MOLECULAR PREVALENCE OF *THEILERIA* INFECTION IN *HYALOMMA ANATOLICUM* INFESTING CATTLE IN DIFFERENT AGRO CLIMATIC ZONES OF INDIA

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ABSTRACT

The present study was conducted to determine the molecular prevalence of *Theileria* organism in vector ticks infesting bovines in four different agro-climatic zones of India. Partially engorged ticks were collected randomly from a total of 100 cattle out of 235 screened. Sampling was done from different age group of animals during the period from November 2021 to October 2022. A pool of two to five ticks collected from individual cattle was processed for the extraction of genomic DNA. Two sets of primers were used in the study for molecular detection of *Theileria* organism in the tick vectors: one set targeting the genus-specific 18S ribosomal RNA (18S *rRNA*) gene, and another set targeting the *Theileria annulata* merozoite surface antigen-1(*Tams*1) gene specific to *T. annulata*. The present study revealed a total tick infestation prevalence of 42.55%, with the Upper Gangetic Plain exhibiting the highest infection rate at 54.5%, followed by the Trans Gangetic Plain (45.0%), Western Dry Region (44.4%) and Western Himalayan Region (22.9%). Using an 18S *rRNA* gene-based PCR test, the study revealed a total molecular prevalence of *Theileria* organisms in a vector tick population of 23.0%. However, the *Tams*1 gene-based PCR assay for *T. annulata* revealed a prevalence of 21.0%. Agroclimatic zone-specific analysis of PCR findings revealed that the Western Dry Region showed the highest prevalence of *Theileria* infection (31.25%), followed by the Trans Gangetic Plain (25.93%), Upper Gangetic Plain (16.67%) and Western Himalayan Region (9.09%). Seasonal studies revealed that the monsoon season had the highest prevalence (28.94%), followed by summer (19.51%) and winter (19.04%). Adult cattle showed the highest incidence (24.48%), followed by calves (20%) and heifers (19.44%). Data generated in the present study provides key information about the prevalence and distribution of *T. annulata* in selected agroclimatic zones of the country, which may be helpful in developing and strategizing effective control measures against this parasite.

Keywords: Cattle, Molecular detection, *Theileria annulata*, Tick, PCR assay

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Bovine Tropical Theileriosis (BTT) caused by *Theileria annulata* is an extremely fatal and debilitating tick-borne disease affecting cattle and is transmitted by *Hyalomma anatolicum* (Ixodid tick), having a complex lifecycle in both vertebrate and invertebrate hosts. This disease is manifested by pyrexia, lymphoid hyperplasia, and digestive disorders and associated pathology due to destruction of erythrocytes. The conventional method of diagnosing *Theileria* organism in the tick vector is based on the staining of the salivary gland by various chemicals including Methyl Green-Pyronin, Giemsa and Feulgen (Voigt *et al.*, 1995). However, the detection by staining is not specific and is unable to differentiate between sporoblasts of different *Theileria* species. Therefore, in recent years, application of molecular diagnostic assays such as polymerase chain reaction (PCR) have been explored as an epidemiological and diagnostic tool for the detection and identification of haemoparasites both in the vertebrate hosts and tick vectors (Bhat *et al.*, 2017; Dehuri *et al.*, 2022). These tools are more sensitive and specific than the other common methods by virtue of amplifying even the minute quantity of parasitic DNA (Mans *et al.*, 2015). Of the many target genes, the18s *rRNA* gene, consisting of both conserved and variable regions is suitable marker for distinguishing closely related species of *Theileria* organisms (Chaisi *et al*., 2011). Conversely, the *Tams*1 gene is widely recognized for its high specificity and sensitivity in identifying *T. annulata*, which makes it a helpful diagnostic market (Jongejan *et al.*, 1994). The diverse climatic zones of India are vastly conducive for survival and propagation of vectors and vector-borne pathogens (Bhattacharjee and Sarmah, 2013). Data pertaining to the epidemiology of tick-borne diseases, such as dynamics of pathogen transmission by the tick vector, is imperative for devising effective control strategies. Keeping in view the importance of tick-borne diseases in bovines, the aim of the present study was to investigate the molecular prevalence of *Theileria* organism in vector ticks i.e. *Hyalomma anatolicum* collected from cattle maintained in different agro-climatic zones of India.

MATERIALS AND METHODS

Study area and duration

The present study was conducted in four agro-climatic zones of India viz. Upper Gangetic Plain (Bareilly, Uttar Pradesh and Udham Singh Nagar, Uttarakhand), Trans Gangetic Plain (Hisar and Ambala; Haryana), Western Dry Region (Bikaner, Rajasthan) and Western Himalayan Region (Nainital, Uttarakhand) during the period from November 2021 to October 2022.

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Screening of animals for tick infestation and sample collection

A total of 235 cattle were screened carefully for tick infestation from different agro-climatic zones of India (Table1) and ticks were collected from a total of 100 animals of different categories (15 calves, 36 heifers and 49 adult) and season of the year (41-summer, 38-monsoon and 21-winter). Stratified random sampling was made keeping in view the probability of animal movements from one place to another.

Collection of ticks from animals

Ticks were manually removed from the body surface of cattle taking precaution not to break any appendages with the help of fine forceps. Ticks were temporarily paralyzed with cotton soaked in ethanol or chloroform for this purpose. A total of 5-10 fully engorged or semi engorged female ticks per animal were collected in sample collection tubes. Collected samples were labelled properly and brought to the laboratory for morphological identification and further downstream application.

Morphological identification and isolation of genomic DNA from *Hyalomma* sp.

Ticks collected from individual animals were identified in laboratory using stereozoom microscope considering the morphological features described by Soulsby (1982). Genomic DNA was extracted from *Hyalomma* spp. using Presto™ DNA/RNA/Protein kit (Geneaid). Partially or fully engorged female ticks recovered from individual cattle were cut in two pieces and pooled (2-5 ticks per sample) before DNA extraction. Each pooled sample was homogenized using the pestle and mortar and after that 400 µL of DR buffer and 4 µl of ß-mercaptoethanol was added and again homogenized. Tissue lysate was sheared through 20-G needle syringe by passing 10 times followed by the incubation step at room temperature of 5 min. Thereafter, sample was centrifuged at 13000 rpm for 2 min and supernatant was transferred in GD column and all other steps were performed according to the protocol provided with kit. In the end, DNA was eluted in a total of 50 µl final volume and it was stored at -20°C till further use.

Optimization of PCR assay:

PCR protocol for detection of *Theileria* organism was optimized in the laboratory using positive DNA of *T. annulata* and two sets of primers (Table 2). PCR reaction was performed in a total volume of 25 µL and reaction mixture was prepared using the following reagents; 10x PCR buffer-2.5 µL, forward and reverse primers (20 pmol/µL)-0.5 µL each, dNTPs-0.5 µL, genomic DNA-3.0 µL, Dream Taq DNA polymerase-0.25 µL. and nuclease free water-17.75 µL. Reaction was run in a thermal cycler (S1000 Gradient Thermal Cycler, Bio-Rad) with the cycling conditions as an initial denaturation of 95°C for 5 min, followed by 34 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec (18S *rRNA* gene) or 58°C for 1 min (Tams1 gene) and extension at 72 °C for 45 sec, and a final extension at 72 °C for 10 min. Thereafter, gDNA samples extracted from ticks (n=100) were screened at the optimized PCR condition as described above. The PCR amplified products were run in 1.2% agarose gel using TAE (Tris-acetate-EDTA) buffer. GeneRulerTM 100 bp Plus DNA ladder (Thermo Scientific, USA) was used to determine the size of amplified PCR product. Amplification was visualized in gel documentation system under the UV transilluminator (GeNei).

Statistical analysis of data:

Agroclimatic zone, season and age wise prevalence data was analyzed using Chai square (χ^2) test (https://www.socscistatistics.com/tests/chisquare/default2.aspx) to determine the significance of data generated in the study.

RESULTS AND DISCUSSION

In the present study, screening of a total of 235 animals revealed overall 42.55% tick infestation rate. The highest infestation rate was recorded in the Upper Gangetic Plain (54.5%), followed by the Trans Gangetic Plain (45.0%), Western Dry Region (44.4%), and Western Himalayan Region (22.9%). According to an estimate, about 84.0% of the world cattle populations are exposed to tick infestation (FAO, 1984). Further, high tick infestation rate in cattle ranging from 58.06% to 86.15% had already been reported from different part of India (Patel *et al.*, 2013; Singh and Rath, 2013; Balasubramanian *et al.*, 2019).

The ticks collected were morphologically identified as *Rhipicephalus* spp. and *Hyalomma* spp. Genomic DNA extracted from the partially or fully engorged *Hyalomma* spp. (n=100) and subjected for PCR amplification targeting the 18S *rRNA* and *Tams*1 gene revealed positive amplification (Figs. 1 & 2) in a total of 23 and 21 samples, respectively. Agroclimatic zone-wise analysis of PCR results (18S *rRNA* gene based) indicated highest prevalence of *Theileria* infection in the Western Dry Region (31.25%) followed by the Trans Gangetic Plain (25.93%), Upper Gangetic Plain (16.67%) and Western Himalayan Region (9.09%) (Table 3 & Fig. 3). Likewise, seasonal analysis revealed highest prevalence during the monsoon season (28.94%) followed by summer (19.51%) and winter season (19.04%) (Fig. 4). Age wise analysis revealed higher prevalence in adult cattle (24.48%) followed by calves

Table 1. Agro climatic zone wise prevalence of *Hyalomma* sp. infestation in cattle

S. No.	Agro climatic zone	No. of cattle screened	No. of cattle positive (% prevalence)
1.	Upper Gangetic Plain	55	30[a] (54.5%)
2.	Trans Gangetic Plain	60	27[a] (45.0%)
3.	Western Dry Region	72	32[a] (44.4%)
4.	Western Himalayan Region	48	11[b] (22.9%)
	Total	235	100 (42.55%)

Note: Superscript (a) denoter non-significant correlation, whereas superscript(b) denotes significant correlation at P<0.05.

Table 2. Primers used for detection of *Theileria* infection in tick vectors

Target gene	Oligomer name and sequences (5'-3')	Amplicon Size	Reference
18S *rRNA*	Theil 18S-For: GCTGCATTGC TTGTGTCCCTCT Theil18S-Rev: CAAGGTGCT GAAGGAGTCGTAAAAAC	440bp	Self-designed
Tams1	Tamulti-For: CCGTTAATGC TGCAAATGAGGAGG Tamulti-Rev: GAGGCGAAGA CTGCAAGGGGAG	751bp	Kundavae *et al.* (2018)

Table 3. Agro-climatic zone wise prevalence of *T. annulata* infection in cattle tick

S. No.	Agro-climatic zone	No. of DNA samples (tick) examined	No. of DNA samples (tick) positive
1.	Upper Gangetic Plain	30	5[a] (16.67%)
2.	Trans Gangetic Plain	27	7[a] (25.93%)
3.	Western Dry Region	32	10[a] (31.25%)
4.	Western Himalayan Region	11	1[a] (09.09%)
	Total	100	23[a] (23.00%)

Note: Superscript (a) denotes non-significant correlation (P<0.05)

(20%) and heifers (19.44%) (Fig. 5). Statistical analysis of agroclimatic zone, season and age wise PCR results could not witness any significant difference (P >0.05).

The present study was performed to determine the molecular prevalence of *Theileria* organism in vector ticks collected from 4 different agroclimatic zones of the country. Study recorded overall 23.0% molecular prevalence of *T. annulata* by using 18S *rRNA* gene based PCR assay and 21.0% prevalence by *Tams*1 gene based PCR assay in vector ticks. Above difference in the detection rate of both PCR assay(s) could by justified by the fact that 18S *rRNA* gene based PCR has ability to amplify *Theileria* genus specific DNA, whereas *Tams*1 gene based PCR is specific for *T. annulata* only. Detection of pathogens in the tick vector utilizing PCR assay may prove beneficial in larger epidemiological investigations.

In the study, molecular prevalence of *T. annulata* has been found reasonably high in Upper Gangetic, Trans Gangetic and Western dry region (16.67% to 31.25%) in

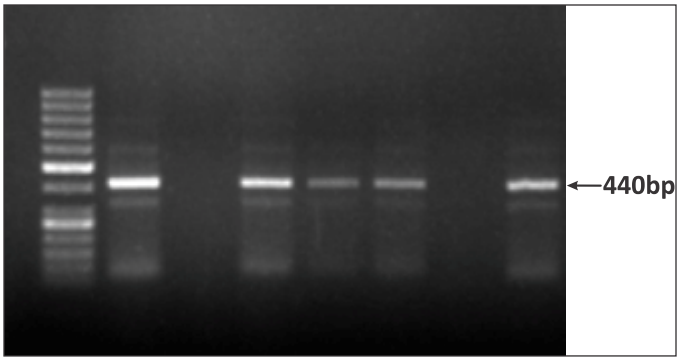

Fig. 1. PCR amplification of 18S *rRNA* gene of *Theileria* from vector tick M- 50 bp plus DNA ladder, L1- positive control, L2- Negative control, L3 to L7- Tick DNA samples

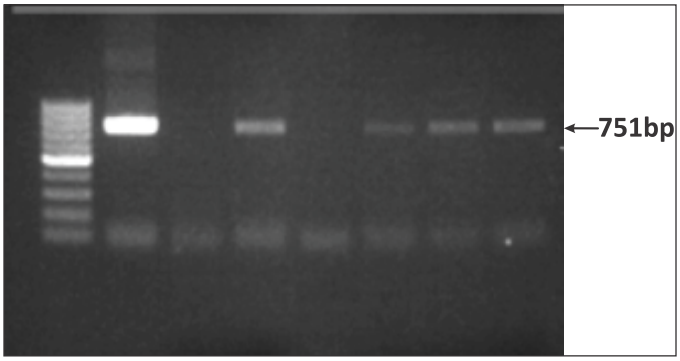

Fig. 2. PCR amplification Tams1gene of *Theileria annulata* from vector tick (M- 100 bp plus DNA ladder, L1- positive control, L2- negative control, L3 to L7- tick DNA samples)

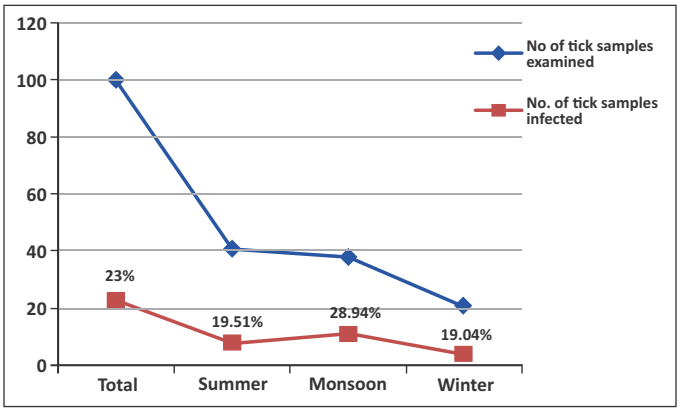

Fig. 4. Season wise prevalence of *Theileria* infections in cattle tick

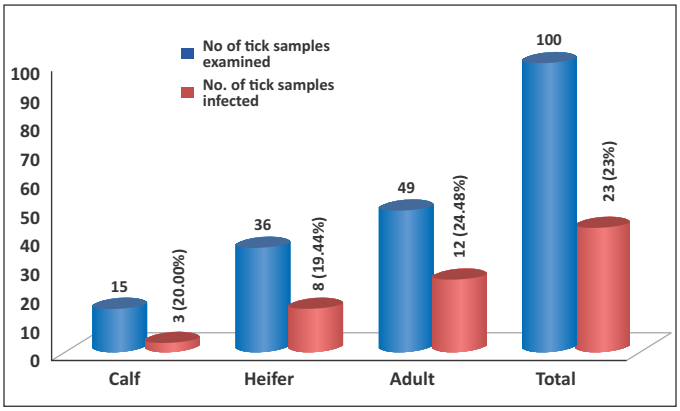

Fig. 5. Age wise prevalence of *Theileria* infections in cattle tick

comparison to the Western Himalayan region (9.09%). Therefore, an in-depth epidemiological investigation is required in these areas involving appropriate sample size of cattle population. Molecular studies conducted by other workers have also reported variable prevalence rate of this parasite in different states of India. Tiwari *et al.* (2015) tested DNA samples extracted from *H. anatolicum* collected from healthy cattle in Punjab and recorded 20.0% prevalence of *T. annulata* using primary PCR and 60.0% prevalence by nested PCR. Another study from Chhattisgarh reported overall 34.24% intra-acarine haemoprotozoan (*Theileria* and *Babesia*) infection (Jadhao *et al.*, 2021). Recently, Dehuri *et al.* (2022) employed a nested PCR assay targeting the *Tams*1 gene and recorded an overall prevalence of 14.6% *T. annulata* infection in vector ticks from Odisha.

In the study, highest prevalence of *T. annulata* was recorded in ticks collected from western dry region, while it was lowest in the Western Himalayan region. Such difference recorded in the prevalence rate in tick vectors seems to be influenced by the prevailing climatic condition in both the region. Hot and humid climate usually favors the survival of *Hyalomma anatolicum*, which is prevailing in Western dry region. Seasonal dynamics revealed highest prevalence rate in vectors during the monsoon season followed by summer and winter seasons. Lower seasonal temperature of the area seems to be detrimental for the life cycle stages of the ixodid ticks, which ultimately results in the low vector population during winter season. Based on the findings of the molecular studies performed in other countries, where theileriosis is endemic, a high prevalence of *Theileria* infection was reported in *Hyalomma* sp. (Khodabandeh and Razmi, 2015). Sporadic reports are also available on the presence of *Theileria* sp. infection in vector tick other than *Hyalomma* sp. (Ionita *et al.*, 2013; Jadho *et al.*, 2022).

CONCLUSION

Molecular detection of *Theileria* organism in vector ticks by PCR assay has been a powerful tool for epidemiological investigation. In the study, *18S rRNA* gene based PCR was found more useful in comparison to the *Tams*1 gene based PCR. Further, agroclimatic zones having considerably high prevalence rate of this parasite in vectors may be targeted as a priority for detail epidemiological investigations. Data generated in the present study provides key information about the distribution of *T. annulata* in the targeted agroclimatic zones, which may be helpful in developing and strategizing effective control measures against this parasite.

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