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Original Article

Molecular epidemiology, associated risk factors, and phylogeny of *Theileria annulata* infecting buffaloes and cattle from different agro-climatic regions of Punjab, Pakistan

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Abstract

Background: Tropical theileriosis is the most prevalent hemoprotozoan disease in Pakistan. **Aims:** The study aimed to investigate the epidemiology and evolutionary relationship of *Theileria annulata* in bovines in diverse agro-climatic regions of Punjab, Pakistan. **Methods:** 800 blood specimens were collected from asymptomatic cattle (n=480) and buffaloes (n=320) using a multistage sampling method from Sargodha (n=400) and Multan (n=400) districts. The samples were assessed for blood smear microscopy and *cytochrome b* gene based PCR. Twenty samples were collected from each union council of each district. **Results:** The overall prevalence of *T. annulata* infection in bovines was 9% and 17.13% as determined by blood smear analysis and PCR, respectively. The disease positivity in cattle and buffaloes was respectively 10.21% and 20.42% by blood smear screening and 7.19%, 12.19% by PCR. The overall PCR based prevalence in the Sargodha and Multan districts was 19% and 15.25%, respectively. Absence of rural poultry, tick infestation, and a history of tick-borne diseases had significant effect in cattle. Tick infestation and age were the main statistically significant disease determinants in buffaloes. The evolutionary analysis of the *cytochrome b* gene showed that the Pakistani isolate infecting buffalo was related to those from Iran, India, Egypt, and Sudan. The isolate from cattle was genetically close to those from Pakistan, India, Iran, Iraq, and Turkey. **Conclusion:** It can be concluded that biotic and abiotic factors contribute to disease occurrence. The current study will help to devise control strategies to prevent substantial economic losses.

Key words: Agro-climatic regions, *Bos taurus*, *Bubalus bubalis*, Pakistan, *Theileria annulata*

Introduction

Tropical theileriosis, a blood-borne protozoal illness caused by *Theileria annulata*, is one of the most prevalent disease in Pakistan and has a significant impact on the global dairy industry (Asif *et al*., 2022; Atif *et al*., 2022). The worldwide economic toll of this disease is estimated to be 7.3 billion USD per head per year (Rodríguez-Hidalgo *et al*., 2017). Due to its location in a tropical region, Pakistan has an ideal climate for the growth and multiplication of ticks, which transmit theileriosis to a range of domestic and wild ruminants (Ghafar *et al*., 2020; Zaman *et al*., 2022; Aslam *et al*., 2023). The transmission of this disease occurs via Ixodid ticks, including species such as *Hyalomma*, *Haemaphysalis*, *Dermacentor*, *Rhipicephalus*, and *Amblyomma* (Caeiro *et al*., 1999; Ferrolho *et al*., 2016; Gomes *et al*., 2016; Abaker *et al*., 2017).

Generally, *Theileria* species are divided into two categories: non-transforming and transforming host cells, based on their ability to manipulate leukocyte transformation and multiply within the host. *T. annulata* is a transforming parasite (Tajeri *et al*., 2021). The clinical symptoms of the disease include fever, restlessness, loss of appetite, swollen lymph nodes, rapid breathing and heartbeat, a sudden decrease in milk

production, and kidney damage, and can be accompanied by hemoglobinuria and persistent diarrhea (Ananda *et al*., 2009). As the illness progresses, anemia and jaundice may develop (Oryan *et al*., 2013; Tumer *et al*., 2023). In most cases, animals recovering from the disease become carriers without exhibiting any symptoms (Selim *et al*., 2020).

Several risk factors, including various agro-climatic regions, animal age, gender, breed, unrestricted use of antiprotozoal/acaricidal drugs, and management practices, contribute to the prevalence of tropical theileriosis. The distribution of vectors and incidence of tick-borne diseases (TBDs) are influenced by agro-ecological regions (Silatsa *et al*., 2019; Ghafar *et al*., 2020; Basit *et al*., 2022). In Pakistan, the incidence of *T. annulata* has been reported to range from 4% to 60% (Shahnawaz *et al*., 2011; Khattak *et al*., 2012; Farooqi *et al*., 2017; Zeb *et al*., 2020; Parveen *et al*., 2021a, b; Al-Hamidhi *et al*., 2022; Asif *et al*., 2022; Mohsin *et al*., 2022). Rapid population growth in Pakistan led to an increased demand for milk and dairy products, which supplement the significance of controlling tropical theileriosis.

The dairy industry in both the public and private sectors raises exotic and crossbred cattle with a higher milk production potential, however, these animals are highly susceptible to ticks and tropical theileriosis (Jabbar *et al*., 2015). Accurate epidemiological information and the identification of genotypes are crucial for disease prevention and control perspectives. This study aims to explore the epidemiology and phylogenetic status of *T. annulata* in cattle and buffalo in different agro-climatic regions of Sargodha and Multan districts in Punjab, Pakistan. No comparative molecular surveillance study targeting *cytochrome b* gene has been conducted in bovines from two agro-ecologically diverse southern (Multan) and central (Sargodha) canal irrigated regions of Punjab, Pakistan yet. We created unique datasets to evaluate the impact of various determinants, such as animal age, breed, species, gender, tick infestation, history of TBDs, body condition, animal sources, herd size, farm type, feeding pattern, frequency of acaricide use, the role of rural poultry, and season on a questionnaire.

Materials and Methods

All research segments were conducted as per the institutional guidelines of the ethical review committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan vide No. DR/1147, dated October 26th, 2017.

Study locations

The study was conducted in Sargodha and Multan districts in Punjab province, Pakistan. Sargodha is situated between Chenab and Jhelum rivers located in Central Punjab at 32° 4’56.88" N, 72° 40' 8.8608‘E with higher cattle (n=574887) and buffaloes (n=687685) population. Whereas Multan is located in Southern Punjab at 30.1575° N, 71.5249° E. These regions were selected because of having distinct agro-climatic region and higher population density of cattle (n=498548) and buffaloes (n=416494) (Fig. 1; Pakistan Livestock

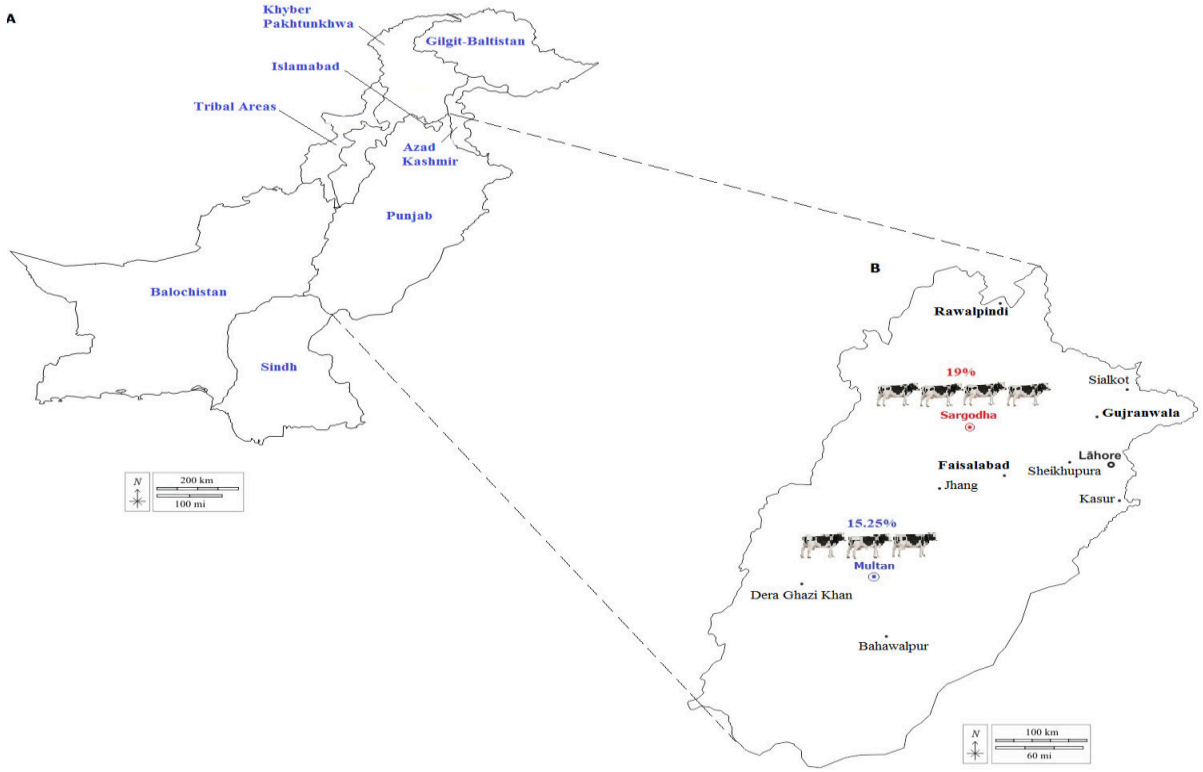

Fig. 1: Map of Pakistan showing investigated Punjab province (**A**) and Sargodha and Multan districts (**B**) along with overall prevalence in bovines. Each animal in a picture is indicating about 5% prevalence

Census, 2006; Atif *et al*., 2013; Asif *et al*., 2022).

Sample collection

A total of 800 blood samples were collected from bovines (cattle, n=480 and buffalo, n=320) from Sargodha (n=400) and Multan (n=400) districts using a multistage sampling technique from March 2019 to June 2020. Twenty union councils were nominated from each district, and samples (n=20) were collected from each union council. Samples were collected from small holder dairy farmers having 2-5 animals. The sampling frame included all union councils of both districts. The sampling unit consisted of asymptomatic buffaloes and cattle related to different ages (cattle: <1, 1-2, >2-4, >4 years and buffalo: <1, 1-3, >3-5, >5 years), sex (male and female), and breeds (cattle: Holstein Friesian, Crossbred, and Indigenous, and buffalo: Nili Ravi, Kundi, and Non-descriptive). Blood samples were aseptically taken from the jugular veins and immediately transferred to EDTA-coated vacutainers. The samples were stored in a styrofoam cooler box containing ice packs for transportation to the laboratory. Each animal was closely examined for tick infestation attached to different animal body parts. Collected blood samples were brought to the College of Veterinary and Animal Sciences, Jhang, Pakistan, for further analysis.

Blood smear examination and estimation of sensitivity and specificity

A thin blood smear was prepared as described by Atif *et al*. (2021). Giemsa stain was performed from commercially available Giemsa powder containing eosin, azure B, and methylene blue. The blood smear was fixed with methanol for 30 s and stained with Giemsa stain solution (5%) for 20-30 min. Later, the smear was washed with water and dried in air. The stained slide was observed for *Theileria* specific inclusion bodies under 100X of oil immersion lens (Atif *et al*., 2021).

DNA extraction and PCR amplification

DNA was extracted with a DNA extraction kit (Catalog No. K0782 and K0721) following the protocol defined by the manufacturer (Thermo Scientific, USA). The amplification of the specific genomic region of *T. annulata* was performed by targeting variable region of *cytochrome b* gene, with 312 bp product, and utilizing Cytob1F (3´-ACT TTG GCC GTA ATG TTA AAC-5´) and Cytob1R (3´-CTC TGG ACC AAC TGT TTG G-5´) in conventional PCR. This gene proved highly sensitive for the detection of carriers for large scale field studies, validated by Bilgic *et al*. (2010) and Bilgic *et al*. (2013). The PCR was carried out in a reaction mixture with a volume of 50 µL, comprising 10 mM of Tris-HCL (pH 8.3), KCl, 50 mM of $MgCl_2$, 1.5 mM of gelatin 0.001%, 250 µM of deoxynucleotide triphosphate, 1 U of AmpliTaq DNA polymerase, 10 pmol of each primer (Cytob1F/Cytob1R) and 2 µL of template DNA. The reaction was performed in an automatic thermal cycler (Bio Rad) and performed initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 95°C for 50 s. Primers were annealed at 55°C for 50 s and extension was performed at 72°C for 1 min, and a final extension step occurred at 72°C for 10 min. The PCR products were maintained at 4°C or stored at -40°C. For electrophoresis, an agarose gel (1.5%) was used for the amplified DNA. The gel was stained with ethidium bromide and visualized with Trans-illuminator (Catalog No. MUVB-112; Major Scientific, USA).

Assessment of risk factors

A pretested questionnaire was used to collect information regarding abiotic (area and season), host-related (tick infestation, history of tick-borne diseases, body condition, breed, age, and gender), and managemental factors (animal sources, herd size, farm type, feeding pattern, frequency of acaricide use, presence of rural poultry).

DNA sequencing and data analysis

To validate the results of PCR, sequencing was performed. Representative *T. annulata* isolates from cattle and buffalo were sequenced in both directions by "First base laboratories SDN BHD (604944-X) Malaysia", utilizing the same primers employed for the PCR intensifications. The reaction was achieved utilizing a traditional "BigDye™ Terminator Cycle Sequencing Ready Reaction" kit (Applied Biosystems, USA) along with an Automatic DNA Sequencer (ABI3730XL, Macrogen Europe, Netherland). Chromatograms were edited with Chromas Lite v 2.01. The DNAMAN program was used to perform multiple sequence alignment of *Cytochrome b* partial sequences (Version 5.2.2; Lynnon Biosoft, Que., Canada) and to compute genetic distances by the maximum composite likelihood method (Tamura and Nei, 1993). Neighbor-joining trees were created using the same software (Saitou and Nei, 1987). Statistical support for internal branches was recognized by bootstrap analysis with 1000 replications.

Statistical analysis

The Chi-square test was performed to find the association between infection rates and different risk factors. The Cohen's kappa test was used to assess the degree of agreement between the results of PCR and blood smear microscopy. The P-values ≤ 0.05 were considered significant using the statistical software SPSS 21. The sensitivity and specificity of blood smear microscopic examination were evaluated against the reference test (PCR) using the following formula (Nayel *et al*., 2012).

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{Number of false positives}} \times 100$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{Number of false negatives}} \times 100$$

Results

Prevalence of *T. annulata* in cattle and buffaloes

Overall prevalence rates of *T. annulata* infection in bovines (cattle and buffaloes) were 9% (72/800) and 17.13% (137/800) based on blood smear and PCR, respectively (Table 1). The relative sensitivity and specificity of blood smear microscopy compared to PCR were estimated at 52.55% and 100%, respectively (Table 1). The kappa coefficient revealed a moderate level of agreement between PCR and microscopy, estimated as 0.64 (Table 2). District-wise overall prevalence was estimated as 19% (76/400) in Sargodha and 15.25% (61/400) in Multan district. The overall PCR based prevalence rate of *T. annulata* in cattle and buffaloes was 20.42% (98/480) and 12.19% (39/320), respectively (Tables 3 and 4). The highest prevalence rate was recorded in spring (21.6%, 16/74) and summer (26.4%, 53/201) in buffaloes and cattle. However, the difference in infection rates between seasons was statistically significant only for cattle (P<0.001) (Tables 3 and 4). In Multan, the statistically highest *T. annulata* infection rate was recorded in bovines in summer (cattle, 23.76%) and spring (buffaloes, 22.58%), respectively (P<0.05, Fig. 2). Similarly, a statistically highest occurrence of *T. annulata* was also recorded in cattle and buffaloes tested, respectively, in summer (29%) and spring (20.93%) in Sargodha district (P<0.05, Fig. 2).

Table 2: Diagnostic comparison of PCR and blood smear microscopy

Diagnostic assay	Blood smear microscopy		*k*-value*
PCR	Positive	Negative	0.64
Positive	72	65	
Negative	0	663	

* Cohen's kappa coefficient

Table 1: Prevalence, specificity, and sensitivity of PCR and blood smear microscopy

Diagnostic assay	Total	Positive (n)	Specificity (%)	Sensitivity (%)	Prevalence (% ± C.I.[1])
PCR	800	137	100	100	17.13* ± 0.025
Blood smear microscopy	800	72	100	52.55	9 ± 0.019

[1] C.I.: Confidence interval. * P=0.00, df=1, and X^2=23.25, Statistically significant difference between the prevalence of two tests based on Chi-square test analysis. P: Value, df: Degree of freedom, and X^2: Chi-square value

Table 3: Molecular prevalence of *Theileria annulata* infection in Pakistani cattle according to different abiotic and management risk factors

Factors	Classes	Number	Positive	Rate (% ± C.I.[1])	P-value (Khi2)
Location	Sargodha	240	55	22.9 ± 0.053	0.174 (1.84)
	Multan	240	43	17.9 ± 0.049	
Season	Summer	201	53	26.4 ± 0.060	0.000* (15.03)
	Autumn	79	11	13.9 ± 0.076	
	Winter	59	2	3.4 ± 0.047	
	Spring	141	32	22.7 ± 0.068	
Animal sources	Home bred	243	50	20.6 ± 0.051	0.983 (0.0004)
	Local purchase	128	24	18.75 ± 0.067	
	Imported foreign	109	24	22.0 ± 0.078	
Herd size	1-5 animals	127	30	23.6 ± 0.074	0.674 (0.177)
	6-10 animals	101	15	14.85 ± 0.069	
	11-20 animals	110	23	20.9 ± 0.076	
	21-40 animals	115	24	20.9 ± 0.074	
	>40 animals	27	6	22.2 ± 0.157	
Farm type	Dairy	319	70	21.9 ± 0.045	0.230 (1.438)
	Beef	26	4	15.4 ± 0.139	
	Dairy and beef	135	24	17.8 ± 0.065	
Acaricide use	<30 days	23	3	13.0 ± 0.137	0.794 (0.068)
	31-60 days	81	21	25.9 ± 0.096	
	>60 days	352	72	20.45 ± 0.041	
	None/Manual picking	24	2	8.3 ± 0.109	
Type of acaricides	Organophosphates	134	23	17.2 ± 0.065	0.058 (3.585)
	Pyrethoids	62	9	14.5 ± 0.088	
	Avermectin	284	66	23.2 ± 0.049	
Rural poultry	Yes	319	51	15.98 ± 0.041	0.000* (11.459)
	No	161	47	29.2 ± 0.071	
Total		480	98	20.42 ± 0.035	

[1] C.I.: 95% confidence interval. * Statistically significant (P<0.05)

Table 4: Molecular prevalence results of *Theileria annulata* infection in Pakistani buffalos according to different abiotic and management risk factors

Factors	Classes	Number	Positive	Rate (% ± C.I.[1])	P-value (Khi2)
Location	Sargodha	160	21	13.1 ± 0.052	0.608 (0.26)
	Multan	160	18	11.3 ± 0.049	
Season	Summer	90	15	16.7 ± 0.076	0.119 (2.427)
	Autumn	61	6	9.8 ± 0.074	
	Winter	95	2	2.1 ± 0.029	
	Spring	74	16	21.6 ± 0.094	
Feeding pattern	Grazing	106	11	10.4 ± 0.058	0.742 (0.108)
	Stall feeding	142	19	13.4 ± 0.056	
	Mix	72	9	12.5 ± 0.076	
Herd size	1-5 animals	120	16	13.3 ± 0.060	0.527 (0.399)
	6-10 animals	90	10	11.1 ± 0.064	
	11-20 animals	58	8	13.8 ± 0.088	
	21-40 animals	37	5	13.5 ± 0.109	
	>40 animals	15	0	0 ± 0	
Farm type	Dairy	180	20	11.1 ± 0.045	0.642 (0.215)
	Beef	48	5	10.4 ± 0.086	
	Dairy and beef	92	14	15.2 ± 0.072	
Acaricide use	<30 days	18	1	5.6 ± 0.105	0.010* (6.588)
	31-60 days	56	8	14.3 ± 0.092	
	>60 days	183	30	16.4 ± 0.052	
	None/Manual picking	63	0	0 ± 0	
Type of acaricides	Organophosphates	42	5	11.9 ± 0.098	0.480 (0.497)
	Pyrethoids	127	10	7.9 ± 0.047	
	Avermectin	151	24	15.9 ± 0.058	
Rural poultry	Yes	206	23	11.2 ± 0.043	0.453 (0.563)
	No	114	16	14.0 ± 0.064	
Total		320	39	12.19 ± 0.035	

[1] C.I.: 95% confidence interval. * Statistically significant (P<0.05)

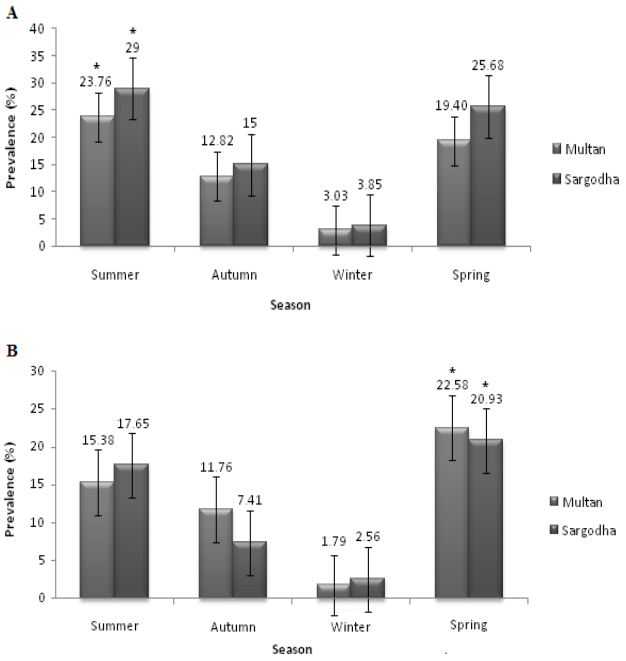

Fig. 2: Seasonal prevalence of *Theileria annulata* infection in Multan and Sargodha districts. Season: (cattle, P=0.000, df=3, X^2=15.03, and buffalo, P=0.119, df=3, X^2=2.427). First A and second B graph indicating seasonal prevalence of cattle and buffaloes, respectively

Assessment of risk factors

For cattle, risk factors analysis revealed that rural poultry (P<0.001, X^2 = 11.459), tick infestation (P=0.001, X^2 = 10.278), and history of TBDs (P<0.001, X^2 = 12.42) act as significant disease determinants. Indeed, cattle not located in rural areas (29.2%, 47/161) were statistically more disease-ridden with *T. annulata* than those in these areas (15.9%, 51/319). In addition, cattle infested with ticks (22.8%, 94/412) were significantly more infected than those not infested (5.9%, 4/68). Moreover, cattle previously infected with TBDs (23.2%, 94/405) were more prevalent with *T. annulata* than without a history of TBDs (5.3%, 4/75) (P<0.001; Tables 3 and 4). For buffaloes, statistical analysis showed that acaricide use (P=0.010, X^2 = 6.588), age (P<0.001, X^2 = 11.178), tick infestation (P<0.001, X^2 = 13.823), and history of TBDs (P<0.001, X^2 = 11.505) were significantly associated with *T. annulata* infection (Tables 5 and 6). Indeed, buffaloes that received acaricide treatment in a period >60 days (16.4%, 30/183) and between 31 and 60 days (14.3%, 8/56) had significantly higher infection compared to those received tick control acaricide <30 days (5.6%, 1/18) or by manual picking (0%, 0/63) (P<0.05, Table 5). Furthermore, buffaloes infested with ticks (18.8%, 31/165) were significantly more infected than those free

Table 5: Molecular prevalence of *Theileria annulata* infection in Pakistani cattle according to different biotic risk factors

Factors	Classes	Number	Positive	Rate (% ± C.I.[1])	P-value (Khi2)
Gender	Male	128	29	22.7 ± 0.072	0.463 (0.537)
	Female	352	69	19.6 ± 0.041	
Age	<1 year	56	22	39.3 ± 0.127	0.300 (1.07)
	1-2 years	76	5	6.6 ± 0.054	
	>2-4 years	152	37	24.3 ± 0.068	
	>4 years	196	34	17.3 ± 0.052	
Breed	Holstein Friesian	158	43	27.2 ± 0.068	0.164 (1.935)
	Crossbred	248	50	20.2 ± 0.049	
	Indigenous	74	5	6.8 ± 0.056	
Tick infestation	Present	412	94	22.8 ± 0.041	0.001* (10.278)
	Absent	68	4	5.9 ± 0.050	
History of TBDs	Present	405	94	23.2 ± 0.041	0.000* (12.42)
	Absent	75	4	5.3 ± 0.050	
Body condition[2]	1	27	5	18.5 ± 0.147	0.570 (0.321)
	2	97	20	20.6 ± 0.080	
	3	235	50	21.3 ± 0.052	
	4	84	17	20.2 ± 0.086	
	5	37	6	16.2 ± 0.119	
Total		480	98	20.4 ± 0.035	

[1] C.I.: 95% confidence interval, and [2] From good to poor body condition, the animal takes a score from 1 to 5. * Statistically significant (P<0.05)

Table 6: Molecular prevalence results of *Theileria annulata* infection in Pakistani buffalos according to different biotic risk factors

Factors	Classes	Number	Positive	Rate (% ± C.I.[1])	P-value (Khi2)
Gender	Male	119	16	13.4 ± 0.0607	0.597 (0.279)
	Female	201	23	11.4 ± 0.043	
Age	<1 year	37	5	13.5 ± 0.109	0.001* (11.178)
	>1 and <3 years	68	18	26.5 ± 0.105	
	>3 and <5 years	71	9	12.7 ± 0.076	
	>5 years	144	7	4.9 ± 0.035	
Breed	Nili Ravi	76	12	15.8 ± 0.082	0.198 (1.6499)
	Kundi	65	9	13.8 ± 0.084	
	Non-descriptive	179	18	10.1 ± 0.043	
Tick infestation	Present	165	31	18.8 ± 0.058	0.000* (13.823)
	Absent	155	8	5.2 ± 0.035	
History of TBDs	Present	200	34	17.0 ± 0.052	0.000* (11.505)
	Absent	120	5	4.2 ± 0.035	
Body condition[2]	1	39	4	10.3 ± 0.096	0.927 (0.008)
	2	51	6	11.8 ± 0.098	
	3	103	13	12.6 ± 0.064	
	4	99	12	12.1 ± 0.064	
	5	28	4	14.3 ± 0.129	
Total		320	39	12.9 ± 0.035	

[1] C.I.: 95% confidence interval, and [2] From good to poor body condition, the animal takes a score from 1 to 5. * Statistically significant (P<0.05)

of ticks (5.2%, 8/155). In addition, buffaloes previously infected with TBDs (17%, 34/200) were found to be statistically more infected with *T. annulata* than those not infected with TBDs (4.2%, 5/120) (P<0.001, Table 6). Upon Chi-square analysis, specie variable revealed as a significant determinant. While area-wise prevalence showed a non-significant effect on tropical theileriosis (Table 7).

Table 7: Molecular prevalence results of *Theileria annulata* infection in Pakistani cattle and buffalos from Multan and Sargodha districts

Factors	Classes	Number	Positive	Rate (%)	P-value (Khi2)
Area	Multan	400	61	15.25	0.159
	Sargodha	400	76	19.00	(1.982)
Specie	Buffalo	320	39	12.19	0.002*
	Cattle	480	98	20.42	(9.161)

* Statistically significant (P<0.05)

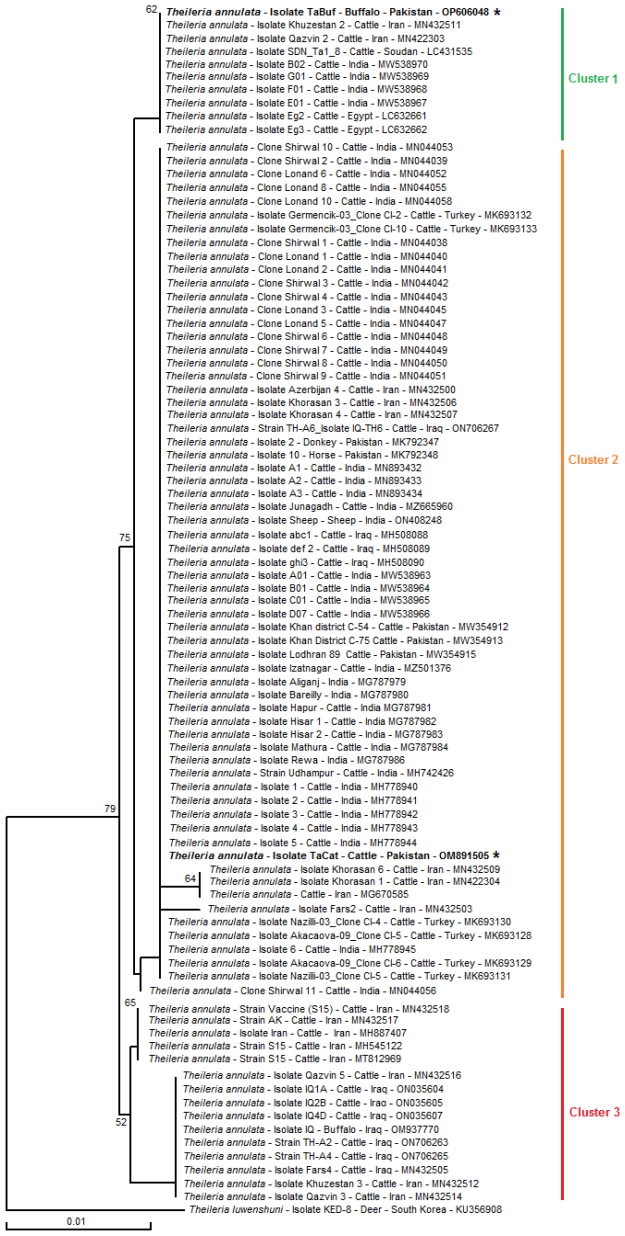

Fig. 3: Phylogenetic tree based on *cytochrome b* partial sequences (272 bp) of *T. annulata* isolated from Pakistani cattle and buffalo in comparison to globally sequences available in GenBank with robustness node rates ≥75%. Numbers in nodes represent the percentage of 1,000 bootstrap iterations supporting the nodes (only percentages greater than 50% are shown). The host, strain or isolate identification, country of origin, and GenBank accession number are indicated in the tree for each sequence. One *T. luwenshuni* was added as an out-group. Our sequences are highlighted in bold with asterisks

Phylogenetic analysis

Sequencing was performed to verify the results of PCR (*cytochrome b*). The representative *T. annulata* partial sequences from two current isolates were deposited to GenBank under accession numbers OM891505 (cattle) and OP606048 (buffalo). Phylogenetic analysis effectuated in the present study revealed three different clusters with robustness node rates ≥75%. The Pakistani strain that infected buffalo (OP606048) was clustered in the first cluster beside strains of Iran (MN432511 and MN422303), India (MW538967, MW538968, MW538969, and MW538970), Egypt (LC632661 and LC632662), and Sudan (LC431535) (Fig. 3). In addition, the phylogenetic study showed that our *T. annulata* sequence isolated from cattle (OM891505) was grouped in the second cluster with those of various isolates infecting cattle, sheep, and donkey located in several neighboring countries, i.e., Iran (MN432500, MN432506, and MN432507), India (MN044052, MN044053, MN044055, and MN044058), Iraq (ON706267), and Turkey (MK693132, and MK693133) (Fig. 3).

Discussion

In this study, we evaluated the prevalence, risk factors, and genetic diversity of *T. annulata* in cattle and buffaloes from agro-ecologically distinct regions of Punjab, Pakistan. The results showed that the PCR and microscopic-based prevalences were 17.13% and 9%, respectively. Asif *et al*. (2022) estimated a comparable prevalence rate of 11.3% in Multan using *Tams-1* gene target for PCR assay. Whereas Rehman *et al*. (2019) recorded a lower rate of 6.67% in arid and semi-arid agro-climatic zones of Punjab, Pakistan. The occurrence of *T. annulata* varies greatly from country to country, such as 61% in Tunisia (Sallemi *et al*., 2018), 31% in Iran (Shahedi *et al*., 2022), 25.26% in China (Hassan *et al*., 2018), 22.78% in India (Patil *et al*., 2019), 5% in Saudi Arabia (*Ghafar et al*., 2019), and 0.59% in Turkey (Kose *et al*., 2022). Despite this variation, bovine theileriosis is widely regarded as one of the most pressing issues among tick-borne diseases.

In addition to polymerase chain reaction, Giemsa staining microscopy was performed to detect *Theileria* piroplasm in cattle and buffaloes. The PCR has been proven highly sensitive for detecting carriers for large scale field studies (Bilgic *et al*., 2010; Bilgic *et al*., 2013). The results showed an overall positivity rate of 9% in bovines (cattle 10.21% and buffaloes 7.19%). Our findings on sensitivity and specificity are supported by Hoghooghi-Rad *et al*. (2011), which suggested that the sensitivity and specificity of this method were reliable in comparison to PCR. However, hemolysis, smear preparation, staining quality, lower parasitemia, carrier infections, and microscopic expertise can affect diagnostic sensitivity (Hoghooghi-Rad *et al*., 2011; Ullah *et al*., 2021).

Nevertheless, we noticed that the incidence of blood-borne pathogens varies across different geographical regions. This is possibly due to the warm and humid climate in the Sargodha district, which is conducive to the growth and proliferation of tick vectors. Our area-wise findings were supported by previous research performed by Atif *et al*. (2012) on this region. However, our results contrast with the findings of Khan *et al*. (2017) who depicted a higher occurrence of *T. annulata* (14.32%) in cattle as revealed through blood smear microscopy in the Dera Ismail Khan district. The higher

density of cattle in the region, combined with poor management practices, may have contributed to the rapid spread of the infection. On the other hand, Soomro *et al*. (2014) found a higher prevalence of natural infections with *A. marginale*, *T. annulata*, and *B. bovis* (62.28, 14.28, and 21.42%, respectively) through the use of Giemsa-stained blood smears in Karachi. This higher incidence of *T. annulata* compared to our findings may be due to a higher number of older animals in the study area, which are more likely to be carriers of the infection.

The present study utilized molecular and microscopic techniques for the detection of *T. annulata*. The results showed that the molecular technique, specifically PCR assay, was more sensitive and specific than microscopic examination. The PCR was a simple, efficient tool for detecting *T. annulata* in the field with higher diversity between isolates (Yousef *et al*., 2020; Shahedi *et al*., 2022). On the other hand, microscopic examination is a cost-effective technique; however, it is less sensitive in detecting the carrier stage of the disease.

Tropical theileriosis is influenced by various factors including breed, age, gender, tick infestation, season, geographical area, and management practices (Ghafar *et al*., 2019; Atif *et al*., 2021; Atif *et al*., 2022). Differences in agro-ecological zones, climatic conditions, housing strategies, and animal populations play a crucial role in the disparities in the occurrence of tick-borne diseases (Sajid *et al*., 2017; Ceylan *et al*., 2021). Analysis of the studied risk factors revealed that season, age, frequency of acaricide use, tick infestation, history of TBDs, and the absence of rural backyard poultry were significant factors associated with *T. annulata* infection, as determined by PCR assay.

The age of animals directly influenced the prevalence of tropical theileriosis, as animals aged <1 year were more susceptible (41.07%) to be infected with *T. annulata* than those belonging to >4 years (25%) and 1-2 years (11.21%) age groups. Our findings suggest that the majority of animals achieved immunity at an early age due to natural infection. Therefore, older animals did not express any acute disease due to acquired immunity as supported by Farooqi *et al*. (2017), Atif *et al*. (2021), and Atif *et al*. (2022). In contrast with our results, Patil *et al*. (2019) found a higher prevalence in elder animals (>2 years) than in young animals (<2 years).

The species of the animals played a significant role in the infection in the present study. Holstein Friesian and Nili Ravi buffaloes were more susceptible at 27.22% and 15.79%, respectively; compared to indigenous cattle and non-descriptive buffaloes. These findings align with previous studies (Farooqi *et al*., 2017; Hassan *et al*., 2018; Atif *et al*., 2021; Atif *et al*., 2022), and the breed-determinant effect was found to be greater in *Bos taurus* cattle compared to buffalo breeds.

The relationship between the presence of ticks and the *T. annulata* infection was also established. The chance of developing *T. annulata* infection was 2.5 times greater in tick infested animals than in non-infested animals (Farooqi *et al*., 2017). The prevalence of theileriosis was greatly influenced by the burden of ticks and mixed farming practices (Haji *et al*., 2022).

The type of acaricide used for tick control was a significant risk factor in the prevalence of tropical theileriosis. Current findings are in covenant with those of Karim *et al*. (2017), mentioning that *Hyalomma* ticks are the main biological vector of *T. annulata*. In addition, we also discovered *H. anatolicum* ticks in both ecological regions. Our study showed that pyrethroids were more effective than avermectin and organophosphates, supported by Atif *et al*. (2021). Furthermore, we found that the use of acaricides within a time frame of less than 30 days was more common than manual tick removal. This observation contradicts the results of Farooqi *et al*. (2017), who reported that animals with no repetition of acaricide use were more prevalent than those with regular acaricide application.

The study compared the occurrence of *T. annulata* in cattle and buffaloes, with and without rural backyard poultry. The results showed that the presence of rural backyard poultry had a lower occurrence of *T. annulata* (13.25% in cattle and 9.64% in buffaloes) compared to the absence of rural poultry (21.91% in cattle and 13.08% in buffaloes). These findings indicate that having rural backyard poultry can lower the risk of tick infestation in cattle and buffaloes. However, a study conducted by Rehman *et al*. (2017) found that the absence of rural backyard poultry was a significant risk factor for higher tick infestation.

Our study results regarding the seasonality of tropical theileriosis are consistent with the reports of Okafor *et al*. (2018), Atif *et al*. (2022), and Hasan *et al*. (2022), as they depicted the highest incidence of tropical theileriosis during the summer season. Additionally, Simuunza *et al*. (2011) reported a higher prevalence of tick-borne diseases (TBDs) during the rainy season. Furthermore, the monsoon season in Pakistan is linked with a higher prevalence of tick-borne diseases due to the increased tick load, relative humidity, and temperature. Our study found that the higher incidence of theileriosis is correlated with the abundance of *Hyalomma anatolicum* ticks.

The results of phylogenetic analysis of current strains from cattle and buffaloes showed a significant level of genetic diversity compared to previous regional and international strains. The *cytochrome b* sequence of *T. annulata* from cattle in this study was found to be more closely related to those reported from India, Pakistan, Iraq, Iran, and Turkey. In addition, mutations in the mitochondrial *cytochrome b* gene that affect the binding site of ubiquinone is associated with buparvaquone resistance in *T. annulata* (Sharifiyazdi *et al*., 2012; Chatanga *et al*., 2019; Hacılarlıoglu *et al*., 2023). On the other hand, the buffalo isolate was determined to be more closely aligned with Iranian, Sudanese, Indian, and Egyptian strains. These findings highlighted the higher diversity of *T. annulata* genetic strains revealed from different agro-climatic regions of Punjab, Pakistan and the need for continued research to understand better the *Theileria* genetic structure and its impact on the health of bovines in these regions. The distribution of diseases in

different agro-climatic regions can likely be attributed to spatial and temporal factors. The identified determinants need to be considered when developing control strategies to alleviate the great economic losses caused by tropical theileriosis in Punjab, Pakistan.

Tropical theileriosis is endemic in bovines of the study area. The PCR offers a more sensitive and accurate method for detecting the parasite. Seasons, absence of rural poultry, and a history of tick-borne diseases were significant predisposing factors for cattle. Whereas tick infestation and age were the major disease determinants associated with tropical theileriosis in buffaloes. Finally, the study underscored the importance of considering agro-climatic and managemental factors in the dissemination of *T. annulata* infections. By considering these factors, we can develop effective and targeted control strategies to reduce the significant economic losses caused by tropical theileriosis in the region.

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Conflicts of interest

The authors declare no competing interests to anyone.

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