Molecular insights into the prevalence and diversity of *Theileria annulata* in *Bos indicus* and crossbred cattle

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Journal of Livestock Science (ISSN online 2277-6214) 17: 462-470
Received on 22/10/25; Accepted on 6/7/26; Published on 10/7/26
doi. 10.33259/JLivestSci.2026.462-470

Abstract

Tropical theileriosis, primarily caused by *Theileria annulata*, is a significant tick-borne hemoprotozoan disease affecting bovines, resulting in substantial economic losses to the livestock sector. This study investigated the detection, hemato-biochemical alterations, and molecular characterization of *T. annulata* infection in cattle from North Gujarat, India. A total of 300 blood samples were analyzed, revealing an overall prevalence of 26.66% (80/300) by microscopic examination and 43.33% (130/300) using PCR. Age-wise analysis indicated comparatively higher PCR prevalence among cattle younger than two years, particularly in HF crossbred cattle. Seasonal analysis indicated that summer recorded the highest prevalence compared to other seasons. Hematological parameters, including hemoglobin (Hb), total erythrocytes count (TEC), packed cell volume (PCV) and while white blood cell (WBC), showed significant reductions, mean corpuscular volume (MCV) counts increased non-significantly. Biochemical parameters exhibited a non-significant increase in HF crossbreds and a non-significant decrease in Kankrej cattle. Phylogenetic and sequence analyses of the *T. annulata* 18S rRNA gene revealed homology with isolates from Telangana, India, and the Netherlands, underscoring its genetic similarity and global distribution.

Keywords: Cattle, Hemato-biochemical alteration, Molecular characterization, PCR, *Theileria annulata*

Introduction

Tropical theileriosis, primarily transmitted by the cattle fever tick *Hyalomma anatolicum* poses a significant threat to the livestock industry (Denizhan et al., 2017). In addition to tropical theileriosis, *H. anatolicum* is a vector for various other diseases, including anaplasmosis, babesiosis, borreliosis, ehrlichiosis, and the potentially fatal Crimean-Congo hemorrhagic fever (CCHF), one of the most severe arboviral infections (Biglari et al., 2018). Among the numerous *Theileria* species infecting cattle such as *Theileria parva*, *T. annulata, T. orientalis*, *T. mutans*, and *T. senegalensis*; *T. annulat*a and *T. parva* are the most pathogenic, causing tropical theileriosis and East Coast fever, respectively.

The cardinal symptoms shown by affected cattle are, high fever and lingering anemia because of the intraerythrocytic parasitism (Gnani Charitha et al., 2025). The affected animals reveal fever, anemia, enlargement of lymph node, anorexia, and progressive loss of body weight, along with alteration in various haematolgical and biochemical parameters (Dua et al., 2012). Theileriosis significantly impacts the economy through mortality and decreased milk production. Hemoprotozoan parasites, predominantly transmitted by ticks, are major economic concerns in Asia and have historically challenged cattle survival in India.

In India, reports of theileriosis have been documented in several states, including Punjab, Haryana, Gujarat, and Kerala, with prevalence rates of 60% in Punjab, 32.8% in Haryana 16% in Gujarat and 37% in Northern Kerala (Tiwari et al., 2015; Vahora et al., 2012). Diagnosing animals with low parasitemia or in carrier states is challenging using traditional microscopic examination of stained blood smears and lymph node biopsies (Khatoon et al., 2015).

Sensitive diagnosis of low-level parasite infections is crucial for epidemiological studies. Conventional diagnostic techniques, including post-mortem lesions, clinical signs, blood and tissue smears, patient histories, and serological tests provide valuable clues but are labour-intensive and often lack sensitivity and specificity (Farhang 2017). PCR and other molecular techniques are effective for detecting low-level parasitemia. Demonstrated that PCR offers high sensitivity in identifying haemoprotozoan infections in carrier cattle. Keeping in view these perspectives the present study was undertaken with an objective to determine the prevalence of *T. annulata*, in North Gujarat, compare microscopic and molecular diagnostic methods, evaluate hemato-biochemical alterations, and characterize circulating isolates using molecular and phylogenetic analyses which will contribute to the development of effective diagnostic and control strategies.

Materials and Methods

Ethical approval

The study was conducted following ethical guidelines and was approved by the Institutional Animal Ethics Committee (IAEC) under protocol number VETCOLL/IAEC/2022/19/Protocol-21.

Study area and sample collection

The study was conducted from April 2021 to March 2022 in four districts of North Gujarat, India: Banaskantha, Sabarkantha, Mehsana, and Patan. A total of 300 (126 Kankrej and 174 Holstein Friesian (HF) cross-bred) bovine blood samples were collected from various sources, including farms, gaushalas, panjrapoles (shelter for animals that are elderly, infirm, or needy), and animals presented at veterinary hospitals. Blood samples were drawn from the jugular veins using serum and EDTA vials.

The cattle sampled exhibited a range of clinical signs, including enlarged prescapular lymph nodes, fever, anemia, lethargy, and visible tick infestations, though they generally appeared healthy (Fig. 1). The age of each animal were recorded. Animals were categorized into two age groups: less than two years old and more than two years old. Samples were collected in winter, summer, and monsoon season of the year.

Microscopic examination

Thin blood smears were prepared, fixed with methanol for five minutes, and stained with a 1:10 Giemsa solution for 30 minutes. The slides were then washed with tap water, air-dried, and examined under an oil immersion microscope at 100x magnification to detect the presence of *Theileria* organisms. Additionally, prescapular lymph nodes were massaged after the administration of 1.0 mL of normal saline. The aspirated fluid from the massage was used to prepare smears, which were subsequently stained and examined under an oil immersion microscope for Koch's Blue Bodies (KBB).

Haemato-biochemical analysis

Hematological parameters were analyzed using an automatic hematology analyzer (Nihon Kohden Haematology Analyzer). The parameters included total leukocyte count (TLC), differential leukocyte count (DLC), mean corpuscular hemoglobin concentration (MCHC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and thrombocyte count (Table 3). Biochemical parameters such as alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total bilirubin, and blood urea nitrogen (BUN) were measured using a semi-auto analyzer (Randox Monaco) with commercial diagnostic kits (Agappe Diagnostics Ltd., India).

DNA extraction and PCR

Genomic DNA was extracted from blood samples using the QIAamp® DNA Blood Mini Kit according to the manufacturer's protocol. The quality and quantity of the extracted DNA were evaluated, and samples were stored at -20°C for further use. Genus-specific primers were used to detect *Theileria* spp., and species-specific primers were employed for the identification of *T. annulata* and *T. orientalis* (Table 1). Amplified PCR products were resolved on a 2% agarose gel. Gel purification was performed using the GeneJET PCR Purification Kit (Catalogue # K0702), and the product size was visualized and photographed using a gel imaging system.

Sequencing and phylogenetic analysis

The PCR products of *T. annulata* were sequenced using the Sanger method, with sequencing services provided by Eurofins Genomics India Pvt. Ltd., Karnataka, India. Sequence analysis and editing were performed using the BioEdit Sequence Alignment Editor Software (v.7.2.5). Phylogenetic trees were constructed using the neighbor-joining distance method in the MEGA X software. The robustness of the tree's branching patterns was evaluated through bootstrap analysis with 1,000 replications.

Statistical analysis

Data collected during the study were compiled, totalled, and analyzed statistically. Hemato-biochemical profiles obtained under various clinical conditions were analyzed using an unpaired t-test. The statistical analysis was performed using SPSS software version 21.0.

Results

Microscopic examination

Microscopic examination of Giemsa-stained thin blood smears revealed the presence of intraerythrocytic morphological forms of *Theileria* organisms within red blood cells (RBCs). (Fig. 2). These forms appeared as small, round to oval-shaped structures typical of *Theileria* piroplasms. Koch's Blue Bodies (KBB), representing schizont stages of *Theileria*, were distinctly observed within lymphocytes. These schizonts were identifiable as irregular, blue-staining bodies consistent with lymphocytic parasitic invasion (Fig. 3). The overall positivity rate for *Theileria* infections was 43.33% (130/300) as determined by PCR assay, compared to 26.66% (80/300) detected by microscopic examination. Among the samples, *Theileria* infection was identified in 19.04% (24/126) of Kankrej cattle and 32.18% (56/174) of HF crossbred cattle

Biochemical analysis

Biochemical analysis revealed a non-significant increase in all serum biochemical parameters, including alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total bilirubin, and blood urea nitrogen (BUN). Detailed values of hematological and biochemical parameters are provided in Tables 2 and 3.

Molecular detection

The molecular detection of *Theileria annulata* was performed using PCR, revealing an overall prevalence of 43.33% (130/300) among the tested bovine samples. Of these, 31.74% (40/126) were from Kankrej cattle, and 51.72% (90/174) were from HF crossbred cattle. *Theileria* genus-specific primers successfully amplified an 881-bp DNA fragment, confirming genus-level positivity. Subsequent amplification using species-specific primers for *T. annulata* yielded a 430-bp DNA fragment, while no amplification was observed for *T. orientalis* in any of the samples. This finding indicates that *T. annulata* was the only species detected in the sampled population (Fig. 4 and 5).

Age-wise prevalence

The age-wise analysis highlighted significant differences in the prevalence of *Theileria annulata* infection. The overall prevalence in cattle below two years and above two years old was 51.22% and 40.37%, respectively by PCR. Among Kankrej cattle younger than two years, the prevalence was 18.75% by microscopy and 37.5% by PCR while in cattle above 2 years, it was 19.14% and 29.78%, respectively, for the same diagnostic methods.

In HF crossbred cattle, infection rates were substantially higher. For cattle under two years old, prevalence was 28% and 60% by microscopy and PCR, respectively. For those over two years old, rates were 33.87% and 48.38%. These findings suggest that younger animals, particularly HF crossbreds, are more vulnerable to *Theileria annulata* infection. The age-wise prevalence data is presented in Table 4.

Season-wise prevalence

The study revealed a significant seasonal variation in the prevalence of *Theileria annulata* infection. The overall prevalence rates during winter, summer, and monsoon were 19.14%, 39.70%, and 11.42%, respectively. When assessed through microscopic examination, the prevalence was highest in summer (38.29%), followed by winter (20%) and monsoon (6.66%). Similarly, PCR assays showed the highest prevalence during summer (58.82%), followed by winter (35%) and monsoon (20%).

In Kankrej cattle, prevalence rates across seasons varied significantly. By microscopic examination, infection rates were 20% in winter, 25% in summer, and 6.66% in monsoon. PCR results showed slightly higher prevalence rates, with 35% in both winter and summer and 20% in monsoon. HF crossbred cattle displayed a

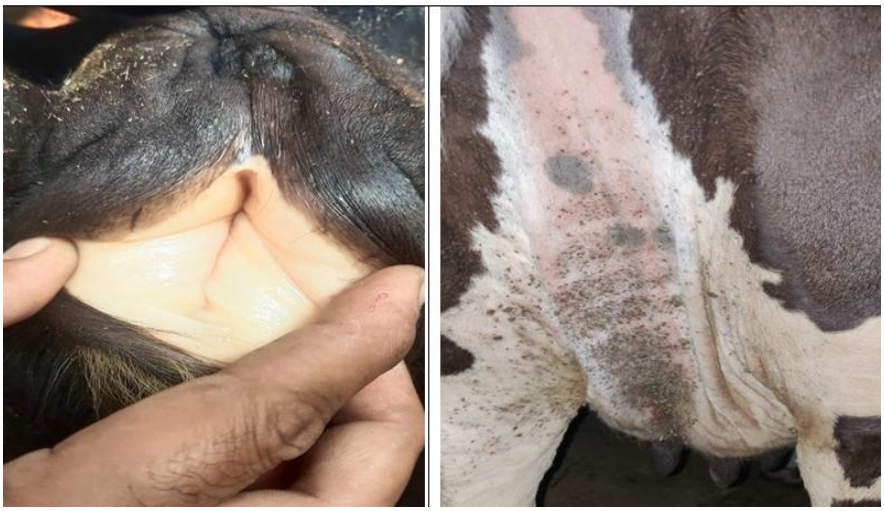


Fig.1. Clinical signs of theileriosis in cattle a. Pale vulvar lip b.Tick infestation in the body

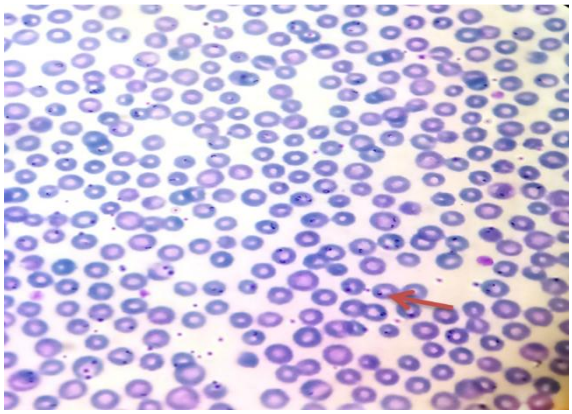


Fig. 2. Peripheral blood smear of naturally infected cattle showing intra-erythrocytic piroplasm of *Theileria annulata* (100x)

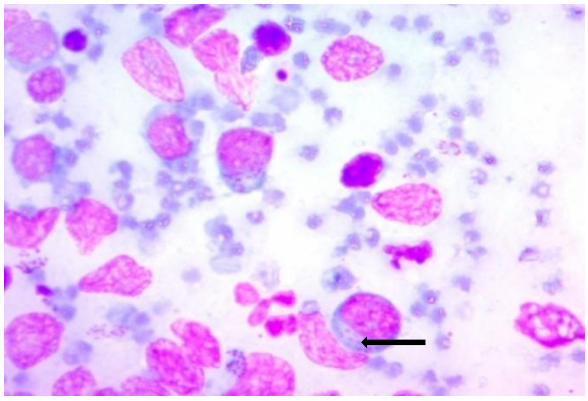
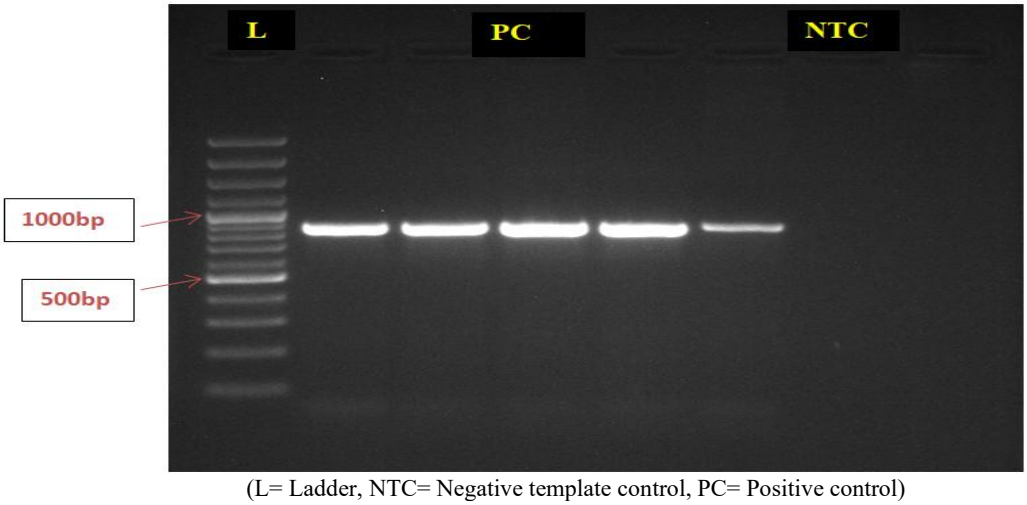
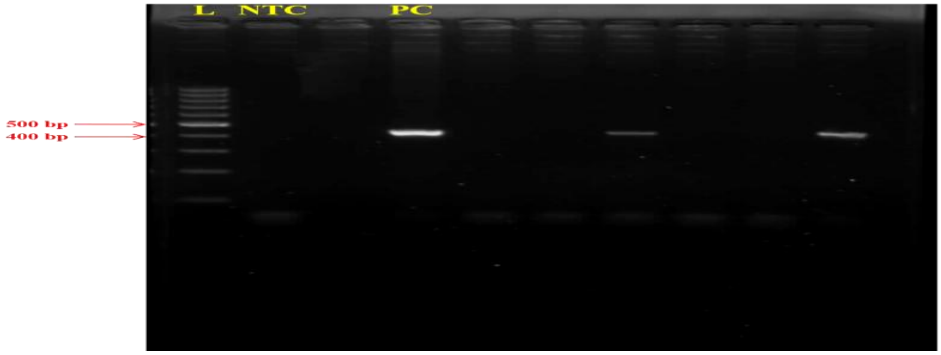


Fig. 3. Koch blue bodies inside lymphocytes in stained lymph node biopsy smear (100x)



(L= Ladder, NTC= Negative template control, PC= Positive control)

Fig. 4. Agarose gel electrophoresis of amplified DNA (881 bp) using genus specific primer of Theileria.



(L= Ladder, NTC= Negative template control, PC= Positive control)

Fig. 5. Agarose gel electrophoresis of amplified DNA (430 bp) of *Theileria annulata*

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
1		91.3	93.0	95.5	47.5	36.8	78.1	78.1	49.7	55.3	81.2	74.4	90.7	87.9	92.7	87.6	1	AF214827.1
2	1.5		84.8	89.0	54.5	43.5	86.0	86.0	56.5	62.1	87.9	82.3	84.0	84.3	84.6	90.2	2	EF092915.1
3	7.4	9.0		92.1	46.3	37.1	76.4	76.4	49.2	53.7	78.9	73.9	93.3	82.0	90.4	93.3	3	AF214844
4	4.6	4.0	8.3		49.7	38.5	80.3	80.3	51.7	57.3	83.1	78.1	92.1	84.0	92.7	86.8	4	AF214796.1
5	22.6	23.3	25.4	17.7		78.9	62.9	62.9	89.9	89.0	61.8	59.0	46.6	47.8	49.7	52.2	5	EF618728.1
6	46.8	48.7	45.9	41.4	40.3		49.7	49.7	81.5	76.7	49.4	47.2	36.5	36.8	37.6	42.4	6	EF618726
7	11.8	11.0	14.1	8.9	15.9	45.1		100.0	64.6	71.6	93.8	86.0	77.5	74.2	81.7	82.3	7	AB690865.1
8	11.8	11.0	14.1	8.9	15.9	45.1	0.0		64.6	71.6	93.8	86.0	77.5	74.2	81.7	82.3	8	AB690864.1
9	8.8	10.0	10.0	4.8	8.3	38.4	3.7	3.7		92.1	64.9	61.8	49.7	49.7	52.0	55.1	9	MW412254
10	11.7	12.8	15.1	8.1	14.7	40.6	4.7	4.7	0.0		72.8	67.7	54.2	55.3	59.0	59.6	10	MF116156.1
11	9.7	10.5	12.7	7.3	14.7	40.6	5.3	5.3	0.0	0.0		87.6	79.2	76.1	86.0	84.8	11	MT113479.1
12	20.9	20.1	21.8	15.8	17.1	42.4	13.3	13.3	2.6	5.6	13.0		74.7	81.5	76.7	80.1	12	OQ230445.1
13	9.9	10.1	7.1	8.3	24.7	47.8	12.6	12.6	8.8	14.0	12.3	20.6		79.2	90.2	87.9	13	MK034704.1
14	13.2	9.7	20.6	18.0	22.0	46.8	17.2	17.2	8.8	11.7	16.5	11.3	24.4		81.7	81.2	14	MK034703.1
15	6.8	8.4	9.4	6.8	16.1	42.3	6.1	6.1	2.7	3.8	2.9	16.7	9.7	20.0		86.0	15	OR256805
16	7.8	9.0	1.5	8.8	24.0	45.9	13.3	13.3	8.8	13.9	11.9	20.5	7.5	15.8	8.9		16	OR256806
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		

Fig. 6. Percent identity and nucleotide divergence matrix of nucleotide sequences of *Theileria annulata* based on 18S rRNA gene.

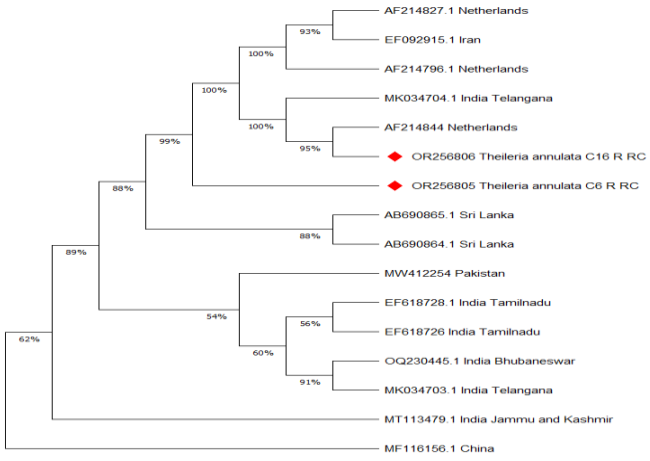


Fig. 7. Phylogenetic tree based on partial gene sequences of *Theileria annulata* isolates constructed using the Neighbor-Joining method**.**

higher seasonal prevalence overall. Using microscopic examination, the prevalence rates were 18.50% in winter, 50% in summer, and 15% in monsoon. By PCR, the infection rates were markedly higher at 40.74% in winter, 75% in summer, and 20% in monsoon. These results emphasize the influence of seasonal factors on the dynamics of *T. annulata* infection in North Gujarat. The seasonal prevalence data is presented in Table 5.

Table 1: Oligoprimer used for detection of *Theileria* parasite at the genus and species level

S. N.	Oligonucleotide primer	Primer sequence (5' to 3')	Amplicon Size	Reference
1	*Thieleria* Forward Primer (TF1)	GCA TTC GTA TTT AAC TGT CAG AGG	881bp	Jalali *et al.* (2014)
	Thieleria Reverse Primer (TF2)	ATA AGG TTC ACA AAA CTT CCC TAG		
2	*T. annulata* Forward Primer (TA430F)	CCAGGACCACCCTCAAGTTC	430bp	Kundave *et al.* (2015)
	T. annulata Reverse Primer (TA430R)	GCATCTAGTT CCTTGGCGGA		
3	*T. orientalis* Forward Primer (TO876F)	CACGCTATGTTGTTCAAGAG	876bp	Tanaka *et al*. (1993)
	T. orientalis Reverse Primer (TO876R)	TGTGAGACTCAATGCGCCTA		

Table 2: Hematological and serum biochemical alteration in HF cross-cattle (*p<0.05= Significant)

Parameters	Infected Animal (N=20)	Non-infected Animals (N=30)
Hematological parameters		
Hb (g/dl)	6.17 ±0.64*	8.79±0.47
RBC $\times 10^6$/µl	2.92±0.32*	4.42±0.21
PCV (%)	15.09±1.36*	18.80±0.96
WBC $\times 10^3$ / µl	6.91±1.30*	3.57±0.33
Lymphocytes %	5.04±2.60	3.35±1.68
Granulocytes %	3.61±1.09	1.94±0.40
Monocytes %	0.71±0.17	0.49±0.13
PLT $\times 10^3$ /µl	191.25±21.13	151.43±18.57
MCV (fl)	51.69±2.82*	42.44±1.17
MCH(pg)	21.26±0.89	19.77±0.56
MCHC (g/dl)	44.93±1.13	45.58±1.76
Serum biochemical parameters		
ALT (IU/L)	17.26±0.97	16.91±0.89
AST (IU/L)	86.00±3.48	78.23±2.37
GGT (IU/L)	17.26±0.97	16.41±1.09
BUN (mg/dl)	17.20±1.20	17.00±0.96
TB (mg/dl)	1.11±0.14	0.87±0.10

Table 3: Hematological and serum biochemical alteration in Kankrej cattle (*p<0.05= Significant)

Parameters	Infected Animal (N=12)	Non infected Animals (N=17)
Hematological parameters		
Hb (g/dl)	8.05±0.49	9.69±0.63
RBC $\times 10^6$/µl	4.14±0.28*	5.95±0.45
PCV (%)	19.17±1.29	24.22±2.07
WBC $\times 10^3$ / µl	6.27±1.07	7.81±0.98
Lymphocytes %	2.92±0.55	3.96±0.56
Granulocytes %	2.57±0.45	3.13±0.67
Monocytes %	0.86±0.18	0.80±0.14
PLT $\times 10^3$ /µl	171.58±40.94	201.88±22.27
MCV (fl)	46.63±1.75	40.48±1.39
MCH(pg)	19.50±0.73	18.27±0.82
MCHC (g/dl)	43.67±0.32	42.23±1.74
Serum biochemical parameters		
ALT (IU/L)	16.75±1.40	17.55±0.77
AST (IU/L)	76.93±3.20	82.51±3.19
GGT (IU/L)	17.26±1.50	16.83±1.18
BUN (mg/dl)	17.76± 1.63	17.94±1.07
TB (mg/dl)	0.96±0.13	1.12±0.15

Table 4: Age-wise prevalence

	< 2 year age			> 2 year age		
	No. of samples	By blood smear examination	By PCR	No. of samples	By blood smear examination	By PCR
Kankrej	32	6 (18.75%)	12(37.5%)	94	18 (19.14%)	28 (29.78%)
HF cross bred	50	14 (28%)	30 (60%)	124	42 (33.87%)	60 (48.38%)

Table 5: Season-wise prevalence

	Season	No. of samples	By blood smear examination	By PCR
Kankrej	**Winter**	40	8 (20%)	14 (35%)
	Summer	56	14 (25%)	20 (35.71%)
	Monsoon	30	2 (6.66%)	6 (20%)
HF cross bred	**Winter**	54	10 (18.50%)	22 (40.74%)
	Summer	80	40 (50%)	60 (75%)
	Monsoon	40	6 (15%)	8 (20%)

Molecular characterization and phylogenetic analysis

The molecular characterization of *T. annulata* was carried out through Sanger sequencing of representative PCR-amplified products. The obtained sequences were submitted to the NCBI GenBank database and assigned the accession numbers OR256805 and OR256806. These sequences were analyzed using BLAST and exhibited high sequence homology with previously reported *T. annulata* isolates from various geographical locations.

Phylogenetic analysis was performed using the neighbor-joining method with 1000 bootstrap replications in MEGA X. The phylogenetic tree revealed the evolutionary relationships of the study sequences with global and regional *T. annulata* isolates (Fig. 6 and 7). The study sequences clustered with isolates from the Netherlands (AF214827.1, AF214844), Telangana, India (MK034704.1), and Iran (EF092915.1) with a high bootstrap value of 100%. This indicates a close genetic relationship and suggests that the isolates from Gujarat are evolutionarily similar to those circulating in Europe and parts of South Asia.

Interestingly, the two study sequences (OR256805 and OR256806) formed a distinct sub-clade with the Netherlands sequence (AF214844), indicating a shared ancestry. The clustering of these sequences with isolates from Telangana, India, further supports the possibility of a common lineage within the Indian subcontinent. These results underscore the genetic variability of *T. annulata* within and across regions.

The study sequences showed moderate divergence from isolates reported in Sri Lanka (AB690865.1, AB690864.1) and Pakistan (MW412254), with bootstrap support of 88%-89%. This divergence could be attributed to regional differences, host-specific adaptations, or environmental factors influencing the evolution of the parasite.

In contrast, the sequences from Tamil Nadu, Bhubaneswar, and Jammu and Kashmir (EF618728.1, OQ230445.1, MT113479.1) exhibited lower sequence similarity (54%-62%) with the study sequences. These findings suggest potential genetic diversification of *T. annulata* across different Indian states, possibly driven by ecological and geographical factors.

Discussion

The present study screened 300 bovine blood samples (126 from Kankrej cattle and 174 from HF crossbred cattle) for *Theileria* spp. using microscopic analysis and PCR assays. The detection rates were 19.04% (24/126) and 31.74% (40/126) in Kankrej cattle and 51.72% (90/174) in HF crossbred cattle, respectively. These findings align with those reported by Kumar et al., 2022, Parveen et al., 2021, Farooq et al., 2018 and Ghosh et al., 2020, who consistently observed higher prevalence rates in crossbred cattle compared to native breeds. The increased susceptibility of crossbred cattle to *Theileria* infection could be attributed to their genetic makeup, which may result in a less robust immune response and native breeds possess specific genes for tolerance that confer greater resilience.

The study revealed a comparatively higher prevalence of *Theileria annulata* infection in cattle younger than two years, particularly in HF crossbred animals. This observation is consistent with the findings of Ghosh et al., 2020 and Brahmbhatt et al., 2019. This increased susceptibility in young animals can be explained by the immaturity of their immune system and the absence of acquired immunity against the parasite.

The highest prevalence of theileriosis was recorded during the summer season, with moderate prevalence during the monsoon and the lowest prevalence during winter. These findings corroborate the observations of Farooq et al., 2018, Swami et al., 2019 and Vandana et al., 2019. The increased prevalence in summer can be attributed to the hot and humid conditions that favour tick reproduction and activity, leading to higher rates of transmission. (Kumar et al., 2022, Brahmbhatt et al., 2019 and Acharya et al., 2017).

Biochemical analysis showed significantly elevated levels of alanine transaminase (ALT) and aspartate aminotransferase (AST), indicative of hepatic tissue damage caused by *Theileria* infection. Histopathological changes such as coagulative necrosis, distortion of hepatic cords, and heavy lymphocyte infiltration in periportal areas likely contribute to these alterations. These findings align with those of Vandana et al., 2019, Kachhawa et al., 2016 and Modi et al., 2015.

The phylogenetic analysis reveals that the *T. annulata* isolates from Gujarat share a close genetic relationship with isolates from Europe and certain parts of India. This indicates potential movement of parasite

strains due to trade, animal migration, or tick dispersal. The observed genetic divergence in isolates from Tamil Nadu, Bhubaneswar, and Jammu and Kashmir highlights the importance of understanding regional variations to design effective control measures.

Conclusion

PCR was more sensitive than microscopy for detecting *T. annulata* infection in cattle. HF crossbred cattle showed higher susceptibility than indigenous (*Bos indicus*) Kankrej cattle, with the highest prevalence recorded during summer. Molecular characterization confirmed close genetic similarity of the study isolates with previously reported *T. annulata* isolates, providing useful information for epidemiological surveillance and disease control.

Acknowledgement

The authors acknowledge the Department of Veterinary Microbiology, Animal Biotechnology, Veterinary Pathology, Animal Genetics and Breeding, and Veterinary Public Health the necessary facilities for the study. The author also acknowledges the Principal College of Veterinary Science & A.H, Sardarkrushinagar and Scheme of Developing High Quality Research (SHODH) of education department, Government of Gujarat, India for giving the required funds for the study.

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