Original Article

Molecular evidence, risk factors analysis, and hematological alterations associated with *Theileria* spp. spillover in captive wild mouflon sheep in Punjab, Pakistan

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Abstract

Background: Landscape anthropization and interaction between domestic and wild animals are the major contributing factors involved in the emergence of new pathogens in wild animals. Theileriosis is an emerging issue of wild ungulates, especially in the tropical and subtropical areas of the globe. **Aims:** The current study investigated the mouflon sheep for *Theileria* infection using molecular methods and hematological analysis. **Methods:** This study was conducted on a total of 103 captive wild mouflon sheep present in eight different recreational zoos, and wildlife parks in Punjab, Pakistan to investigate the genotypic prevalence of *Theileria* spp. by targeting *18S rRNA* and molecular evidence for *Theileria* spillover between domestic and wild mouflon sheep by phylogenetic analysis. The association of assumed risk factors and the effect of *Theileria* spp. on various hematological parameters were also assessed. **Results:** The results depicted that *Theileria* spp. was prevalent in 8 (7.77%, CI 95%: 3.99-14.59%), and 11 (10.68%, CI 95%: 06.07-18.12%) animals based on microscopy, and PCR, respectively. The phylogenetic analysis of the *18S rRNA* gene of *Theileria* spp. from mouflon revealed a close resemblance with *T. annulata* from domestic animals. The risk factor analysis revealed that tick infestation, enclosure hygiene, previous tick infestation history, and the presence of wooden logs in the enclosure were significantly ($P<0.05$) associated with the occurrence of *Theileria* spp. infection in the captive mouflon sheep of Pakistan. Furthermore, a significant reduction in blood parameters like PCV, RBCs count, Hb, and platelets was observed in *Theileria*-positive animals. **Conclusion:** This study is the first evidence at the molecular level to characterize the spillover of *Theileria* spp. between the captive wild mouflon sheep and domestic animals of Pakistan, and it will be useful in developing control strategies for emerging theileriosis in captive wild animals.

Key words: Emerging disease, Mouflon sheep, Phylogenetic analysis, Theileriosis, Wildlife

Introduction

Wildlife parks, breeding centers under captivity, and related recreational sanctuaries parks have been designated as key tools to protect the exposed, and threatened wild animal species (Ali *et al*., 2011). The diseases of wild animals have a huge impact on the national economy, biodiversity, public health, and wildlife conservation (Oludairo *et al*., 2016). The ecological coexistence of wild and domestic animals elevates the risk of bilateral pathogen transmission among different species (González-Barrio *et al*., 2022). The population of mouflon sheep (*Ovis orientalis*), an endangered wild species, is decreasing rapidly due to hunting, stress, deadly infections, parasitic, and hemoparasitic diseases like fasciolosis, muelleriosis, theileriosis, and anaplasmosis (Benfenatki *et al*., 2016; Adjou Moumouni *et al*., 2018). Over the last three decades, the mouflon sheep death rate has increased by 30% (Valdez, 2008) while the conflict between human and sympatric impacts as well as the disease load poses a bigger threat to wildlife refuges (Shabbir and Khan, 2010; Posada-Guzm *et al*., 2015).

In Pakistan, a total of 225 captive mouflon sheep are present in various wildlife parks and recreational zoos in Punjab province according to a recent report by the Wildlife Department. These parks include Safari Zoo Lahore, Wildlife Park Jallo, Lahore Zoo, Wildlife Park Gutwala-Faisalabad, Wildlife Park Changa Manga, Wildlife Park Kamalia, Wildlife Park Lohi Bher Rawalpindi, D.G. Khan Zoo, and Wildlife Park Vehari. Blood-borne parasites are of severe concern in domestic

as well as wild animals in Pakistan. *Theileria* spp., *Babesia* spp., *Ehrlichia* spp. and *Anaplasm*a spp. are important transboundary tick-borne pathogens of animals throughout the world (Ito *et al*., 2013; Batool, 2019; Basit *et al*., 2022). Among the tick-borne hemoparasitic diseases of mouflon sheep, theileriosis is one of the most prevalent disease of domestic and wild animals living in the subtropical and tropical areas of the globe including Bangladesh, Pakistan, and India. Theileriosis is caused by an obligate intracellular protozoan, belonging to the genus *Theileria* (Heidarpour Bami *et al*., 2010; Ghauri *et al*., 2019; Ali *et al*., 2020; Abid *et al*., 2021). In wild ungulates, subclinical infection is caused by the diverse species of *Theileria* and is transmitted by arthropod vectors between domestic and wild animals (Shabbir and Khan, 2010; Alvarado-Rybak *et al*., 2016). Theileriosis is mainly transmitted by the Ixodid ticks i. e. *Hyalomma anatolicum*, *H. impeltatum*, *H. rufipes*, *H. dromedarii*, *Rhipicephalus evertsi*, *R. decoloratus*, *R. sanguineus*, and *Amblyomma lepidum* (Hussain *et al*., 2014; Mans *et al*., 2015; Boucher *et al*., 2020; Hayati *et al*., 2020; Ola-Fadunsin *et al*., 2020). The infected animals exhibit clinical signs including a rise in body temperature, reduced appetite, yellowing of mucous membrane, labored breathing, nasal and conjunctival discharge, coughing, and enlarged lymph nodes (Tanveer *et al*., 2022). For diagnostic purposes, blood smear examination can be performed. However, the most reliable and expedient tool for the diagnosis of theileriosis is polymerase chain reaction (PCR) (Hassan *et al*., 2019).

The epidemiological investigation of disease-causing pathogens is required in captive wild animals to minimize the losses in the terms of deteriorating health and mortalities of precious wild animals (da Silveira *et al*., 2011). Moreover, a pathogen spillover among domestic and wild animals should be considered an important emerging issue that threatens people's domestic economy based on animal production, public health concerns in zoonosis case, national animal health security in the context of transboundary animal diseases, and the success of integrated conservation and development initiatives in case of wild animal protection scenario (Caron *et al*., 2013). In Pakistan, a large number of captive wild animals including mouflon sheep suffer from tick-borne diseases every year (Ghafar *et al*., 2020; AbouLaila *et al*., 2021). In Pakistan, increased prevalence of theileriosis in various domestic animals including small ruminants (Naz *et al*., 2012; Zaman *et al*., 2022), bovines (Farooqi *et al*., 2017), and equines (Ali *et al*., 2019; Shah *et al*., 2020) have been reported, and wildlife practitioners showed concerns regarding hemoparasites and their diagnosis in wild animals (Ishaq *et al*., 2022). In this regard, this study was designed to investigate the molecular prevalence of *Theileria* spp. among domestic animals, and captive mouflon sheep of Pakistan.

Materials and Methods

This study was approved by the Advanced Studies and Research Board, University of Veterinary and Animal Sciences, Lahore (Letter No. DAS/8437; Dated 21-11-2019).

Samples collection and initial screening

The present study was conducted on a total of 103 captive wild mouflon sheep kept in eight different recreational zoos and wildlife parks in Punjab, Pakistan (Fig. 1), from March to September 2020. The study was approved by the Advanced Studies and Research Board, University of Veterinary and Animal Sciences, Lahore (Letter No. DAS/8437; Dated 21-11-2019), and consent was taken from the relevant authorities for the sampling process. The mouflon sheep manifesting clinical signs like tick infestation, pyrexia, anorexia, pale mucous membrane, jaundice, or anemia were incorporated in the study. A data capture form was designed to access probable theileriosis risk factors like sex, age, tick infestation, the previous history of tick infestation, the entrance of new wild animals, enclosure hygiene, and the presence of wooden logs. For sampling, animals were captured using a capturing net irrespective of age and sex, and 3 ml of blood was aseptically collected from the jugular vein, and stored in EDTA-coated vacutainers. The blood smears of all samples in triplets were also prepared for the microscopic examination. All samples were transferred to Medicine Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, maintaining a cold chain. For initial screening, Giemsa-staining was performed on blood smears, and all stained smears were observed under the microscope for intra-cytoplasmic inclusion bodies resembling *Theileria* spp. in blood cells (Radostits *et al*., 2006). For confirmation, blood samples were subjected to molecular diagnosis protocol.

DNA isolation and PCR

The DNA extraction of all blood samples was performed utilizing a Exgene™ Blood SV DNA extraction kit (GeneAll®, South Korea) as per manufacturer's recommendations. Extracted DNA from the samples was subjected to purity and concentration measurements using the NanoDrop at 260/280 nm. Forward primer 5´-GAC ACA GGG AGG TAG TGA CAA G-3´ and reverse primer 5´-CTA AGA ATT TCA CCT CTG ACA GT-3´ were utilized for the amplification of 450 bp *18S rRNA* gene fragment (Nijhof *et al*., 2003). The PCR recipe was prepared by mixing master mix (2X PCRTaq Master Mix, Bioshop) (10 µL), DEPC-treated water (6.6 µL), forward primer (0.7 µL), reverse primer (0.7 µL), and sample DNA (2 µL). The conditions for PCR reaction include one step of 5 min of initial denaturation at 95°C, followed by 35 cycles of denaturation (95°C for 30 s), primers annealing (58°C for 30 s), and extension (72°C for 1 min). The final extension was performed with one step at 72°C for 10 min. Gel electrophoresis of 1.5% ethidium bromide-stained agarose gel was conducted at 120 volts, and 200 milliamperes for 35 min. After gel electrophoresis, PCR products were seen on UV illuminator for detecting

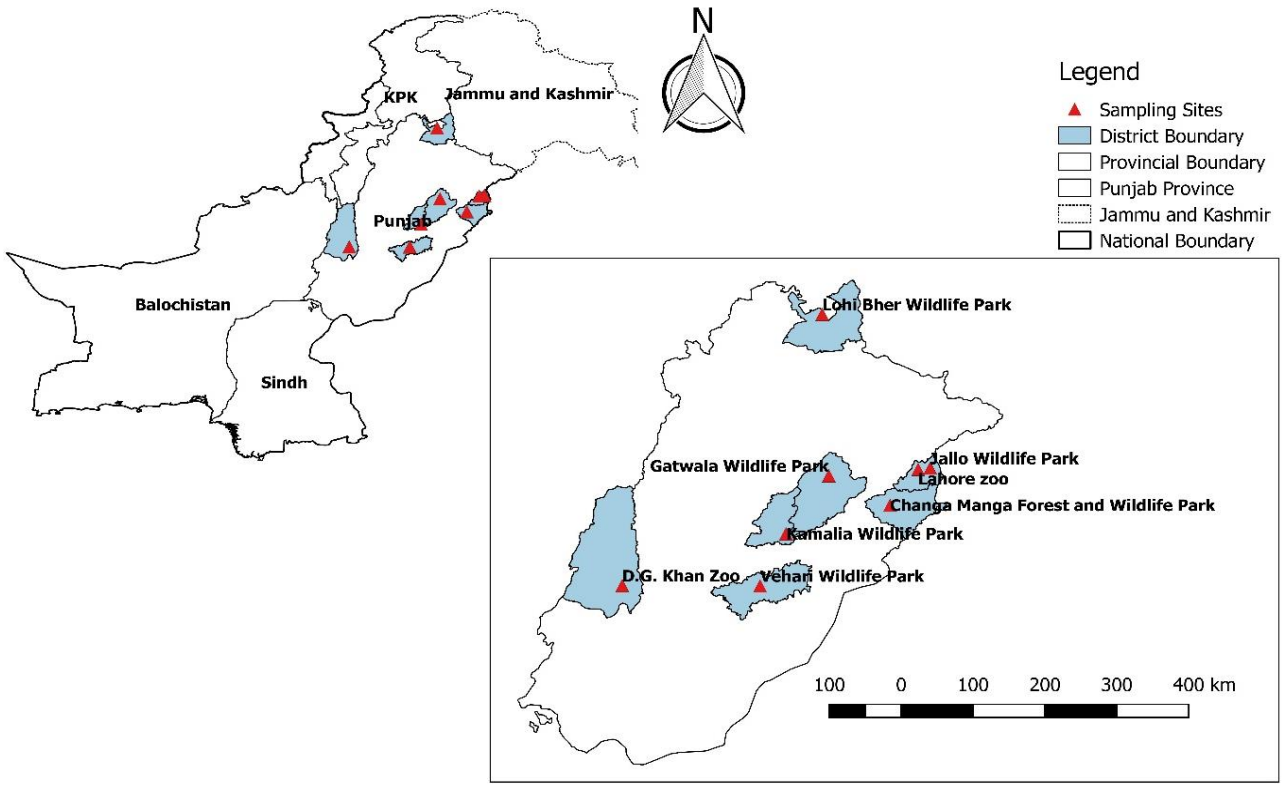

Fig. 1: Map formed by Quantum Geographic Information Systems (QGIS) showing recreational zoos and wildlife parks of Punjab, Pakistan

positive bands at 450 bp position alongside 100 bp molecular mass marker. The PCR positive bands after gel purification using Expin™ Gel SV gel extraction kit (GeneAll®, South Korea) were shipped to 1st BASE sequence lab Singapore, for sequencing.

Molecular evidence for *Theileria* spp. spillover by phylogenetic analysis

The representative nucleotide sequence of *Theileria* spp. *18S rRNA* gene was evaluated using the basic local alignment search tool (BLAST), and phylogenetically analyzed to find out the pathogen spillover among domestic and wild animals at a molecular level in the country. Firstly, the gene sequence of *18S rRNA* of *T. annulata* reported from domestic cattle, camel, buffalo, goat, and sheep of Pakistan, and from neighboring countries like China, and India were retrived from NCBI database. Then multiple sequence alignment was done by BioEdit Software using the CLUSTAL W method. Similarities or differences among the gene sequences were observed at the nucleotide level to figure out the pathogen resemblance between wild and domestic animals. Finally, phylogenetic tree was constructed using the bootstrap phylogeny testing at 1000 replications by maximum likelihood (ML) method through Mega X Software (Ahmed *et al*., 2020).

Hematological analysis

To pinpoint the difference in the hematological parameter of *Theileria*-positive and *Theileria*-negative mouflon sheep, a comparative hematological analysis was performed. A total of four PCR-based *Theileria*-positive and four healthy mouflon sheep (*Theileria*-negative) were selected, and various hematological parameters of them were assessed using an automated hematology analyzer, Abacus Junior Vet (Diatron® Vienna, Austria). The hematological parameters include platelet count, RBCs count, WBCs count, packed cell volume (PCV), and hemoglobin (Hb) to evaluate hematological changes in diseased animals (Penzhorn, 2006; Ahmed *et al*., 2020).

Statistical analysis

The prevalence of the *Theileria* spp. infection was calculated by a formula already reported (Thrusfield, 2007). The data of the current study was analyzed by statistical software SPSS (ver. 20) at a 5% probability level. A Chi-square test was performed to evaluate the association of risk factors with *Theileria* spp. infection. To compare the means of different hematological parameters of *Theileria*-positive and *Theileria*-negative mouflon sheep, an independent sample t-test was applied. The variables showing a p-value less than "0.05" were assumed to be significant determinants of disease occurrence.

Results

Prevalence of *Theileria* spp. infection in mouflon sheep

On microscopic examination, out of 103 mouflon

sheep only 8 sheep (7.77%, CI 95%: 3.99-14.59%) were positive for *Theileria* spp. Molecular test revealed that 11 (10.68%, CI 95%: 06.07-18.12%) out of 103 animals were positive for *Theileria* spp. from various parks in Pakistan including Jallo Wildlife Park, Lahore Zoo, Changa Manga Forest, and Wildlife Park. Among these 11 animals, eight animals were found positive by both microscopy and PCR while three animals were found positive only by molecular test.

Risk factor analysis

The risk factors assessment revealed that tick-infested animals showed a higher prevalence (19%) of *Theileria* spp. compared to tick-free animals (4.9%). Similarly, animals with the previous history of tick infestation or any tick-borne disease were at more risk of *Theileria* spp. infection. Both tick infestation and previous tick infestation history were proved to be significantly associated (P<0.05) with *Theileria* spp. infection in mouflon sheep. Also, the presence of wooden logs, being a good place for ticks, was assumed a risk factor (P<0.05) for the disease. On the other hand, the animals living in enclosures were at less risk of developing theileriosis significantly (P=0.001).

Risk factors like sex, age, and introduction of new animals were not found statistically significant (P>0.05) associated with the occurrence of disease (Table 1).

Molecular evidence of *Theileria* spp. spillover

The BLAST search of *18S rRNA* partial sequences revealed 99% to 100% similarity with *T. annulata* sequences. These similar *T. annulata 18S rRNA* sequences were reported from cattle, buffalo, sheep, goat, and ticks (Fig. 2). Furthermore, the phylogenetic tree revealed that the current sequence shares more evolutionary relation with sequences from camel (MW392284), goat (MT318160) (Niaz *et al*., 2021), buffalo (MN726546) (Ghafar *et al*., 2020) and sheep (MK838106) (Adegoke *et al*., 2020) than the sequences from cattle (MT893655, MG599095), and ticks (MZ452896) (Fig. 3).

Effect of *Theileria* spp. infection on hematological parameters

Hematological analysis exhibited a significant (P<0.05) decline in the RBCs, platelets, hemoglobin (Hb), and PCV in *Theileria*-positive animals in comparison to mouflon sheep negative for *Theileria* spp. A non-significant (P>0.05) decrease in WBCs was also observed in mouflon sheep positive for *Theileria* spp. in comparison to the healthy animals (Table 2).

Table 1: Chi-square analysis of assumed risk factors associated with *Theileria* infection in mouflon sheep

Study variable	Category	No. samples	Positive (%)	P-value
Sex	Male	43	4 (09.30)	0.7
	Female	60	7 (11.67)	
Age	≤ 4 Year	25	3 (12.00)	0.8
	>4 Year	78	8 (10.26)	
Tick infestation	Yes	42	8 (19.04)	0.023*
	No	61	3 (04.91)	
Previous tick history	Yes	28	9 (32.14)	<0.001*
	No	75	2 (02.67)	
Enclosure hygiene	Yes	59	1 (01.69)	0.001*
	No	44	10 (22.72)	
Presence of wooden logs	Yes	37	7 (18.91)	0.043*
	No	66	4 (6.06)	
Introduction of new wild animals	Yes	33	4 (12.12)	0.7
	No	70	7 (10.00)	

* Showing P<0.05 which indicates significant results

Table 2: Comparative hematological analysis of *Theileria*-positive and *Theileria*-negative mouflon sheep by independent sample t-test

Parameter	Unit	*Theileria*-positive animals (mean±SD)	*Theileria*-negative animals (mean±SD)	F-value	Mean difference	Confidence interval	P-value
WBCs	$\times 10^3/\mu L$	9.67 ± 2.957^a	7.5 ± 2.642^a	0.228	2.175	2.67-7.02	0.315
RBCs	$\times 10^6/\mu L$	11.75 ± 2.125^a	5.375 ± 1.869^b	0.084	6.375	2.91-9.83	0.004*
Hemoglobin	g/dL	12.1 ± 2.551^a	5.175 ± 1.909^b	0.144	6.925	3.02-10.82	0.005*
PCV	%	33.575 ± 6.232^a	18.625 ± 2.357^b	6.56	14.95	6.79-23.10	0.004*
Platelets	$\times 10^3/\mu L$	513.25 ± 73.89^a	319.25 ± 95.45^b	1.52	194	46.32-341.68	0.018*

Different lowercase letters indicate significant statistical differences between the mean values of different hematological parameters of *Theileria*-positive and *Theileria*-negative mouflon sheep, and * Indicates the significant association of blood parameters with the infection

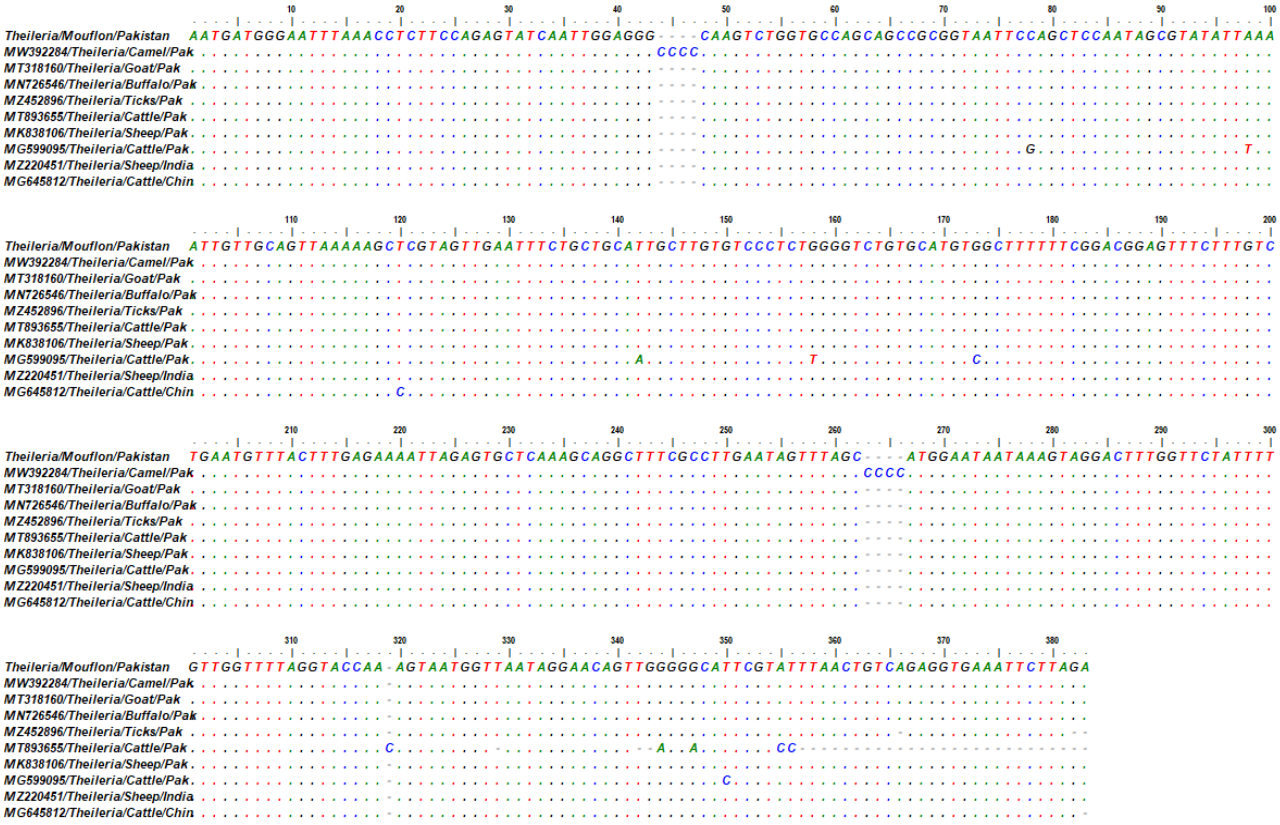

Fig. 2: Clustal W multiple alignments of the study isolate with reported sequences of *T. annulata* isolated from domestic animals

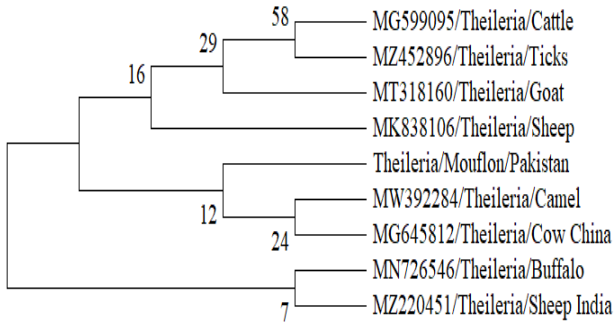

Fig. 3: Phylogenetic tree showing comparison for the study isolate of *Theileria* spp. with reported sequences isolated from domestic animals

Discussion

The microscopy-based *Theileria* spp. prevalence found in the current study was similar to the findings of Zaeemi *et al*. (2011), and Zobba *et al*. (2020) who reported the prevalence of 9.2%, and 10.4% in domestic sheep, and goats, respectively. Microscopic evaluation of blood smears revealed a 10% prevalence of theileriosis in Sudanese sheep (Salih *et al*., 2003). The current findings were not supported by the study conducted on domestic sheep from Khyber Pakhtunkhwa (KPK) province, Pakistan showing a lower molecular prevalence of 4.5% (Saeed *et al*., 2015). The different molecular studies conducted in Pakistan showed theileriosis prevalence of 7.8%, 13.5%, and 14% in domestic sheep which were in line with the current study findings (Taha *et al*., 2013; Nasreen *et al*., 2020; Giangaspero *et al*., 2015).

Contradictory to the findings of this study, a higher prevalence (35%) of *Theileria* spp. was reported from domestic sheep in district Lahore (Durrani *et al*., 2011). Most currently, a prevalence of 6.1% and 1.2% for *T. ovis* and *T. lestoquardi* has been reported in the sheep population of Punjab, Pakistan (Tanveer *et al*., 2022). Another study conducted in Pakistan found a 10.6% molecular prevalence of *T. ovis* in domestic sheep (Abid *et al*., 2021). In other countries, two higher prevalence (32.8% and 54.03%) of *Theileria* spp. were also reported in Iran, and Turkey using nested PCR (Altay *et al*., 2005; Zaeemi *et al*., 2011). Also, the high prevalence (15.50%, 57.73%, and 58.79%) of *T. ovis* in sheep was reported from Turkey (Altay *et al*., 2004; Aktaş *et al*., 2005; Chen *et al*., 2014). Worldwide, the difference in the prevalence of the disease can be associated with the differences in infection status, vector distribution, socioeconomic factors, climate and the type of PCR used to estimate the infection (nested PCR, conventional PCR, and real-time PCR) (Ramos *et al*., 2009; Cicuttin *et al*., 2015).

In current study, both tick infestation and previous tick infestation history were proved to be significantly associated ($P<0.05$) with *Theileria* spp. infection in mouflon sheep. The risk factor of tick infestation history found in this study were also found in other studies (Al-Fahdi *et al*., 2017; Boucher *et al*., 2020; Hayati *et al*., 2020; Ceylan *et al*., 2021). Also, the presence of wooden logs, being a good place for ticks, was assumed a risk factor ($P<0.05$) for the disease. On the other hand, the

animals living in enclosures were at less risk of developing theileriosis significantly (P=0.001). The probable reason could be the better hygienic measures for sheep in the enclosures.

In this study, female sheep showed a non-significant increase in prevalence (11.7%) as compared to male sheep (9.3%) which was in agreement with Gebrekidan *et al*. study (2014), reported a lower infection rate in males (38.1%) in comparison to females (61.9%). The higher infection rate in female sheep may be associated with more stress, particularly during lactation and pregnancy. Similarly, the infection rate in adult sheep (> 4 years) (10.3%) was lower as compared to younger sheep (< 4 years) (12%) which was in agreement with Shabbir and Khan study (2010), reported a non-significantly higher prevalence in younger sheep (17.3%) as compared to adult sheep (16.2%). However, the results of current study were not following a study showed a higher prevalence in adult sheep (79.7%) as compared to younger sheep (20.3%) (Gebrekidan *et al*., 2014). The findings were also in concurrence with other studies on *Theileria* spp. infections in sheep and goats (Razmi *et al*., 2006; Weir *et al*., 2011). Mouflon sheep with tick infestation was more prone (19%) to diseases as compared to those without tick infestation (4.9%) which was in line with the findings of Saeed *et al*. study (2015).

The sequence similarity of study isolates with reported isolates suggest the transmission of the pathogen at the livestock/wildlife interface. As a vector-borne disease, the ecological co-existence of the same vector on both domestic and wild animal, and high prevalence of theileriosis among domestic animals in the country could assume as evidences for pathogen transmission between domestic and wild ungulates. The analysis also revealed a similar gene pattern with sequences reported from China and India as neighboring countries, involved in the transboundary trade of animals and related things. However, the minor differences among the gene sequences as compared to reported sequences could be due to the genetic recombination of an organism at the vector-host interface during concurrent infections (Kurtenbach *et al*., 2006). The possible genetic variation between the extracellular (in ticks) and intracellular (host) phase and the occurrence of more than one *Theileria* genotype in ticks during infection could be involved in the pathogen's sequence variation (Al-Hamidhi *et al*., 2022).

The hematological test is a considerable way to differentiate between healthy and unhealthy animals in veterinary studies (Riaz and Tasawar, 2017). The results obtained in this study were supported by studies showing lower Hb contents, RBCs count, and PCV (%) in *Theileria* spp. infected sheep (Bell-Sakyi *et al*., 2004; Nazifi *et al*., 2011; Razavi *et al*., 2011). The decline in RBCs number might be due to erythrophagocytosis during theileriosis and resulted in increased oxygen radicals causing anemia (Shiono *et al*., 2004). Furthermore, various other studies have also supported the current findings (Hussein *et al*., 2007; Nazifi *et al*., 2011; Oryan *et al*., 2013; AL-Mayah and Abdul-Karim, 2020). The considerable decline in Hb, PCV, and total RBCs count reported in the current study agreed with most hematological studies (Hussein *et al*., 2007; Nazifi *et al*., 2011; Mohammed *et al*., 2014; Al-Saad *et al*., 2015; Kundave *et al*., 2015). All these changes could occur as a result of persistent loss of blood, and anemia caused by permanent tick feeding (Durrani *et al*., 2011; Ito *et al*., 2013). The current study showed an insignificant reduction in total WBCs, as reported by Hussein *et al*. (2007), and Ramos *et al*. (2009). The decrease in WBCs count might be explained by the demolition of WBCs especially lymphocytes due to the disease (Sandhu *et al*., 1998; Omer *et al*., 2002).

Rapid ecological change, increasing globalization, and the co-existence of domestic and wild animals lead to the re-emergence of old and the emergence of new diseases in wild animals. The current study is the first report regarding the epidemiology, related risk factors, and molecular evidence for *Theileria* spp. spillover in the mouflon sheep of Punjab, Pakistan. The results proposed that PCR was a more sensitive and specific technique compared to microscopy for the diagnosis of theileriosis in wild mouflon sheep. Phylogenetic analysis revealed similarity among *18S rRNA* sequence of *T. annulata* from domestic animals and wild mouflon sheep which shows possible spillover of the pathogen. Risk factors assessment revealed that tick infestation, enclosure hygiene, previous tick history, and presence of wooden logs were significantly associated with disease occurrence. Furthermore, hematological parameters like RBCs, PCV, Hb, and platelets were significantly decreased in mouflon sheep infected with *T. annulata*. Further research can be conducted on the tick species involved in the transmission of this disease. The results of this preliminary study could aid in the establishment of better strategies for diagnosis, prevention, and control of theileriosis in the future.

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Conflicts of interest

The authors declare no conflict of interest.

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