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RESEARCH ARTICLE

Molecular Epidemiology and Detection of Mutation in *Cytochrome b* Gene of *Theileria annulata* Associated with Buparvaquone Resistance in Bovines from Punjab, Pakistan

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A B S T R A C T

Drug resistance is an emerging international concern. The detailed knowledge on the epidemiology of bovine tropical theileriosis with respect to buparvaquone resistance is lacking in Punjab, Pakistan. During November 2022 to October 2023, a total of 400 blood samples were gathered from symptomatic cattle (n=200) and buffaloes (n=200) using a multistage cluster sampling technique belonging to the Jhang and Bhakkar districts. The samples were processed through PCR targeting the *cytochrome b* (*cyt b*) gene of *Theileria annulata*. A questionnaire was administered to gather data regarding disease determinants. The positivity of tropical theileriosis in Jhang and Bhakkar was 24.5% and 29%. Chi square-based analysis revealed that age, tick infestation, acaricide use, herd size and season were significantly associated common determinants of tropical theileriosis in cattle and buffaloes. Out of 107 positive samples, 86 (cattle n=54; buffalo n=32) were treated with buparvaquone, and 21 animals were non-treated (cattle n=13; buffalo n=8). Animals selected for treatment were based on parasitemia (≥10%) and PCR positivity. It was observed that out of 86 animals, 72% (n=62) responded (cattle n=39; buffalo n=23) to buparvaquone and 28% (n=24) were declared resistant (cattle n=19; buffalo n=5), confirmed after amplifying 1092 base pairs of the *cyt b* gene. The analysis of *cytb* revealed point mutation at seven points including two non-synonymous point mutations. Phylogenetic insights revealed that current isolates exhibited higher homology with genotypes from India, Turkey, Iran, Egypt, Tunisia and Sudan isolated from cattle. It can be concluded that buparvaquone resistance exists in the study districts. This could be the important limiting factor for the treatment against tropical theileriosis. There is a necessity to conduct large-scale surveillance studies to develop effective control strategies to reduce the antitheilerial resistance.

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INTRODUCTION

Bovine tropical theileriosis, a tick-borne hemo-protozoal disease infected by *Theileria (T.) annulata* (Mohsin *et al.,* 2022). *T. annulata* is the most common disease impacting the cattle in tropical regions (Ullah *et al.,* 2021). Tropical theileriosis is characterized by high fever, anemia, and inflammation of superficial lymph nodes. Other clinical manifestations include watery nasal and eye discharge, dullness, depression, and pale mucous membrane (Hassan *et al.,* 2018). Theileriosis causes substantial economic loss in bovines due to a reduction in milk, meat production, treatment costs, and application of tick control measures (Jabbar *et al.,* 2015; Mohsin *et al.,* 2022). The estimated losses caused by theileriosis range from 5 to 25 percent of total farm losses (Rashid *et al.,* 2018). Several parts of the Middle East and Asian countries have been severely affected by the disease, and approximately one-sixth of the total cattle population is at risk (Kumar *et al.,* 2018).

Pakistan's position in a tropical area makes it an ideal environment for the multiplication and growth of ticks

(Jabbar *et al.*, 2015). In Pakistan, molecular methods have determined the prevalence of theileriosis to range from 17 to 66.1% (Khattak *et al*., 2012; Jabbar *et al*., 2015; Ullah *et al*., 2021; Atif *et al*., 2022; Atif *et al*., 2024). The local breeds of cattle have a lower incidence (20%) of tropical theileriosis than crossbred or exotic animals (80%) (Moumouni *et al.*, 2018). Most of the time, the disease is subclinical, which causes substantial financial losses (Moumouni *et al.*, 2018). The diagnosis of tropical theileriosis is typically rely on clinical symptoms, blood smear microscopy, serology, and confirmation through PCR (Hassan *et al.*, 2018; Krishnamoorthy *et al.*, 2021).

Among antitheilerial drugs, buparvaquone is a drug of choice for the treatment of *T. annulata* in Pakistan (Qayyum *et al.*, 2010). The mechanism of action of Buparvaquone has not been clarified yet, but it has been found that the drug inhibits parasitic mitochondrial respiration by binding to cytochrome b (Müller *et al.*, 2016; Ali *et al.*, 2022).

Buparvaquone resistance is causing a serious threat to efficient production in the cattle industry due to high mortality in spite of treatment (Ali *et al.*, 2022). Numerous studies on antiprotozoal drug resistance have been conducted that have shown that a single mutation or gene deletion is adequate to cause sensitivity loss followed by secondary mutations to increase resistance (Munday *et al.*, 2015). Earlier studies from Iran, Tunisia, Sudan, Egypt, and Turkey revealed that the mutation on cytochrome b is associated with the failure of buparvaquone treatment (Sharifiyazdi *et al.*, 2012; Mhadhbi *et al.*, 2015; Chatanga *et al.*, 2019; Yousef *et al.*, 2020a; Hacılarlıoglu *et al.*, 2023). The detailed knowledge and understanding of the epidemiology of tropical theileriosis with respect to antitheilerial resistance is lacking in Pakistan. Therefore, the current study assessed the molecular prevalence and occurrence of mutation in the *cytochrome b* gene from agro-ecologically diverse districts of Punjab, Pakistan.

MATERIALS AND METHODS

Study area and sample collection: The study was conducted in the Jhang and Bhakkar districts of Punjab from November 2022 to October 2023. Comparatively, these districts have distinct agro-climatic conditions (temperature, rainfall, and humidity). The district of Jhang is located at the bank of the Chenab River at 31°16'40.9656N and 72°18'42.3360E. Whereas, Bhakkar is located in the west of Jhang at 31°37'59.9988' N and 71°3'59.9976'E (Fig. 1). A total of 400 blood samples were collected from symptomatic cattle (n=200) and buffaloes (n=200) from Jhang (n=200) and Bhakkar (n=200) districts using the multistage cluster sampling technique, assuming 50% prevalence. The sampling unit was symptomatic bovines belonging to different genders, ages, and breeds. Blood samples were acquired from healthy, diseased, buparvaquone treated, and non-treated animals. Cattle of different ages [cattle (<1, 1-2, >2-5, >5 years), buffaloes (<2, 2-4, >4-6, >6 years)], sex (male, female), and breeds [(Cattle: exotic, indigenous, crossbred; Buffalo: Nili Ravi and Kundi)] were selected for blood collection. The screening of animals for treatment was made based on single *T. annulata* infection with clinical signs, percent parasitized erythrocytes ≥10% (Coetzee and Apley, 2006; Atif *et al*., 2024), and PCR positivity (Bilgic *et al*., 2010). The selected animals were treated with two doses of Butalex, ICI Pakistan LTD (buparvaquone) @ 2.5mg per kg body weight intramuscularly at 48h interval (Yousef *et al*., 2020b). Blood samples were taken in a 10 ml disposable plastic syringe from the jugular vein of each animal. After collection, the blood was immediately transferred into EDTA-coated vacutainers. These samples were stored in an icebox containing ice pack and brought to the Postgraduate Laboratory of Medicine, College of Veterinary and Animal Sciences (CVAS), Jhang, Pakistan. The Ethical Review Committee of CVAS, Jhang has approved the study vide letter no. CVAS/ERC- 111, dated July 17, 2023.

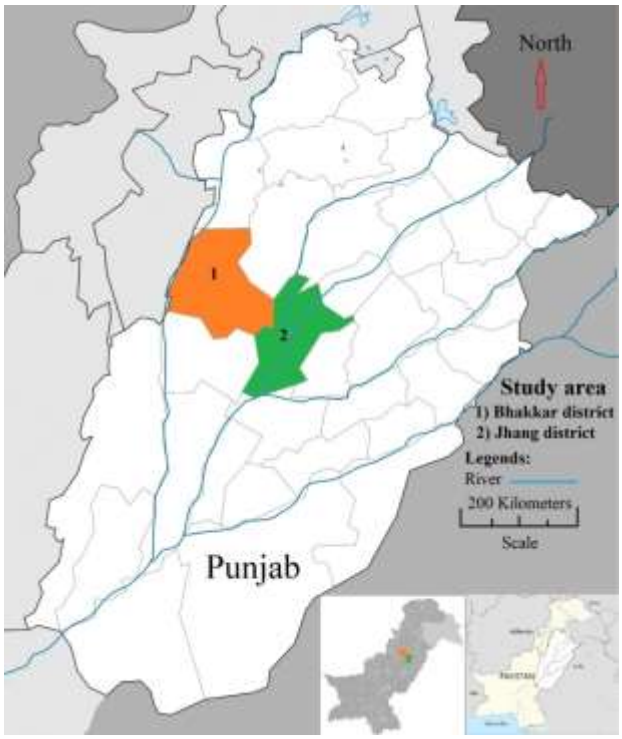

Fig. 1: Map of the study districts.

DNA extraction and PCR amplification: The DNA was removed using Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit, observing the protocol as mentioned by the manufacturer, and preserved at -20°C for further studies. Cyt b gene PCR amplification was conducted with the reported primer pair: forward (5'CAGGGCTTTAACCTACAAATTAAC3') and reverse (5' CCCCTCCACTAAGCGTCTTTCGACAC3') (Bilgic *et al*., 2010). The PCR was performed in a reaction mixture with a volume of 50µL, having 10mM of Tris-HCL at pH8.3, KCl, 50mM of MgCl, 1.5mM of gelatin 0.001%, 1U of AmpliTaq DNA polymerase, 250µM of deoxynucleotide triphosphate, 10pmol of each primer (Cytob1F/Cytob1R), and 2µL of template DNA (Atif *et al*., 2023). The PCR reaction was performed using an automatic thermal cycler (BioRad T-100), including an initial denaturation step at 94°C for 3min., followed by a series of 35 denaturation cycles at 95°C for 50sec, primers annealing was performed at 55°C (50sec), primers extension at 72°C (1min.), and concluding extension took

place at 72°C (10min). The samples were detained at 4°C or stored at -40°C. The PCR products were resolved using 1.5% agarose gel as well as stained with ethidium bromide, and observed under UV light.

Questionnaire survey: A questionnaire was administered to collect information concerning area, host, and management factors. The data regarding age [cattle (<1, 1-2, >2-5, >5 years), buffaloes (<2, 2-4, >4-6, >6 years)], breed [cattle: exotic, indigenous, crossbred; buffalo: Nili Ravi and Kundi], seasons (summer, winter, spring, and autumn), tick infestation (high, moderate, and less), gender (male and female), and management practices like feeding system (stall feeding and free grazing), floor (cemented and non-cemented), acaricide used (yes and no), herd size (1-10, 10-30, and >30), and hygienic measures (unhygienic and hygienic) was collected. The collected data were analyzed to identify the disease determinants associated with the *T. annulata* infection.

Sequencing, molecular basis of resistance, and phylogenetic analysis: The DNA sequencing was done for non-responding animals, and sequences were aligned using BLAST. The sequences were compared with the reference sequence (*T. annulata* Ankara strain accession no. XM949625). A phylogenetic tree was created using resistant amino acid sequences of *T. annulata* from Egypt (MK390360, MK390361, MK390362, MK390363 and MK390364), Iran (JQ308837, JQ308838, and JQ308839), Turkey (MK693134, and MK693135), Tunisia (KF732024, KF732025, KF732026, KF732027, KF732028, KF732030, and LC431530), Sudan (LC431531, LC431532 and LC431534), and India (PP619427). Whereas, the *T. lestoquardi* sequence was used as out-group. Further comparison was made with other available resistant strains from different countries. The phylogeny was inferred using the maximum likelihood method and the Jones-Taylor-Thornton model of amino acid substitutions (Jones *et al*., 1992) with the highest log likelihood (-876.89). The associated taxa were clustered together at 1,000 replicates (Felsenstein, 1985). The initial tree was designated by selecting the tree with the superior log-likelihood between a Neighbor-Joining (NJ) tree and a Maximum Parsimony (MP) tree using a matrix of pairwise distances computed using the p-distance (Saitou and Nei, 1987; Nei and Kumar, 2000). The analytical procedure encompassed 25 amino acid sequences. Evolutionary analyses were built in MEGA12 using the procedure described by Kumar *et al*. (2024).

Statistical analysis: A chi-square test was conducted to determine the association between the occurrence of tropical theileriosis and various disease determinants. A P-value of 0.05 or less was considered significant. The statistical data were analyzed using IBM SPSS Statistics 26.

RESULTS

Prevalence and determinants of tropical theileriosis: Among 400 samples, 107 were found positive for *T. annulata* by using PCR, amplified 1092 base pairs of cytochrome b gene of *T. annulata*. The positive animals showed clinical signs of fever, enlarged lymph nodes, lacrimation and salivation. The peripheral blood smear revealed single Theileria infection. Out of 107 positive samples 86 were treated (parasitemia $\geq$10) and 21 animals were not treated with buparvaquone. The animals with mix infection were ruled out from treatment/resistance trial. As far as the cattle disease determinants are concerned. The results indicated that cattle of 1-2 years of age were infected higher (41.03%) compared to <1 years of age (13.89%) (P=0.00724, X^2=12.04, df=3). The breed-wise prevalence showed that Holstein Friesian (33.33%) and Crossbred animals (38.71%), were more infected (39.25%) than local breed (14.71%) (P=0.0317, X^2=6.9021, df=2). The prevalence was highest in female (37.41%) than male (24.57%). The animals with higher tick infestation load were significantly infected (56.34%) at higher frequency compared to lower tick infested animals (3.23%) (P<0.00001, X^2=32.957, df=2). In the current study, different managemental practices were observed that had a significant effect on *Theileria* infection. The animals that were applied with regular acaricide have lesser infection rate (15.79%) than those animals with irregular acaricide use (44.35%) (P=0.00003, X^2=17.25, df=1). The prevalence was highest in animals with herd size of 30 animals (54.39%), followed by 45.28% in herd size of >10-30 animals, and 13.33% in animals with herd size of 1-10 animals (P=0.00001, X^2=30.8947, df=2). The free grazing cattle were infected to a lesser extent (25.35%) than stall fed animals (37.98%) (P=0.701, X^2=3.2804, df=1). The animals that were kept on non-cemented floor were more infected (49.28%) than those kept on cemented floor (25.19%) (P=0.0006, X^2=11.767, df=1). The animals that were kept in good hygienic conditions were lesser infected with tropical theileriosis (22.58%) than those who were kept in unhygienic environment (38.41%) (P=0.0283, X^2=4.809, df=1). Season had a significant effect on the occurrence of disease. The infection was highest in Spring (56.86%), followed by Summer (55.74%), Autumn (6.38%) and lowest in Winter (2.44%) (P<0.00001, X^2=53.306, df=3) (Table 1). The chi-square based analysis for buffaloes revealed that variables of age, tick infestation, acaricide used, herd size, and season revealed as statistically significant disease determinants (Table 2).

It was observed that out of 86 treated animals, 62 (72.1%) bovines (cattle n=39; buffalo n=23) responded to two doses of Butalax (buparvaquone) @ 2.5mg per kg body weight intramuscular at an interval of 48 hours with significant improvement in clinical signs and reduction of parasitemia. While 24 animals (27.9%) did not respond to the treatment. To determine the evidence of buparvaquone resistance the positive samples were further confirmed by PCR, amplifying 1092 base pair product of cytochrome b gene of *T. annulata*. The parasitemia levels in one of the non-responding cows increased by 19% and an animal was died within 7 days despite treatment. Over all, there was a non-significant decrease in parasitemia in non-responders. Whereas, the responding cattle were cured and significant lowering of parasitemia level within 48 hours post treatment with two doses of buparvaquone was noticed (Table 3).

Table 1: Chi square based analysis of determinants linked with molecular occurrence of tropical theileriosis among cattle from Jhang and Bhakkar districts of Punjab, Pakistan

Variables	Categories	Total	Positive	Prevalence (%)	Chi-square (X^2)	*p*-value	df
Area	Jhang	100	31	31.00%	0.561	0.454	1
	Bhakkar	100	36	36.00%			
Gender	Male	61	15	24.59%			1
	Female	139	52	37.41%	3.128	0.770	
Age	<1	36	19	52.78%	28.44	<0.00001*	3
	1 to 2	39	14	35.90%			
	> 2 to 4	56	27	48.21%			
	>4	69	7	10.14%			
Breed	Holstein Friesian	42	14	33.33%			2
	Crossbred	124	48	38.71%			
	Local	34	5	14.71%	6.902	0.032*	
Tick infestation	High	71	40	56.34%			2
	Moderate	98	26	26.53%			
	Low	31	1	3.23%	31.513	<0.00001*	
Acaricide used	Yes	76	12	15.79%			1
	No	124	55	44.35%	17.259	0.00003*	
Herd Size	1 to 10	90	12	13.33%			2
	>10 to 30	53	24	45.28%	30.894	<0.00001*	
	>30	57	31	54.39%			
Feeding system	Stall feeding	129	49	37.98%			1
	Free grazing	71	18	25.35%	3.280	0.7011	
Floor	Cemented	131	33	25.19%			1
	Non-cemented	69	34	49.28%	11.767	0.0006*	
Hygienic measures	Unhygienic	138	53	38.41%			1
	Hygienic	62	14	22.58%	4.809	0.0283*	
Season	Summer	61	34	55.74%			3
	Winter	41	1	2.44%			
	Spring	51	29	56.86%	59.306	<0.00001*	
	Autumn	47	3	6.38%			

*Statistically significant.

Table 2: Chi square based analysis of determinants linked with molecular occurrence of tropical theileriosis among buffaloes from Jhang and Bhakkar districts of Punjab, Pakistan

Variables	Categories	Total	Positive	Prevalence (%)	Chi-square (X^2)	*p*-value	df
Area	Jhang	100	18	18.00%	0.50	0.476	1
	Bhakkar	100	22	22.00%			
Gender	Male	39	7	17.95%			1
	Female	161	33	20.50%	0.13	0.721	
Age	< 2 year	34	11	32.35%	8.26	0.0410*	3
	2 to 4 year	41	4	9.76%			
	> 6 year	58	15	25.86%			
	>4	67	10	14.93%			
Breed	Nili Ravi	132	27	20.45%			2
	Kundi	68	13	19.12%	0.05	0.823	
Tick infestation	High	41	14	34.15%			2
	Moderate	127	25	19.69%			
	Low	32	1	3.13%	7.48	<0.24*	
Acaricide used	Yes	65	4	6.15%			1
	No	135	36	26.67%	11.54	0.00068*	
Herd Size	1 to 10	90	5	5.56%			2
	>10 to 30	57	20	35.09%	22.13	<0.00016*	
	>30	53	15	28.30%			
Feeding system	Stall feeding	132	25	18.94%			1
	Free grazing	68	15	22.06%	0.27	0.6014	
Floor	Cemented	111	18	16.22%			1
	Non-cemented	89	22	24.72%	2.23	0.1351	
Hygienic measures	Unhygienic	131	29	22.14%			1
	Hygienic	69	11	15.94.58%	1.08	0.2978	
Season	Summer	61	20	32.79%			3
	Winter	41	1	2.44%			
	Spring	51	14	27.45%	18.48	0.00035*	
	Autumn	47	5	10.64%			

*Statistically significant.

Point mutation in the *cytochrome b gene*: The analysis of 1092bp of *cytochrome b* gene revealed point mutation at seven points (78, 116, 139, 143, 146, 203 and 290). Out of which two point mutations were non-synonymous. The analysis of Q drug binding sites revealed two mutations; one at codon 146 that led to the formation of alanine to threonine and second mutation was observed at codon 203 leading to formation of isoleucine to valine. The mutation within the Q01 (130-148) binding site was observed in all the resistant isolates (Table 4).

Sequences and phylogenetic analysis: The non-responding animals that did not show any evidence of significant improvement even after two doses of buparvaquone were enrolled for sequencing to assess the confirmation of mutation in *cytochrome b* gene. The *T. annulata* sequences were aligned and compared with the reference sequence (XM949625). The representative sequences acquired in this study were deposited to GenBank and we successfully got with accession numbers

OR130523, OR136502, and OR168940 with percent identity score of 99.82, 99.36 and 99.36 with reference strain, respectively. The Isolate (OR130523) of the current study collected from buparvaquone resistant cattle and isolates from Iran, Egypt, Turkey were included in Cluster-I. Furthermore, isolates from Tunisia and Sudan were included in Cluster-II. Nonetheless, present study's isolates OR136502 (cattle), OR168940 (buffalo), and Indian cattle isolates were grouped in Cluster-III. Our isolates showed 98.90-100% similarity with isolates from different countries. The phylogenetic insights revealed that our isolates showed higher similarity with isolates from India (PP619427), Iran (JQ308837, JQ308838 and JQ308839), Turkey (MK390360, MK390361, MK390362, MK390363, and MK390364), and Egypt (MK390360, MK390361, MK390362, MK390363, and MK390364). Whereas, lesser homology with Tunisian (KF732024, KF732025, KF732026, KF732027, KF732028, KF732030, and LC431530) and Sudanese resistant bovine isolates (LC431531, LC431532, and LC431534). Whereas *T. lestoquardi* (NC053925) acted as out-group (Fig. 2).

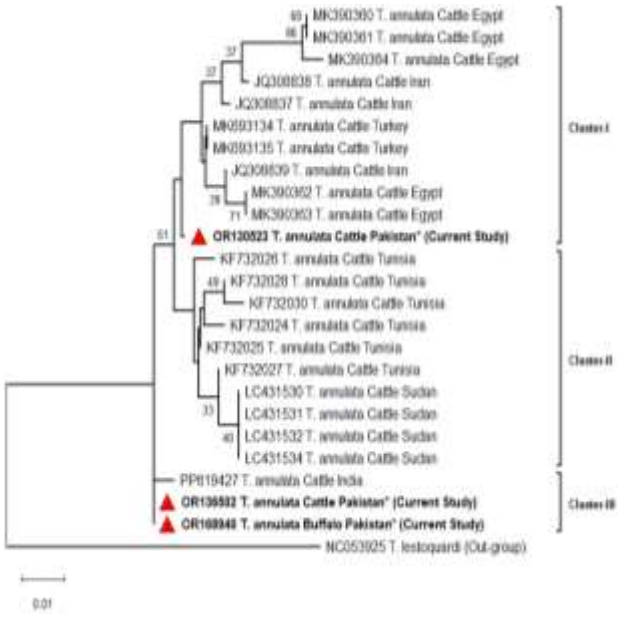

Fig. 2: The phylogenetic tree based on amino acid sequences of *Cyt b* gene of resistant *T. annulata* strains. Pakistani isolates of current study are represented with red triangle in bold font with asterisks.

Table 3: Parasitemia percentage of *T. annulata infection* before and after treatment

Recovered animals			
Sr. No.	Animal number (total)	Parasitemia (%)	
		Pre-treatment ± SE	Post-treatment ± SE
1.	1 -- 10 (10)	11.1±0.64	0.2±0.13
2.	11 – 20 (10)	12.2±0.51	0.4±0.12
3.	21 – 30 (10)	11.6±0.40	0.2±0.13
4.	31 – 40 (10)	12.1±0.34	0.3±0.13
5.	41 – 50 (10)	12.8±0.24	0.18±0.14
6.	51 – 62 (12)	10.5±0.33	0.05±0.09
Non-recovered animals			
Sr. No.	Animal number (total)	Parasitemia (%)	
		Pre-treatment ± SE	Post-treatment ± SE
1	1-12 (12)	10.3±0.33	8.9±1.27
2	12-24 (12)	9.7±0.41	8.2±0.71

S.E= Standard error.

Table 4: Mutations in the *Cyt b* gene isolated in the current study compared to reference *T. annulata* sequence

GenBank accession numbers	Nucleotide	234	348	417	429	436	607	870
	Codon	78	116	139	143	146*	203*	290
XM949625 (Reference)		TCG	ACT	TTA	TTC	GCT	ATA	GTA
OR130523**		---	---	---	--T	A--	---	---
OR136502**		--A	--C	--G	--T	A--	G--	--G
OR168940**		--A	--C	--G	--T	-T-	-C-	---

* Non-synonymous mutation; **Isolates of present study.

DISCUSSION

The detailed knowledge and understanding of the epidemiology of tropical theileriosis with respect to antitheilerial resistance is lacking in Pakistan. In the current study, the predisposing factors of age, tick infestation load, acaricide use, herd size, and season were identified as common determinants of cattle and buffaloes. The data analysis revealed that cattle calves of less than 1 year of age were more susceptible to theileriosis. The determinant of age showed a significant relationship. The highest infection was noticed in calves of less than 1 year (52.78%), followed by the animals of 2-4 years, >4 years and lowest in 1-2 years age group. Our results were in agreement with the earlier studies of Simuunza *et al.* (2011) and Mohammad-Ahmed *et al.* (2018). Whereas Ullah and colleagues have mentioned that the owner of the herd may have taken better care of adult cattle, especially dairy cows, while neglecting young animals, this has accounted for higher incidence in young cattle (Ullah *et al.*, 2021). The possible reason for the higher occurrence in young animals is attributed to the fact that young cattle had not developed immune system to fight infection. On the other hand, there is a reduced infection rate of tropical theileriosis in older animals owing to the fact that their lifetime development of concurrent immunity and several repeated infections. On the contrary, Kundave and associates mentioned that the higher prevalence rate in cattle of >5 years is due to the increased likelihood of tick exposure, and the lowest prevalence in calves of <1 year is due to the active immunity acquired by colostrum in young calves (Kundave *et al.*, 2015). The cattle and buffaloes have different age groups for different species based on different life expectancies, gestation and puberty ages. The disease occurrence was higher in crossbred and Holstein Friesian and lower in local cattle breeds, which showed a significant relationship ($P<0.00001$). The lower incidence of disease in exotic breeds could be justified by variations in management practices and hygienic measures used by the owner of Holstein Friesian (Kumar *et al.*, 2018; Singh *et al.*, 2022). Research findings by Parveen *et al.* (2021) from Dera Ismail Khan and Lodhran districts depicted a higher disease positivity rate in Sahiwal cattle. This difference might have occurred due to difference of cattle enrolled in the study.

It was revealed that the occurrence of disease was considerably higher in females compared to males. The high prevalence in females was also reported by Ullah *et al.* (2021). The dams may be more susceptible to *T. annulata* due to hormonal fluctuations, impaired immune function during pregnancy or lactation (Tuli *et al.*, 2015; Sray *et al.*, 2021). These factors may contribute to the increased infection of *T. annulata* in dams. Conversely, Khawale and colleagues reported higher prevalence in

males (40%) than in females (20.96%) (Khawale *et al.*, 2019). The management practices like tick load, acaricide use, herd size, feeding system, floor and hygienic measures also had a significant impact on the infection rate. In the present study, the scrutiny of data revealed that the prevalence of *T. annulata* was higher in heavily tick-infested cattle than moderate and lightly infested cattle. The dynamics of *T. annulata* transmission were significantly linked to tick infestation. Our findings are consistent with previously published research from Pakistan and elsewhere in the world on tick infestation and its ability to affect the prevalence pattern. According to published research, *T. annulata* transmission occurs more frequently when an appropriate vector is present and an infected host is infested by *Hyalomma anatolicum* in cattle (Zeb *et al.*, 2020).

The acaricide use on cattle was substantially associated with tropical theileriosis. When compared to those animals that were frequently treated with acaricides, the infection was higher in cattle without treatment. Our results were supported by earlier researchers that have published reports from other regions of the country and abroad. These indicated that a lower infection rates were recorded in herds where cattle were routinely use acaricidal treatment. The evaluation of the review of literature from other agro-climatic regions of the country revealed that regular use of acaricides on cattle could control tick vectors and ultimately *T. annulata* frequency in widespread region (Zeb *et al.*, 2020; Ullah *et al*., 2021).

During the current study, it was noticed that the cattle kept at a herd size of 1 to 5 cattle had a greater disease outcome (36.17%), followed by 29.13% in a herd size of 6 to 10 animals and 14.39% in a herd size of >20 bovines. It might be due to better management practices at the farm level and feeding practices in animals of greater herd size. These findings are coherent with Mohsin *et al.* (2022), but contrary to Ashraf *et al.* (2024), they depicted that animals of large herd size were more prone to theileriosis.

It was noticed that free-grazing animals have higher infectivity (33.73%) than stall- feeding (15.23%). Tropical theileriosis is highly prevalent in free-grazing animals compared to cattle kept on farms with access to fodder. These results are in line with data from other researchers who analyze the impact of grazing on tropical theileriosis. Research suggested that certain farmers let their cattle graze freely, increasing the frequency of interaction with animals from other herds and allowing ticks to spread more easily, leading to an increase in *T. annulata* infection. However, cattle housed in stalls and kept in farms or fences rather than being free to wander causing decreased exposure to tick-infested animals, questing ticks, and grasses in grazing animals (Ullah *et al.*, 2021; Ullah *et al*., 2022).

Animals kept on cemented floors have less disease incidence than those animals that were kept on non-cemented floor. Cattle kept in unhygienic conditions were at higher risk of disease (32.75%) due to the chance of more spread of tick vectors than the animals kept in hygienic conditions. Season had a significant impact on the disease outcome. In the current study the prevalence was recorded highest during summer (38.14%), followed by 26.23% during spring season, 16.67% in autumn and the lowest, 8.79% in winter. Our study is in line with the findings of Akhtar *et al.* (2019) and Atif *et al.* (2022), while disagree with the results of Hassan *et al.* (2018), who depicted the highest occurrence during autumn, followed by winter, summer, and spring. Whereas, statistical analysis revealed that age, tick infestation, acaricide use, herd size, and season were significantly associated determinants of tropical theileriosis in buffaloes. The different age groups were selected for cattle (<1 year, 1-2 years, >2-4 years, >4 years) and buffaloes (<2 years, 2-4 years, >4-6 years, >6 years) based on different puberty, gestation and life expectancy. The buffaloes are relatively less susceptible and lesser determinants for tropical theileriosis compared to cattle (Atif *et al*., 2023).

Buparvaquone is the preferred treatment for tropical theileriosis, demonstrating 92% efficacy when administered intramuscularly at a dose of 2.5mg/kg. It has shown equal effectiveness against both the schizont and piroplasm stages (Gharbi and Darghouth, 2015). Buparvaquone, along with other hydroxyl naphthoquinones, is believed to function by competitive inhibition of the binding of coenzyme Q (ubiquinone) to the mitochondrial cytochrome bc1 complex. This inhibition interrupts the electron transport chain, leading to the failure of the membrane potential at the two drug binding sites [Q01 (130-148) and Q02 (244-266)], which ultimately hinders the parasite's respiration and pyrimidine biosynthesis (Mhadhbi *et al*., 2010).

The enrolled bovines for the investigation of resistance in the current study expressed typical signs of bovine theileriosis. Despite treatment at the early stage of the disease, the animals did not show any sign of improvement after 48 hours of two doses of buparvaquone. On clinical examination, it was observed that the temperature remained high and the parasitemia level was not reduced, instead, it was increased and reached 19% in affected cattle. This finding is endorsed by earlier research by Mhadhbi *et al.* (2010) and Yousef *et al.* (2020b).

The most important factor restricting treatment options for protozoan diseases is the emergence of drug resistance in certain hemoprotozoan parasites, including *T. annulata* (de Koning 2017). The genetic basis of mutation that can lead to the existence of resistance in protozoan parasites includes point mutations, gene deletions, copy number variations, insertions or deletions of base pair in the target gene and the development of chimeric genes via recombination (Munday *et al.*, 2015; Chatanga *et al.*, 2019).

The sequences were matched and aligned with the standard sequence of *T. annulata* (XM949625) as well as isolates from Tunisia, Sudan, Iran, India, Turkey, and Egypt isolated from cattle**.** The analysis of 1092 bp of the *cytb* gene revealed point mutations at seven points: 78, 116, 139, 143, 146, 203, and 290**.** Out of which two point mutations were non-synonymous. The scrutiny of Q drug binding sites revealed two mutations at codon 146 (alanine to threonine) and at codon 203 (isoleucine to valine). The mutation within the Q01 (130-148) binding site was observed in all the resistant isolates.

In previous studies, mutations in the cytochrome b gene have been depicted from Pakistan, Germany, Sudan, China, Turkey, Iran, Egypt, and Tunisia (Rashid *et al*., 2024). The analysis of the *cytochrome b* gene reveals a point mutation at the 146th codon site, which is coherent with the previous studies indicating a mutation at codon 146 observed in both sensitive and resistant clones in

Tunisia and resistant clones from Sudan (Mhadhbi *et al.*, 2010; Chatanga *et al.*, 2019).

Conclusions: It can be concluded that drug resistance associated with buparvaquone against the *T. annulata cytb* gene exists in the study districts, which could be the important limiting factor for the treatment against tropical theileriosis. There is a necessity to demeanor large-scale surveillance studies to evaluate the extent of the issue in order to develop effective prevention and control measures aimed at reducing buparvaquone-associated antitheilerial resistance in the region.

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Authors contribution: FAA contributed to conceptualization, funding acquisition, involved in project administration as well as provision of resources, utilized software, supervised, contributed in writing review and editing of the draft. SA conducted formal analysis, created resources, utilized software and contributed in writing of draft. MI played its role in validation, review, and editing. MA contributed to validation, supervision, and editing.

REFERENCES

Ashraf S, Hikal WM, Almahallawi R, *et al*., 2024. Molecular prevalence, associated risk factors and phylogenetic evaluation of Theileria lestoquardi in the blood samples of small ruminants. Plos One 19:e0306697.

Ali Q, Zahid O, Mhadhbi M, *et al*., 2022. Genetic characterisation of the Theileria annulata cytochrome b locus and its impact on buparvaquone resistance in bovine. Int J Parasitol Drugs Drug Resist 20:65-75.

Atif FA, Abbas RZ, Mehnaz S, *et al*., 2022. First report on molecular surveillance based on duplex detection of Anaplasma marginale and Theileria annulata in dairy cattle from Punjab, Pakistan. Trop Anim Health Prod 54:155.

Atif FA, Nazir MU, Abbas RZ, *et al*., 2023. Molecular epidemiology, associated risk factors, and phylogeny of *Theileria annulata* infecting buffaloes and cattle from different agro-climatic regions of Punjab, Pakistan. Iranian J Vet Res 24:247.

Atif FA, Nazir MU, Roheen T, *et al*., 2024. Antitheilerial efficacy of juglone, buparvaquone and oxytetracycline against tropical theileriosis in naturally infected crossbred cattle. Pak Vet J 44:129-34.Bilgic HB, Karagenç T, Shiels B, *et al*., 2010. Evaluation of Cytochrome b as a sensitive target for PCR based detection of T. annulata carrier animals. Vet Parasitol 174:341–347.

Chatanga E, Mosssad E, Abubaker HA, *et al*., 2019. Evidence of multiple point mutations in *Theileria annulata* cytochrome b gene incriminated in buparvaquone treatment failure. Acta Trop 191:128-132.

Coetzee JF, Apley MD, 2006. Efficacy of enrofloxacin against severe experimental *Anaplasma marginale* infections in splenectomized calves. Vet Ther 7:319.

de Koning HP, 2017. Drug resistance in protozoan parasites. Emerg Top Life Sci 1:627-632.

Gharbi M and Darghouth MA, 2015. Control of tropical theileriosis (Theileria annulata infection in cattle) in North Africa. Asian Pac J Trop Dis 5:505-510.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evol 39:783-91.

Hacılarlıoglu S, Bilgic HB, Bakırcı S, *et al*., 2023. Selection of genotypes harbouring mutations in the cytochrome b gene of *Theileria annulata* is associated with resistance to buparvaquone. PLoS One 18:e0279925.

Hassan MA, Liu J, Rashid M, *et al*., 2018. Molecular survey of piroplasm species from selected areas of China and Pakistan. Parasites Vectors 11:1-7.

Jabbar A, Abbas T, Sandhu Z-u-D, *et al*., 2015. Tick-borne diseases of bovines in Pakistan: major scope for future research and improved control. Parasites Vectors 8:1-13.

Jones DT, Taylor WR, Thornton JM, 1992. The rapid generation of mutation data matrices from protein sequences. Bioinform 8:275-82.

Khattak RM, Rabib M, Khan Z, *et al*., 2012. A comparison of two different techniques for the detection of blood parasite, Theileria annulata, in cattle from two districts in Khyber Pukhtoon Khwa Province (Pakistan). Parasite 19:91.

Khawale TS, Siddiqui MF, Borikar ST, *et al*., 2019. Study of occurrence of theileriosis in cattle from Parbhani district, Maharashtra, India. Pharma Innov 8:171-3.

Krishnamoorthy P, Akshatha L, Jacob S, *et al*., 2021. Theileriosis prevalence status in cattle and buffaloes in India established by systematic review and meta-analysis. Indian J Anim Sci. 91:269-79.

Kumar S, Mohmad A, Parthasarathi BC, *et al*., 2018. Epidemiological Status of Bovine Theileriosis in Uttar Pradesh a state of India. Med J Clin. Trials Case Stud 2:1-7.

Kumar S, Stecher G, Suleski M, *et al*., 2024. MEGA12: Molecular Evolutionary Genetic Analysis version 12 for adaptive and green computing. Mol Biol Evol 41:msae263.

Kundave VR, Patel, AK, Patel, PV, *et al*., 2015. Detection of theileriosis in cattle and buffaloes by polymerase chain reaction. J Parasitic Dis 39:508-513.

Mhadhbi M, Chaouch M, Ajroud K, *et al*., 2015. Sequence polymorphism of cytochrome b gene in *Theileria annulata* Tunisian isolates and its association with buparvaquone treatment failure. PLoS One 10:e0129678.

Mhadhbi M, Naouach A, Boumiza A, *et al*., 2010. In vivo evidence for the resistance of *Theileria annulata* to buparvaquone. Vet Parasitol 169:241-247.

Mohsin M, Hameed K, Kamal M, *et al*., 2022. Prevalence and risk factors assessment of theileriosis in livestock of Malakand Division, Pakistan. J Saudi Soc Agric Sci 21:242-247.

Moumouni PFA, Aplogan GL, Katahira H, *et al*., 2018. Prevalence, risk factors, and genetic diversity of veterinary important tick-borne pathogens in cattle from *Rhipicephalus microplus*-invaded and non-invaded areas of Benin. Ticks Tick Borne Dis 9:450-464.

Müller J, Aguado-Martínez A, Manser V, *et al*., 2016. Repurposing of antiparasitic drugs: the hydroxy-naphthoquinone buparvaquone inhibits vertical transmission in the pregnant neosporosis mouse model. Vet Res 47(1):1-8.

Munday JC, Tagoe DN, Eze AA, *et al*., 2015. Functional analysis of drug resistance-associated mutations in the *Trypanosoma brucei* adenosine transporter 1 (TbAT 1) and the proposal of a structural model for the protein. Mol Microbiol 96:887-900.

Saitou N and Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4:406-25.

Parveen A, Alkhaibari AM, Asif M, *et al*., 2021. Molecular epidemiology of Theileria annulata in cattle from two districts in Punjab (Pakistan). Animals 11:3443.

Rashid M, Liu J, Iqbal N, Hassan MA, *et al*., 2018. Molecular survey of piroplasm species from selected areas of China and Pakistan. Parasit Vectors 11:1-7.

Qayyum M, Farooq U, Samad H, *et al*., 2010. Prevalence, clinicotherapeutic and prophylactic studies on theileriosis in district Sahiwal (Pakistan). J Anim Plant Sci 20:266-270.

Rashid M, Hayat MH, Zahra N, *et al*., 2024. Systematic review on buparvaquone resistance associated with non-synonymous mutation in drug binding genes site of *Theileria annulate*. Vet Parasitol 9:110321.

Sray AHK, 2021. First Serological Detection of Theileria annulata in buffaloes in Iraq. Ann. Rom. Soc. Cell Biol 25:1262–1273.

Saitou N, Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-25.

Sharifiyazdi H, Namazi F, Oryan A, *et al*., 2012. Point mutations in the *Theileria annulata* cytochrome b gene is associated with buparvaquone treatment failure. Vet Parasitol 187:431-435.

Simuunza M, Weir W, Courcier E, *et al*., 2011. Epidemiological analysis of tick-borne diseases in Zambia. Vet Parasitol 175(3-4):331-42.

Singh K, Kumar S, Sharma AK, *et al*., 2022. Economic impact of predominant ticks and tick-borne diseases on Indian dairy production systems. Exp Parasitol 243:108408.

Ullah R, Shams S, Khan MA, *et al*., 2021. Epidemiology and molecular characterization of *Theileria annulata* in cattle from central Khyber Pakhtunkhwa, Pakistan. PLoS One 16:e0249417.

Tuli A, Da Singla L, Sharma A, *et al*., 2015. Molecular epidemiology, risk factors and hematochemical alterations induced by *Theileria annulata* in bovines of Punjab (India) Acta Parasitol 60:378–390.

Ullah K, Numan M, Alouffi A, *et al*., 2022. Molecular characterization and assessment of risk factors associated with *Theileria annulata* infection. Microorganisms 10:1614.

Yousef SG, El Balkemy FA, El Damaty HM, 2020a. Mutations in *Theileria annulata* cytochrome b gene associated with buparvaquone resistance in cattle, Egypt. Pak Vet J 40: 261-263.

Yousef SG, El-Balkemy FA, El-Shazly YA, *et al*., 2020b. Clinical picture and haemogram profile associated with Theileria annulata infection in cattle before and after therapeutic intervention. Adv Anim Vet Sci 8:290-296.

Zeb J, Shams S, Din IU, *et al*, 2020. Molecular epidemiology and associated risk factors of Anaplasma marginale and *Theileria annulata* in cattle from North-western Pakistan. Vet Parasitol 279:109044.