



# Molecular epidemiology of *Theileria annulata* infection of cattle in Layyah District, Pakistan

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## Abstract

*Theileria annulata* is the cause of tropical theileriosis in cattle in Pakistan, where it has a significant impact on the cattle industry. Here we report the molecular detection and seasonal prevalence and blood parameters of *T. annulata* infection in crossbred, Holstein Frisian and Sahiwal breed in Layyah District in the Punjab. In total, 844 blood samples (cross = 244, Holstein Frisian = 300, Sahiwal breed = 300) collected in 2017 and 2018 were tested. Blood smear screening revealed 125/844 (15%) of cattle positive for *Theileria* species. PCR amplification of cytochrome b gene indicated an overall *T. annulata* prevalence of 21% (174/844). The highest prevalence was observed in autumn season (53%), followed by winter (20%), summer (14%) and spring (3%). Crossbred cattle were the most susceptible to *T. annulata* (28%) followed by Sahiwal (19%) and Holstein Frisian. Representative partial cytochrome b gene sequences of *T. annulata* revealed phylogenetic similarities with sequences submitted from India, Iran, China, Turkey and Spain. Small numbers of ticks, including *Hyalomma anatolicum*, *Hyalomma excavatum*, *Rhipicephalus microplus*, and *Haemaphysalis punctata*, were identified from cattle but none of them was found PCR positive for the presence of *T. annulata*. Analysis of the hematology data indicated that red blood cell, hemoglobin, mean cell hemoglobin, mean corpuscular hemoglobin, lymphocyte (%), monocyte (%) and platelet count were significantly altered in *T. annulata*-positive cattle of all three breeds. Screening of cattle by PCR for the detection of *T. annulata* is recommended for diagnosis and treatment.

**Keywords** Cytochrome b gene · PCR · *Theileria annulata* · Phylogenetic analysis · Cattle

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## Introduction

The most important role of livestock is the production of high-quality animal protein for human consumption through the supply of milk and meat (Asif et al. 2020). Pakistan has 15 indigenous breeds of cattle belonging to zebu (one-humped) breed (*Bos indicus*), comprising 43% of the cattle population (Saeed et al. 2016). In addition to these local breeds Pakistan has exotic breeds (Holstein Frisian and Jersey) as well as crossbreeds (Zulfiqar et al. 2012). Local breeds like Sahiwal and Cholistan are famous for heat and tick resistance whereas exotic dairy cattle like Holstein Frisian has genetic potential of high milk production (Ashraf et al. 2021).

The productivity of cattle is known to be greatly diminished by tick-borne parasitic diseases, particularly theileriosis, causing considerable economic losses on both local and global scales with several complications (Ahmed et al. 2002; Elsify et al. 2015). Ticks not only transmit different pathogens causing number of threatening diseases in cattle but are blood suckers that damage skin and hides causing physical damage to livestock (Aslam et al. 2015). The arid region of Punjab in Pakistan is notorious for the presence of the vector tick (Acari: Ixodidae) and tick-borne diseases of domesticated animals such as theileriosis and babesiosis (Hassan et al. 2018). This hot and humid climate is highly conducive and favors the multiplication and survivability of ticks (Khan et al. 2004; Kohli et al. 2014).

Bovine theileriosis is caused by the protozoan parasite of *Theileria* species which are round, ovoid rod-like or irregular shaped organism found in lymphocytes, histiocytes and erythrocytes (Bhatnagar et al. 2015). Globally, *Theileria annulata* and *Theileria parva* are the most economically important tick-transmitted pathogenic species causing bovine theileriosis (Gebrekidan et al. 2016). *Theileria annulata* is transmitted by *Hyalomma* spp. ticks (Durrani et al. 2010) and its infection is characterized by a marked rise in body temperature, reaching 40–41.5 °C, depression, lacrimation, nasal discharge, swelling of the superficial lymph nodes and anemia (Saeed et al. 2016). Strategies to control tropical theileriosis are controlling tick infestation by acaricides, immunization using live vaccines and treatment of infected cattle (Akat et al. 2014). Present study was designed for the season-based detection of *T. annulata* in three breeds of cattle (cross, Holstein Frisian and Sahiwal) from District Layyah through conventional (smear screening) and modern tool (polymerase chain reaction, PCR) and to report the association of parasite prevalence with epidemiological factors and complete blood count parameters, if any.

## Materials and methods

### Study area and design

An active epidemiological survey was conducted to determine the prevalence of *T. annulata* in apparently healthy cattle (N=844; 300 each of Sahiwal and crossbreed and 244 of Holstein Frisian breed) from randomly selected villages in district Layyah of Punjab (Pakistan) on seasonal basis [summer (May–July), autumn (August–October), winter (November–January) and spring (February–April)] After the informed consent of owners, all the animals were examined by veterinarians on the sampling sites and a blood sample (10 ml) was collected from a jugular vein of each animal and immediately preserved in a sterile tube containing 1 ml of 0.5 M EDTA solution as an anticoagulant to be used for DNA

extraction and complete blood count analysis. A questionnaire was filled for each animal on sampling site to gather information about characters of animals and herds in order to calculate risk factors associated with the prevalence of *T. annulata*. All the animal handling procedures and lab protocols were approved by the ethical committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Pakistan.

### Blood smear formation and screening

Geimsa-stained blood smear of each animal was prepared and examined under the oil immersion lens of a microscope for the detection of *T. annulata* following Takihi and Sandes (2013).

### Collection and morpho-taxonomic identification of ticks

Whole body of each animal was searched thoroughly for the presence of ticks. Collected ticks were preserved in 70% ethanol. Tick samples were identified morphologically using tick morpho-taxonomic characters under stereozoom microscope according to the standard relevant identification keys (Apanaskevich and Horak 2010; Madder et al. 2014; Caetano et al. 2017).

### DNA extraction from blood and tick samples

DNA extraction from blood samples was carried out by inorganic method following Saeed et al. (2016) whereas DNA from ticks was extracted by the ammonium hydroxide method described by Ammazalorso et al. (2015). The quality of the DNA extracted was assessed by measuring the optical density at 260/280 nm (O.R.I. Reinbeker, Hamburg) and by using submerged gel electrophoresis.

### PCR amplification

Polymerase chain reaction (PCR) was performed for the detection of *T. annulata* in blood and tick samples following Bilgic et al. (2013). Primers F 5'ACT TTG GCC GTA ATG TTA AAC 3' and R 5'CTC TGG ACC AAC TGT TTG G' 3' were used to amplify 312 base pair fragment from Cytochrome b gene of *T. annulata*. Reaction mixture was prepared in a final volume of 25 µl containing 13 mM Tris-HCl (pH 8.3), 65 mM KCl, 2 mM MgCl<sub>2</sub>, 0.0013% gelatin, 300 µM of each dNTP, 1 U of AmpliTaq DNA polymerase, 0.5 µM of each primer, and 2 µl of template DNA. Reaction conditions comprised of an initial denaturation step of 94 °C for 5 min followed by 30 cycles of denaturation 95 °C for 50 s, primer annealing 56 °C for 50 s and extension 65 °C for 50 s. A final extension at 65 °C for 5 min was performed (Bilgic et al. 2013).

*Theileria annulata* positive sample, provided by Prof. Dr. Munir Aktas from University of Firat, Turkey, and negative samples (reaction mixture without DNA) were also amplified during each PCR reaction as positive and negative controls, respectively.

## DNA sequencing and phylogenetic analysis for *Theileria annulata*

To confirm the PCR results of *T. annulata*, three representative PCR products were randomly selected for DNA sequencing. PCR products were purified from agarose gel slices with NZYGelpure® (Nzytech, Portugal), and subsequently sequenced (First Base Sequencing Service, Malaysia) with the same primers used for DNA amplification. The obtained DNA sequences were deposited at GenBank.

Phylogenetic analysis was conducted by using cytochrome b sequences of *T. annulata*, and tree was created in MEGA v.7.0 (Kumar et al. 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992). The tree with the highest log likelihood (– 316.25) is documented here. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Non-coding. All positions containing gaps and missing data were eliminated. There were, in total, 106 positions in the final dataset.

## Complete blood count analysis

Complete blood count parameters in all cattle blood samples were analyzed by using automated complete blood count analyzer following Saeed et al. (2016).

## Statistical analysis

Statistical analysis was conducted with the statistical package Minitab (v.16; State College, PA, USA). Fischer exact test was calculated to correlate parasite prevalence with the studied epidemiological factors. Two sample t-tests were calculated to compare complete blood count parameters between parasite positive and negative animals. Significance levels were set at  $\alpha=0.05$ .

## Results

### Blood smear screening-based prevalence of *Theileria annulata*

Giemsa-stained blood smear examination revealed an overall prevalence of 15% for *T. annulata*. Breed-wise analysis indicated an overall 23, 10 and 16% prevalence of *T. annulata* for crossbred, Holstein Frisian and Sahiwal cattle, respectively (data not shown). Season-wise screen analysis demonstrated 2.2, 12, 43 and 9.5% overall prevalence of *T. annulata* for spring, summer, autumn and winter season, respectively (data not shown).

**Table 1** Overall and seasonal prevalence of *Theileria annulata* among crossbreed, Frisian and Sahiwal breed blood samples collected during present study through PCR amplification of Cytochrome b gene

Season (no. collected samples)	Crossbreed	Frisian breed	Sahiwal breed	Total samples (% prevalence)
Spring (225)	3	2	2	7 (3)
Summer (225)	6	15	11	32 (14)
Autumn (169)	58	6	26	90 (53)
Winter (225)	17	10	18	45 (20)
Total samples (% prevalence)	84/300 (28)	33/244 (14)	57/300 (19)	174/844 (20.6)

### PCR based prevalence of *Theileria annulata*

Polymerase chain reaction (PCR) amplified a 312-base pair amplicon specific for cytochrome b gene of *T. annulata* in 21% of total animals enrolled in the present investigation. Seasonal analysis of prevalence data indicated that the highest rate of *T. annulata* infection was observed during autumn (53%) followed by winter (20%), summer (14%) and spring (3%) (Table 1). Crossbred cattle was found to be the most highly *T. annulata*-infected breed (28%) followed by Sahiwal (19%) and Holstein Frisian (14%) (Table 1). We were unable to find any tick positive for *T. annulata* through PCR analysis.

### DNA sequencing and phylogenetic analysis

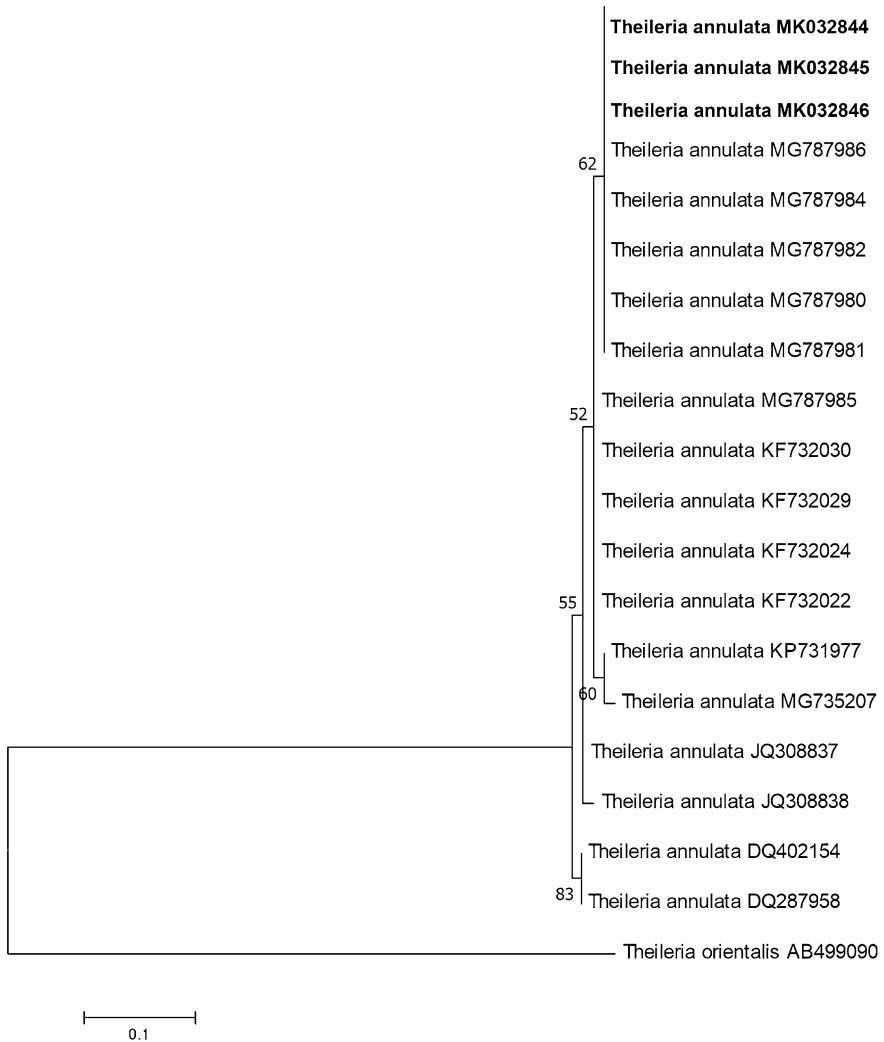
Three represented amplicons of 312 bp from cytochrome b gene sequence were confirmed by DNA sequencing and were submitted to the GenBank database under the accession numbers MK032844, MK032845 and MK032846. A BLAST analysis revealed nucleotide sequence identities of 97–99% with homologous sequences of *T. annulata* isolates registered in GenBank. The phylogenetic analysis of the obtained DNA sequence from *T. annulata* placed it in a stable monophyletic cluster along with cytochrome b gene of *T. annulata* downloaded from public databases (Fig. 1).

### Morpho-taxonomic identification of ticks

A limited number of ticks (N = 24) was collected during summer from crossbred cattle only as most of the enrolled animals were farmed and hygienically well maintained by the owners. In the present study, various tick species including *Hyalomma anatolicum*, *Hy. excavatum*, *Rhipicephalus microplus*, and *Haemaphysalis punctata* were identified. The morphological structures analyzed included the capitulum, Haller's organ, adanal plates, spiracles, festoons and other aspects of general morphology (data not shown).

### Epidemiology factors analysis

In crossbreed, analysis of results revealed that female animals were more susceptible to *T. annulata* infection than males during autumn and winter (Table 2). In summer, farms



**Fig. 1** Phylogenetic tree based on partial cytochrome b sequences from *Theileria annulata* isolates from cows in Pakistan and cattle worldwide, available in GenBank. The evolutionary history was inferred using the maximum likelihood method based on the Tamura 3-parameter model. The three new sequences of *T. annulata* obtained in the present study are represented in bold

supplied with pool water had more *T. annulata* infection than those having water pump-based water supply. Farms where dogs were present during autumn had a tick load with more *T. annulata* infection (Table 2).

In Sahiwal cattle, it was observed that female animals ( $P=0.02$ ), cattle with tick load ( $P=0.04$ ) and farms supplied with water pumps ( $P=0.03$ ) were more infected with *T. annulata*. Cattle with tick load had also significantly higher *T. annulata* infection in winter ( $P=0.02$ ) and summer ( $P=0.03$ ) (Table S1).

In Holstein Frisian cattle, male cattle in spring ( $P=0.05$ ) and female cattle in summer ( $P=0.05$ ) were more infected with *T. annulata* than animals of the other gender. Farms

**Table 2** Association of epidemiological parameters of crossbred cattle collected in the four seasons in District Layyah with the presence of *Theileria annulata*

Parameters	Autumn (%)	P	Winter (%)	P	Spring (%)	P	Summer (%)	P
<b>Sex</b>								
Male	0/13 (0)	0.03	0/12 (0)	0.05	0/0 (0)	–	1/13 (8)	1
Female	17/62 (27)		17/63 (27)		3/75 (4)		5/62 (8)	
<b>Water resource</b>								
Pump	12/58 (21)	0.5	14/63 (22)	0.1	3/75 (4)	–	1/47 (2)	0.02
Pool	5/17 (29)		3/12 (25)		0/0 (0)		5/28 (18)	
<b>Tick load</b>								
Yes	14/62 (23)	1	15/61 (25)	0.5	1/40 (3)	0.5	5/66 (8)	0.5
No	3/13 (23)		2/14 (14)		2/35 (6)		1/9 (11)	
<b>Other dairy animals at farm</b>								
Yes	5/18 (28)	0.5	11/39 (28)	0.2	2/43 (4)	1	1/31 (3)	0.3
No	12/57 (21)		6/36 (17)		1/32 (3)		5/44 (11)	
<b>Dogs at farm</b>								
Yes	15/44 (34)	0.005	14/64 (22)	0.7	3/49 (6)	0.5	5/60 (8)	1
No	2/31 (6)		3/11 (27)		0/26 (0)		1/15 (7)	
<b>Tick load on dogs</b>								
Yes	12/35 (34)	0.02	16/66 (24)	0.6	0/10 (0)	1	5/60 (8)	1
No	5/40 (13)		1/9 (11)		3/65 (46)		1/15 (7)	

Prevalence (%) of *T. annulata* is given in parenthesis. P-values are based on Fisher's tests

supplied with water through pools had higher *T. annulata* infection than farms supplied with water through pumps ( $P=0.02$ ). In autumn, animals without a tick load ( $P=0.01$ ) and farms with dogs with ticks ( $P=0.003$ ) had higher *T. annulata* infection. In winter, farms having other dairy animals in addition to cattle ( $P=0.02$ ) and farms with dogs without a tick burden ( $P=0.03$ ) had higher *T. annulata* infestation rates (Table S2).

### Complete blood count analysis

For crossbred cattle, in samples collected in spring a significant increase in mean corpuscular hemoglobin concentration and significant decrease in monocytes (%) and hemoglobin concentration was observed in *T. annulata* positive blood samples compared to *T. annulata* negative samples (Table 3). In autumn, mean cell volume, mean cell hemoglobin and mean corpuscular hemoglobin concentration were decreased in *T. annulata* positive crossbred cattle (Table 3).

For Sahiwal cattle, blood samples collected during spring showed an increase in lymphocytes (%) ( $P=0.001$ ) and mean corpuscular hemoglobin concentration ( $P=0.04$ ) in *T. annulata* positive relative to negative samples, whereas hemoglobin concentration was decreased ( $P=0.03$ ) (Table S3). In summer, *T. annulata* positive cattle had higher mean cell hemoglobin ( $P=0.02$ ) and mean corpuscular hemoglobin concentration ( $P=0.04$ ). An increase in lymphocytes (%) in autumn ( $P=0.05$ ) and in winter, a decrease in red blood

**Table 3** Mean ( $\pm$  SEM) complete blood count parameters compared between *Theileria annulata* positive vs. *T. annulata* negative blood samples of crossbred cattle collected in the four seasons in District Layyah during 2017–2018

Parameters	Spring		Summer		Autumn		Winter	
	Positive (N=3)	Negative (N=72)	Positive (N=6)	Negative (N=69)	Positive (N=17)	Negative (N=58)	Positive (N=58)	Negative (N=17)
White blood cell ( $\times 10^3 \mu\text{L}^{-1}$ )	8.50 $\pm$ 2.0	10.06 $\pm$ 3.5	7.73 $\pm$ 0.97	8.18 $\pm$ 0.89	128.0 $\pm$ 3.6	10.71 $\pm$ 1.2	9.16 $\pm$ 0.83	8.13 $\pm$ 0.26
Lymphocytes (%)	44.5 $\pm$ 8.7	55.9 $\pm$ 2.6	75.33 $\pm$ 2.2	77.38 $\pm$ 0.70	24.0 $\pm$ 3.8	23.2 $\pm$ 1.5	33.73 $\pm$ 2.3	35.7 $\pm$ 1.7
Monocytes (%)	4.50 $\pm$ 0.78	4.72 $\pm$ 0.24*	4.50 $\pm$ 0.34	3.62 $\pm$ 0.10*	3.059 $\pm$ 0.10	3.138 $\pm$ 0.057	4.53 $\pm$ 0.75	5.21 $\pm$ 0.51
Red blood cells ( $\times 10^6 \mu\text{L}^{-1}$ )	5.28 $\pm$ 0.52	5.79 $\pm$ 0.14	4.56 $\pm$ 0.36	4.49 $\pm$ 0.14	4.50 $\pm$ 0.21	4.39 $\pm$ 0.11	4.64 $\pm$ 0.16	4.74 $\pm$ 0.10
Hemoglobin (g $\text{dL}^{-1}$ )	8.02 $\pm$ 1.2	8.98 $\pm$ 0.19*	8.73 $\pm$ 0.54	9.13 $\pm$ 0.16	11.14 $\pm$ 0.39	11.07 $\pm$ 0.24	8.60 $\pm$ 0.22	8.70 $\pm$ 0.12
Cell volume (fL)	42.9 $\pm$ 1	44.2 $\pm$ 0.52	76.0 $\pm$ 6.6	76.6 $\pm$ 1.9	77.8 $\pm$ 1.3	82.5 $\pm$ 0.77**	74.62 $\pm$ 2.2	76.76 $\pm$ 1.1
Hematocrit (%)	22.67 $\pm$ 2.5	26.04 $\pm$ 0.6	36.5 $\pm$ 4.8	32.4 $\pm$ 0.86	34.76 $\pm$ 1.7	35.5 $\pm$ 0.91	37.7 $\pm$ 1.4	39.6 $\pm$ 1.1
Cell hemoglobin (pg)	14.7 $\pm$ 0.96	15.0 $\pm$ 0.16	19.67 $\pm$ 2.8	20.24 $\pm$ 0.66	27.6 $\pm$ 0.56	28.9 $\pm$ 0.29*	29.9 $\pm$ 0.56	30.4 $\pm$ 0.62
Corpuscular hemoglobin concentration (g $\text{dL}^{-1}$ )	36.57 $\pm$ 1.8	34.1 $\pm$ 0.49*	25.83 $\pm$ 1.3	29.5 $\pm$ 0.54*	32.82 $\pm$ 0.35	33.9 $\pm$ 0.30*	33.5 $\pm$ 0.68	32.7 $\pm$ 0.33
Platelets ( $\times 10^3 \mu\text{L}^{-1}$ )	300.3 $\pm$ 42	247.0 $\pm$ 17	137.3 $\pm$ 27	224.2 $\pm$ 12*	249.5 $\pm$ 22	237.0 $\pm$ 17	270.8 $\pm$ 12	288.4 $\pm$ 11

P-values are based on two-sample t-tests



cell count ( $P=0.02$ ) and hematocrit ( $P=0.04$ ) was observed in *T. annulata* positive compared to negative cattle (Table S3).

For Holstein Frisian cattle, a decrease in lymphocytes (%) was observed during spring ( $P=0.002$ ), summer ( $P=0.04$ ) and winter ( $P=0.04$ ), whereas an increase was observed during autumn ( $P=0.05$ ) in *T. annulata* positive compared to negative animals. Mean cell volume in autumn ( $P=0.03$ ), monocytes in summer ( $P=0.02$ ) and platelets in spring ( $P=0.001$ ) were increased, whereas hematocrit in spring ( $P=0.03$ ) was decreased in *T. annulata* positive Holstein Frisian cattle (Table S4).

## Discussion

Tropical theileriosis has been recognized as a burden to the development of the dairy industry and causes major economic losses (Mohamed et al. 2018). The prevalence of theileriosis depends upon geographical region, tick density, climatic conditions, age, gender, management practices, immunity of the host animal (Gul et al. 2015) and cattle breed as cattle usually differ in tick resistance and innate susceptibility to infection (Muhammad et al. 2008). In the present study, ticks belonging to *Hyalomma*, *Rhipicephalus* and *Haemaphysalis* were identified morphologically. Our observations are in line with previous findings as it has been reported that *Theileria* spp. are transmitted by a variety of ixodid ticks of the genera *Rhipicephalus*, *Hyalomma*, *Amblyomma*, and *Haemaphysalis* (Bishop et al. 2009). A tick infestation study in Holstein Frisian cattle from Kasur district of Pakistan indicated that *Hyalomma* ticks (15%) were most frequently collected followed by *Boophilus* (12%), *Haemaphysalis* (5%) and *Rhipicephalus* (3%) (Durrani and Kamal 2008).

In the present study Giemsa-stained smear examination and PCR were compared for the detection of theileriosis. Blood smear screening is a conventional method for the detection of blood borne parasites; several studies using this method have reported the prevalence of *Theileria* spp. in cattle in Pakistan. In this study, blood smear screening revealed that 15% samples were positive for *Theileria* species. Durrani et al. (2010) had collected 500 blood samples from three districts of Punjab and found that 5% Sahiwal cattle were positive in blood smears for *Theileria* as against 7% samples of Holstein Frisian cattle. Khattak et al. (2012) reported that 5.2% cattle blood smear collected from Kohat and Peshawar districts of Khyber Pukhtoon Khwa (Pakistan) were found positive for *Theileria* spp. PCR-based techniques are standard nowadays for the detection of *T. annulata*. PCR is more sensitive than blood smear screening for the species-specific blood parasite detection (Durrani et al. 2010; Shahnawaz et al. 2011; Khattak et al. 2012; Saeed et al. 2016). Our results also showed that 15% blood smear were found positive for *Theileria* species as compared to 21% cattle blood samples in which cytochrome b gene of *T. annulata* was amplified through PCR (Table 1). A similar comparison was made by Durrani et al. (2010) in Sahiwal (Pakistan) and found 23% prevalence for *T. annulata* in cattle by PCR as compared to 5% prevalence detected by blood smear examination. Also Shahnawaz et al. (2011) compared PCR amplification and screening with Giemsa-stained slides and found that PCR amplification is a more sensitive tool compared to smear scanning (19 vs. 3%) for the detection of *T. annulata*.

The highest prevalence (53%) in our study of *T. annulata* in cattle was observed during autumn (post monsoon) (Table 1). This is in accordance with Dharaneshha et al. (2017) who reported that theileriosis was more prevalent in rainy season (90.9%) than in winter (87.6%). These results indicate that *T. annulata* infection is associated with the rainy

season probably due to change in environmental factors, such as temperature, humidity and rainfall leading to increased tick reproduction and activity (Dharanesha et al. 2017). Our results are also in agreement with Vahora et al. (2012) who had reported higher incidence of theileriosis in crossbred cattle during the monsoon season from Gujarat (India). In a similar study from Patna (India), Kala et al. (2018) also reported highest prevalence of *T. annulata* infection in cattle (11.1%) during post-monsoon (October–November) followed by August–September (9.8%) and June–July (6.3%); they postulated that this higher prevalence in post monsoon season might be due to abundance of vector ticks that developed during summer.

Among the three cattle breeds in the present study, incidence of *T. annulata* infection was highest in crossbreed (Table 1). This is in agreement with Vahora et al. (2012) who reported 37% positive cases of haemoprotozoan infection in crossbred cattle. Annand and Ross (2001) also reported high incidences of tropical theileriosis in crossbred cattle during summer and monsoon. Saeed et al. (2016) also reported breed-wise prevalence of theileriosis and recorded highest incidence of *T. annulata* infection in crossbred cattle (23.9%) followed by Cholistani (19.3%) and Sahiwal (14.4%) cattle. Durrani et al. (2010) demonstrated the PCR prevalence of *Theileria* was 40.2% in crossbreed as compared to 23% in Sahiwal.

Data on genetic diversity of *T. annulata* from Pakistan are very scarce. We performed phylogenetic analysis based on a 312 bp amplicon from cytochrome b gene obtained in this study. Sequences obtained were clustered with the previously reported sequences from India (MG787981-86) indicating that these are closely related genetic variants (Fig. 1). The partial sequences from various regions of the world indicate that there are considerable genetic differences between *T. annulata*. The variation depends not only on geographical location, but also on tick species involved in parasite transmission (Yukari and Umar 2002).

Farm management systems and epidemiological factors were found to be correlated to the prevalence of *T. annulata*. Water supply source, presence of other dairy animals on farm and presence and tick burden of dogs on farms were affecting the prevalence of *T. annulata* (Table 2). Our findings are contradictory to those of Khattak et al. (2012) and Inci et al. (2008), who found that none of the studied epidemiological parameters were associated with prevalence of *T. annulata* in cattle blood samples collected from Khyber Pukhtoon Khwa (Pakistan) and Cappadocia (Turkey), respectively. Salih et al. (2007) and Farooqi et al. (2017) declared that prevalence of *T. annulata* varies with the farm management system and various aspects of management are potential risk factors for the spread of theileriosis.

We found higher prevalence of *T. annulata* infection in females than in males of all three breeds of cattle during the various seasons. Our results are in accordance with those of Inci et al. (2008), Khattak et al. (2012) and Saeed et al. (2016) as they all reported higher prevalence of *T. annulata* in female cattle. Saeed et al. (2016) suggested that a comparatively weak immune response and more hormonal fluctuations in females increase the incidence of infection.

Complete blood counts revealed that hemoglobin, mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), lymphocyte (%) and monocyte (%) count were disturbed in *T. annulata* positive blood samples from the various cattle breeds (Table 3). Our results are in line with findings of Durrani et al. (2010), Khan et al. (2011), Khattak et al. (2012), Qayyum et al. (2010) and Shahnawaz et al. (2011), who documented a decrease in red blood cell count and hemoglobin concentrations in *T. annulata* positive compared to negative cattle. These alterations have been attributed to toxic effects of metabolites of

*Theileria* sp., loss of blood through permanently sucking ticks, parasitemia-induced anemia, immune mediated erythrophagocytosis and Tumour-Necrosis Factor- $\alpha$  (Geerts et al. 2001; Khan et al. 2011). We observed increased MCV in Holstein Frisian cattle infected with *T. annulata*. Haroon et al. (2014) reported that MCV was higher in cattle with higher parasitemia than in cattle with lower parasitemia, signifying an increase in the size of erythrocytes (macrocytosis). This agrees with the findings of Ghanem et al. (2013) and Durrani et al. (2010), where macrocytosis was only observed in animals clinically infected with theileriosis but not in control animals.

In the present study, decrease was observed in lymphocyte in Sahiwal and Holstein Frisian breed cattle. These findings are consistent with those reported by Omer et al. (2002) and Col and Uslu (2006) who reported decreases in white blood cell, lymphocyte, neutrophil, monocyte, eosinophil, and basophil counts during hematological analysis in *T. annulata*-infected cattle. It has been reported that *T. annulata*-induced leucopenia is mainly mediated by TNF- $\alpha$  (7). This decrease is related to the destruction of lymphocytes in lymphoid organs and infiltration of these cells into various organs (Omer et al. 2002).

In conclusion, we reported the prevalence of *T. annulata* infection in three cattle breeds from Pakistan with cross breed being most susceptible to this infection. PCR was a more reliable and sensitive technique for *T. annulata* detection than blood smear screening. Parasite prevalence varied with the sampling season and presence of *T. annulata* had significant effects on the hematological profile of all cattle breeds under investigation. These data will pave the way for the control of tropical theileriosis in Pakistan.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10493-021-00595-6>.

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## Compliance with ethical standards

**Conflict of interest** Authors have no conflict of interest of any sort with anyone.

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