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Prevalence, molecular signature and risk analysis of tropical theileriosis in Gir cattle in south-western region of Gujarat, India

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The high milk yielding Gir cattle breed produces an average of 2110 kg of milk per lactation with a standard yield ranging from 800 to 3300 kg. It is preferred for dairy farming due to its high yield, calm and cooperative temperament in south-western region of Gujarat, India (Patbandha *et al.* 2020). The production of such popular breed is strongly compromised by haemoprotozoan diseases such as theileriosis, babesiosis, trypanosomiasis and anaplasmosis (Bharti *et al.* 2022). Bovine tropical theileriosis is a serious threat to the dairy industry in endemic regions such as India. The infection is caused by the protozoan parasite *Theileria annulata* and is transmitted cyclically by the bite tick *Hyalomma anatolicum anatolicum*, resulting in lethal phases in exotic cattle and astounding mortality in crossbred and zebu cattle. The disease is prevalent in numerous regions worldwide, spanning South Europe, Asia and North Africa (Pereira *et al.* 2018). Clinical signs of theileriosis in cattle typically appear in 7 to 15 days after biting of vector ticks. Various diagnostic approaches can be used to recognize *Theileria* parasites (McFadden *et al.* 2011). Direct microscopic smear examination is a quick and inexpensive method but is not highly sensitive (Calleja-Bueno *et al.* 2017). In contrast, PCR-based assay is highly sensitive and can also identify the species of parasite, making it highly suitable for accurate diagnosis (Zaeemi *et al.* 2007). Previous studies reported prevalence of theileriosis in zebu cattle (Kumar *et al.* 2022, Velusamy *et al.* 2023), but the information regarding risk factors and clinical markers specific to Gir cattle infected with tropical theileriosis is lacking. Hence, the present study was designed to investigate the prevalence, risk factors, and clinical phenotypes associated with tropical theileriosis in Gir cattle.

Clinical examination and sample collection: In our study, a total of 702 blood samples were collected in K_3EDTA (Tripotassium Ethylene Diamine Tetraacetic Acid) containing tubes from Gir cattle at the veterinary hospital, ambulatory clinical services, and nearby Gaushalas in and around Junagadh, Gujarat, India during the period of 2022 to 2023. Each of the animals underwent a comprehensive clinical evaluation to record various clinical phenotypes. The study was approved by Institutional Animal Ethics Committee (IAEC) of College of Veterinary Science and Animal Husbandry with approval no. KU-JVC-IAEC-LA-88-22.

Microscopic examination: All collected blood samples were microscopically screened to detect intra-erythrocytic piroplasms form of *Theileria* and other haemoparasites. For each sample, a thin blood smear was drawn, air-dried, and fixed with 96% methanol for 5 minutes; subsequently, 5% Giemsa stain was applied for 30 minutes. The identification of parasites was performed according to specific morphological characteristics (Soulsby 2006).

Molecular diagnosis: The PCR assay was limited to microscopic positive samples for *Theileria* organism to identify the species through species-specific primers pair (Cyt b1/Cyt b2). Genomic DNA was extracted from a 200 µL whole-blood sample using a Blood DNA Isolation Kit (Thermo Scientific, Lithuania; Quigen, Germany) according to the manufacturer's protocol. In the present study, PCR analysis was carried out to target the *Cytochrome b* gene of *T. annulata* by utilizing the forward primer *Cyt b1* (5′-ACTTTGGCCGTAATGTTAAAC-3′) and reverse primer *Cyt b2* (5′-CTCTGGACCAACTGTTTGG-3′) (Bilgic *et al.* 2010). PCR was performed in a 25 µL total volume comprising 12.5 µL of Dream Taq Green PCR master mix (2×) (Thermo Scientific, Germany), 1 µL of each primer, 8.5 µL of nuclease-free water and 2 µL of extracted DNA. The PCR procedure commenced with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation (94°C for 20 sec), annealing (60°C for 30 sec), and extension (72°C for 30 sec). The final extension was performed at 72°C for 5 min. Subsequently, the PCR products were separated and amplicons were visualized through electrophoresis.

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Statistical analysis: The data concerning to age, sex, season, stages of lactation, tick infestation (presence or absence of ticks on the body of the infected animal) and the frequency of acaricidal treatment in infected animals were analysed by using the chi-square test to know the factors affecting prevalence. Parameters that had a significant effect on prevalence were further subjected to multivariate logistic regression models to estimate the strength of association between the potential risk factors and prevalence. The statistical analysis was carried out with SPSS software version 21.0. (IBM Corporation, Armonk, New York, USA).

Prevalence: In the present study, microscopic examination revealed that a total of 263 blood samples from Gir cattle only exhibited various forms of *Theileria* piroplasms (Fig. 1) and no other haemoparsites were observed. Subsequent molecular analysis of all 263 positive samples was carried out using the *Cyt b1/Cyt b2* primer pair targeting the 312 base pair size of amplicons specific to *Theileria annulata* (Fig. 2). Differentiation of various *Theileria* species based on the structure of their piroplasms through microscopy is not a reliable method due to morphological similarity among these parasites (Razmi *et al*. 2006, Zaitoun *et al.* 2019). To identify the species of *Theileria* organism it is essential to employ sensitive and specific diagnostic tools such as PCR because amplification of DNA is regarded as one of the most powerful tools for detection of theileriosis (Heidarpour Bami *et al.* 2009, Amarasiri *et al.*, 2024). Overall, 37.46% (263/702) of the Gir animals were infected with *T. annulata* (Table 1). This present finding corroborates with the previous observations in terms of percent prevalence of the disease. (Dadhich *et al.* 2017, Kumar *et al.* 2022, Velusamy *et al.* 2023). Among Gir cattle, significantly ($p<0.001$) higher prevalence was observed in older animals more than 5 years of age (57.73%), followed by animals of 1-5 years of age (21.61%), and in animals younger than 1 year of age (18.75%). Age wise prevalence of this disease partially agrees with the earlier report (Kundave *et al.* 2015). The probable reason for higher prevalence of *T. annulata* infection in cattle above 5 years may be due to physiological factors like estrus, pregnancy and lactation. These physiological factors temporarily supress the immunity of older cattle and may increase risk of *T. annulata* infection. Further, the calves get passive immunity through colostrum which may protect them against *T. annulata* infection leading to lower infections (Radostits *et al.* 2010). The sex wise prevalence between male and female was statistically insignificant ($p=0.752$). This counterparts disclose similarity with previous research (Selim *et al.* 2020, Selim *et al.* 2022). The season-wise occurrence of *T. annulata* infection was highest in summer (49.08%), followed by monsoon (34.92%) and winter (23.20%). The suffering of most animals during the summer and monsoon months could be attributed towards abundance of vectors in these seasons of the year (Kumar *et al.* 2015, Velusamy *et al.* 2014). The significantly ($p<0.001$) higher occurrence of *T. annulata* infection in Gir Cattle was during early lactation (61.45%) as compared to late-lactation and lowest was recorded during mid-lactation. These findings were aligning with previous studies (Bhosale *et al.* 2020, Selim *et al.* 2020, Selim *et al.* 2022). Association between tick infestations and the prevalence of tropical theileriosis was worked out. Infection was significantly ($p<0.001$) higher (58.58%) in tick infested animals compared to animals without tick infestation (17.86%). The prevalence of *T. annulata* infection in relation to frequency of acaricidal application for tick control was significantly ($p<0.001$) lower in cattle that were consistently treated at quarterly interval of the year with acaricides (6.98%) than those which did not receive acaricidal treatment (62.80%) or received irregular treatment during the year (19.59%). The current findings were not inconsistent with previous reports (Selim *et al.* 2020, Selim *et al.* 2022, Ullah *et al.* 2021). Ticks and tick-borne diseases constitute a major threat for animal health worldwide with increasing incidence due to climate change, distribution of tick hosts, and ecological and anthropogenically induced changes (Socha *et al.* 2022). Ticks affect host health status directly through manipulation of immune and metabolic processes and transmission of tick-borne pathogens (Vaz-Rodrigues *et al.*

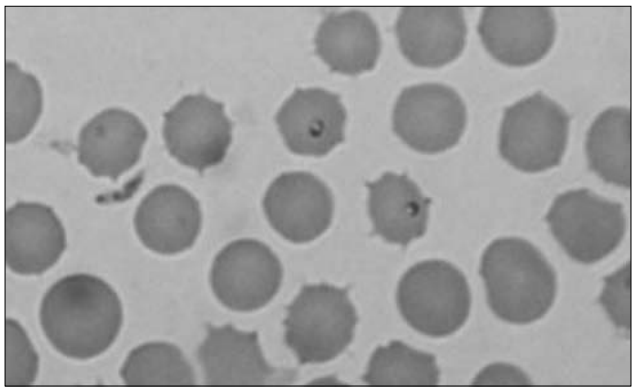

Fig. 1. Microscopic examination of blood film showing signet ring of *Theileria* piroplasms in the erythrocytes.

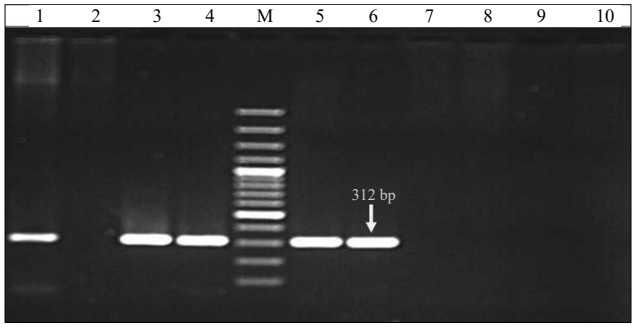

Fig. 2. PCR amplification of Cytochrome b gene of *Theileria annulata*
1 – Positive Control
3, 4, 5, 6 - Positive Sample
M – Markers (100 bp plus DNA ladder)
2 – Negative Control
7, 8, 9, 10 – Negative Sample

Table 1 Prevalence of tropical theileriosis in Gir cattle

Factor	Category	No. of animal screened	No of animal positive
Age	Below 1 year	112	21 (18.75%)
	1 to 5 years	273	59 (21.61%)
	Above 5 years	317	183 (57.73%)
	$\chi 2$ value	101.60	
	p value	p<0.001	
Sex	Male	108	39 (36.11%)
	Female	594	224 (37.71%)
	$\chi 2$ value	0.1	
	p value	0.752	
Season	Winter	177	41 (23.16%)
	Summer	273	134 (49.08%)
	Monsoon	252	88 (34.92%)
	$\chi 2$ value	31.879	
	p value	p<0.001	
Stages of lactation	Early lactation	83	51 (61.45%)
	Mid lactation	119	32 (26.89%)
	Late lactation	141	61 (43.26)
	$\chi 2$ value	24.131	
	p value	p<0.001	
Status of tick infestation	Present	338	198 (58.58%)
	Absent	364	65 (17.86%)
	$\chi 2$ value	124.1	
	p value	p<0.001	
Acaricidal Application	Regular	129	9 (6.98%)
	Irregular	245	48 (19.59%)
	No application	328	206 (62.80%)
	$\chi 2$ value	174.5	
	p value	p<0.001	
Overall prevalence		702	263 (37.46%)

2022). So, integrated pest management is used for tick control adapted to a geographical area and with acaricides as major control intervention (Kunz and Kemp 1994).

Risk analysis: The multivariate logistic regression models (Table 2) demonstrated a significantly (P=0.001) greater likelihood of testing positive for *T. annulata* infection in cattle aged 5 years and older (OR=5.19, 95% CI = 3.504-9.996) than in those aged younger than 1 year. The risk of *T. annulata* infection was significantly higher in summer (OR=3.19, 95% CI = 2.096-4.878, P=0.001) and monsoon (OR=1.78, 95% CI = 1.152-2.74, P=0.009) than in winter. The chances of occurrence of *T. annulata* infection

Table 2 Potential risk factors associated with tropical theileriosis in Gir cattle.

Parameter	Estimates	SEM	P value	OR	95% CI
		Age			
1-to-5-year vs Below 1 year	0.178	0.283	0.530	1.195	0.686-2.081
Above 5-year vs Below 1 year	1.778	0.267	0.001	5.918	3.504-9.996
		Season			
Summer vs Winter	1.162	0.215	0.001	3.198	2.096-4.878
Monsoon vs Winter	0.577	0.222	0.009	1.780	1.152-2.74
		Stages of lactation			
Early vs. Mid	0.733	0.282	0.009	2.090	1.202-3.635
Mid vs Late	-0.729	0.268	0.006	0.482	0.285-0.815
		Tick infestation			
Present vs Absent	1.873	0.176	0.001	6.506	4.609-9.183
		Frequency of acaricidal application			
Regular vs Nil	-3.114	0.364	0.001	0.044	0.022-0.091
Irregular vs Nil	-1.936	0.197	0.001	0.144	0.098-0.212

SEM, Standard error mean; OR, odds ratio; CI, confidence interval

were significantly higher in early lactation (P= 0.001, OR= 2.09, 95% CI= 1.202-3.635) than in mid lactation. Multivariate analysis revealed that cattle experiencing heavy tick infestations were likely to be more at risk for tropical theileriosis (OR= 6.5, 95% CI= 4.609-9.183, P= 0.001) than non-infested. Cattle subjected to regular acaricide treatment (OR= 0.04, 95% CI= 0.022-0.091, P= 0.001) exhibited an approximately 96% lower likelihood of *T. annulata* infection followed by irregular acaricide treatment (OR=0.14, 95% CI= 0.098-0.212, P=0.001) and those did not receive acaricide treatment. Therefore, we conclude that, the risk of *T. annulata* infection in Gir cattle is potentially greater in animals older than 5 years (older animals), in the summer season, in cattle with tick infestations and in those who did not receive or receive irregular acaricidal treatment (Selim *et al.* 2020, 2022).

Clinical phenotypes: In the present study, more than 75% of infected Gir cattle showed pyrexia, prescapular lymphadenopathy, inappetence to anorexia, nasal discharge, ocular discharge, and pale mucous membrane were considered as the most significant clinical phenotypes (Table 3). Additionally, the infected animals also displayed various clinical presentation in tropical theileriosis. The presence and severity of various clinical signs in patients with tropical theileriosis primarily rely on several factors, including the virulence of the specific strain involved, the degree of susceptibility exhibited by the host, and the extent of proliferation of the *Theileria* parasite within the lymphatic tissue (Aktas *et al.* 2006). The production of cytokines, specifically TNF-α, IL-1, and IL-6, by infected mononuclear cells plays a significant role in the development of various clinical symptoms observed in patients with tropical theileriosis (Graham *et al.* 2001). These symptoms include depression, pyrexia (fever), anorexia, cachexia, and disseminated hemorrhages. During the early stages of the disease, *T. annulata*-infected cells undergo clonal expansion, leading to lymphoproliferation and lymphadenopathy (Woods *et al.* 2013). Anaemia results from several contributing factors, including immune-mediated haemolysis, overproduction of cytokines, and generation of reactive oxygen species (Nazifi *et al.* 2009, Saleh *et al.* 2011). *Theileria* parasites primarily infect and multiply within white blood cells, leading to systemic inflammation and immune responses. These responses can affect various organs and tissues, leading to nasal discharge, ocular discharge, corneal opacity, diarrhea, melena, hypersalivation, *etc*. The *Theileria* species may also affect the mediastinal lymph nodes located around the base of the cranial and caudal vena cava, which are responsible for returning blood to the heart. The pressure exerted by the affected lymph nodes can impede blood flow, leading to fluid retention and subsequent swelling in the brisket region. The presence of icterus, or jaundice, and hemoglobinuria observed in tropical theileriosis can be attributed to hemolysis induced by the merozoites of *T. annulata*, a parasite responsible for the disease (Gharbi *et al.* 2012). Chronic constipation/impaction in tropical theileriosis is caused by insufficient bile salt production due to hemolytic anaemia and impaired digestion (Ziam *et al.* 2020).

Table 3. Various clinical phenotypes exhibited by Gir cattle (n=263) affected with tropical theileriosis.

Clinical marker	Number of animals	Percentage (%)
Fever/Pyrexia	235	89.35
Prescapular lymphadenopathy	238	90.49
Pale mucus membrane	202	76.81
Congested mucus membrane	47	17.87
Ticks on body	198	75.29
Inappetence/Anorexia	245	93.16
Impaction/Constipation	60	22.81
Diarrhoea	45	17.11
Melena	29	11.03
Weakness/Emaciation	63	23.95
Dullness/Depression	182	69.20
Hypersalivation	38	14.45
Jaundice	14	5.32
Nasal discharge	230	87.45
Lachrymal discharge	220	83.65
Haemoglobinuria	10	3.80
Oedema	3	1.14
Recumbency	64	24.33
Corneal opacity	7	2.66

In conclusion, this study represents on epidemiological aspects, its molecular detection and risk factors and key clinical phenotypes of tropical theileriosis specifically in Gir cattle from the south-western region of Gujarat, India. These findings have the potential to inform the development of preventive strategies and facilitate clinical diagnosis under field conditions, benefiting clinicians and livestock holders since this study has added information on age wise prevalence of the disease. We claim that, this particular finding is an important step to control the disease by means of this sensitive test to identify and cure the infected animals.

SUMMARY

The study signifies pioneering effort for development of preventive strategies of tropical theileriosis in Gir Cattle by shedding the light on epidemiological information regarding age, season, lactational status, tick infestation and importance of tick control. On the other hand, key clinical observations *viz.*, pyrexia, lymphadenopathy, altered appetite, ocular and nasal discharge, pale mucous membrane, and tick infestations of the study provide the platform for early diagnosis of disease in field conditions to veterinary professionals.

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