Research Article

Parasitological and Molecular Survey on Theileriosis of Sheep and Goats and the Related Risk Factors in Musa Pak Shaheed Town, Multan, Pakistan

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Abstract | The current survey was designed to study the infections of *Theileria* spp. (*Theileria lestoquardi* and *T. ovis)* in small ruminants through PCR and microscopy in Musa Pak Shaheed, Multan district, Pakistan amidst 2013. A total of 200 small ruminants (93 sheep and 107 goats) without any apparent signs of theileriosis were examined to diagnose *Theileria* piroplasms. During sampling, blood was amassed by puncturing the jugular vein in eppendorf comprising EDTA as anticoagulant coated tubes for the detection of *Theileria* piroplasms. DNA was extracted from collected samples and subjected to PCR amplification to determine ovine and caprine theileriosis. Through a questionnaire, the data regarding animals as well as herds characteristics were gathered to define the risk factors favors the spread of *Theileria* spp. infection. 24% and 8.5% *Theileria* infection identified through PCR and microscopic examination during present investigation. The PCR detected 14.5% and 9.5% *Theileria* piroplasms infection in sheep and goats, respectively. Among *Theileria* species, PCR identified the incidence of *T. lestoquardi* and *T.* ovis was 16.5% and 5% respectively while 6% blood samples revealed mixed infection of both species in overall small ruminants. The infection rate of *T. lestoquardi* was higher (9.5% and 7%) than *T. ovis* (3% and 2%) in sheep and goats respectively. Statistically significant correlation revealed between theileriosis and animal species, different age groups, presence of ticks, herd size and herd composition in small ruminants. The outcomes of the current survey ratified PCR amplification is discernible, specific and sensitive assay for diagnosis of ovine and caprine theileriosis.

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Introduction

Livestock sector is an imperative tool of national economy in developing countries like Pakistan, being a source of foodstuff, energy resources and organic fertilizers for crops growth (Ahmad *et al*., 2007; Manan *et al*., 2006). Livestock sector in Pakistan subsidized to agriculture worth 56.3 percent although it contributes 11.8 % to the gross domestic product (GDP) during 2014-15. Livestock sector added Rs. 778.3 billion during 2013-14 to Rs. 801.3 billion during 2014-15 to the national income, a rise in 3.0 % recorded related to prior year (Economic Survey, 2014-15). Livestock role, in rural community can be professed that 30-35 million rural inhabitants are involved in livestock production having 2-3

cattle/buffaloes and 5-6 small ruminants (sheep or goats) per family having household fortune, helping to derive 30-40 % of their income (Durrani *et al.*, 2006; Irshad *et al.*, 2010). Sheep and goats constitute 2 from 3 billion total ruminants throughout the world (Ahmad *et al.*, 2011). In Pakistan nearly 25 goats's breed and at least two dozen sheep's breed are raised throughout the country (Khan *et al.*, 2006). Small ruminant rearing is favorable as a capital asset for generating breeding inventory and food product (Idrees *et al.*, 2007). Comparing small ruminant hoisting with cattle, the former require considerably less capital per head. Generally lower productivity of livestock in Pakistan ascribed to many factors containing health problems caused by nutritional deficiencies, parasitic infestation and contagious infections dissonantly affect the ruminants which resulted in low productivity and high mortality (Zulfiqar *et al.*, 2012). Theileriosis in small ruminants caused clinical illness and higher mortalities reported in Southern Europe, Middle East, China and Indian Subcontinent (Rao *et al.*, 1991). Ovine and caprine theileriosis occurs due to six *Theileria* species, from these species, *T. lestoquardi, T. luwenshuni* and *T. uilenbergi* are deliberated extremely pathogenic while *T. separata, T. ovis* and *T. recondite* are in general less pathogenic in sheep and goats (Razmi *et al.*, 2003). *Theileria* ovis and *T. lestoquardi* are whispered to cause ovine and caprine theileriosis in Pakistan (Durrani *et al.*, 2011). *T. lestoquardi* causes pathogenic infection in sheep (Yin *et al.*, 2007) and in goats (Jianxun and Hong, 1997) which also termed as malignant ovine theileriosis. Malignant ovine theileriosis has three forms: Acute, sub acute and chronic. Acute form may lead to death after a short course of fever and one or more of clinical symptoms that may appear: depression, inappetance, fever, swelling of superficial lymph nodes, respiratory manifestations and appearance of pale visible mucous membranes, jaundice and oedema of the throat (Rehman *et al.*, 2010; Woldehiwet, 2007).

During earlier studies in Pakistan, *Theileria* spp. infection investigated through presence of clinical symptoms and examination of stained smears of bloods and lymphnodes. Microscopic examination and presence of clinical symptoms are trustworthy only in critical cases but deficient in carrier animals due to morphological resemblances among piroplasms (Inci *et al.*, 2010; Telmadarraiy *et al.*, 2012). Subclinical infections are also identified by serological methods such as indirect florescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA) (Leemans *et al.*, 1999) but these methods are not impeccable due to low parasitemia level and cross-reactions with other related pathogens (Altay *et al.*, 2012). Polymerase chain reaction (PCR) is sensitive and specific than microscopy and IFAT for the diagnosis of *Theileria* piroplasms in small ruminants (Altay *et al.*, 2005; Durrani *et al.*, 2012). Bovine theileriosis had been extensively studied throughout the country However, the epidemiological attributes of theileriosis in small ruminants oats in Pakistan are poorly understood throughout the country and further studies are required to determine the real status of theileriosis and development of better methods to control ovine and caprine theileriosis (Naz *et al.*, 2012). Currently, PCR amplification has been considered the preferential technique for diagnosis of ovine caprine theileriosis than conventional methods due to sensitive and specific as well as detected piroplasms at low parasitaemia level. This present investigation was aimed to study the indubitable status of theileriosis in sheep and goats from district Multan, Pakistan and adjoining areas by microscopy and PCR amplification method.

Materials and Methods

Study area

The study was conducted in Musapak Shaheed town of district Multan which is a one of the major cities in Punjab, Pakistan (Figure 1). The district Multan is located between 29'-22'north latitude and 71'-4' east longitude with an extreme temperature 49 °C during summer and 1°C in winter and an average rainfall is 127 mm. The Musa pak shaheed town is located eastern to the Multan district, Pakistan. Total area of Musa pak shaheed town is about 322 sq., kilometers containing 24 union councils. Sheep and goats rearing is significant occupation of Musa pak shaheed town. Mostly small ruminants are reared collectively with average size of herd ranges between 50-100 animals/herd.

Blood sampling

Blood was collected from jugular vein of 200 apparently healthy small ruminants containing sheep (N= 93) and goats (N= 107) arbitrarily selected from various herds during 2013 located in Musapak Shaheed Town, Multan district, Pakistan. The blood samples were preserved in 5 ml glass tubes containing a drop of 0.5 M EDTA as anticoagulant. Through

questionnaire, data regarding characteristics of animals and herds were gathered during sampling.

Figure 1: *Physical Map of Pakistan showing district Multan, the area under study.*

Blood smear examination

Thin smears of blood were prepared in field during sampling, and then air dried, fixed in methanol which used as preservative. 10% giemsa solution was used for staining the smears. Binocular microscope was used to examine smears under oil immersion lens at 1000 x magnification (Altey *et al*., 2005).

DNA extraction

The extraction of DNA was carried out by inorganic method followed (Shaikh *et al*., 2004).

Briefly, 750µl blood was taken in eppendorf tubes and 750µl of TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8.0) was added and mixed by vertex, then centrifuged at 13,000 rpm for 5 min. The supernatant was wasted and TE washing was repeated for 2–3 times to make the pellet of WBCs totally hemoglobin free. Then add 6oo ml TNE buffer (10 mM Tris HCl, 2 mM EDTA, 400 mM NaCl) and 20 µl of 10% sodium dodecyl sulfate and 1 µl Proteinase K for protein digestion was added in pellets and incubated overnight.

After this add 1 ml of 5 M NaCl in eppendorf, shaked and chilled for 15 min on ice for precipitation of digested proteins which were pelleted by centrifugation for 5 min. and removed. Now the supernatant was shifted to new eppendorf and equal volume of chilled isopropanol was added, DNA in the form of fiber was appeared in the eppendorf tubes and pelleted by centrifugation for 5 min. After this DNA pellet was washed (70% ethanol) and dissolved in TE buffer. The extracted DNA was quantified through gel electrophoresis using 1.0% agarose gel.

PCR amplification

A touchdown thermocycler (BIORAD) was used for PCR amplification with a 50 µl total reaction volume having 5 µl of 10 X PCR buffer (100 mMTris–HCl (pH 9), 500 mMKCl, 1% Triton X-100), 250M each of the four dNTPs, 2 U Taq DNA polymerase, each primer 10 pg and 5 µl of template DNA. Three primers sets were used for amplification of the parasitic DNAs through PCR during present study described in Table 1. Professor Urike Seitzer (VIIRC, Borstel, Germany) provided positive control DNA of *T. ovis* and *T. lestoquardi.* Thermo profile for PCR of *Theileria* specific was containing at 94°C for 3 min., followed by 35 cycles, 1 min. at 94°C for denaturation, 1 min. at 60°C for annealing and 1 min. at 72°C for extension, with a final extension step of 72°C for 7 min. (Allossp *et al*., 1993) Thermo profile for *T. lestoquardi* was comprising at 94°C for 3 min., followed by 35 cycles at 94°C for 1 min., 56°C for 1 min. and 72°C for 1 min. with a final extension step of 72°C for 7 min. (Kirver *et al*., 1998). Thermo profile of *T. ovis* was consisting for 3 min. at 96°C, was followed by 5 cycles, 30s at 94°C, 30s at 56°C and of 1 min. at 72°C. These 5 cycles were followed by 30 cycles. Each cycle consisted of 30s at 94°C, 30s at 54°C and 1 min. at 72°C (Altay *et al*., 2005). The PCR program was ended with a final extension step of 7 min. at 72°C. Gel electrophoresis was used for documentation of PCR amplified fragments from extracted DNA of blood samples. 10 µl PCR amplified products with loading buffer run on 1.5% gel for 30 minutes and observed under UV illuminator for valuation and record.

Statistical analysis

For statistical analysis small ruminants were divided into different age catagories: ≤ 1 year, 1-2 years and > 2 years old. Herds were divided in three different size categories containing animals 1-30, 31-60 and > 60 animals. Based on composition, the animal's herd was categorized into three groups containing sheep only, goats only or mixed containing both sheep and goats. Chi square and Fisher's exact test was used for data analysis regarding *Theileria* spp. infection and potential risk factors responsible involved in outbreak of theileriosis in studied population of sheep and

goats. MiniTab (Version 16) used to statistically analyze the recorded data of small ruminants.

Results and Discussion

Prevalence of Theileria infection in small ruminants

Theileria piroplasms were detected in 8.5% (17/200) small ruminants based on microscopic screening having ribbon or curved shaped observed under binocular microscope as described by Urquhart *et al.* (1996) as shown in Figure 2. Sheep revealed higher infection (5%) compared to goats (3.5%) based on microscopic examination. To evaluate the true status of *Theileria* spp. infection, the blood samples were subjected to PCR amplification which revealed significantly higher prevalence 24% (48/200) of *Theileria* species by the amplification of a 1098-bp DNA fragment of 18SSU ribosomal RNA (Allsopp *et al.*, 1993) as depicted in Figure 3. *Theileria* infection found significantly higher (14.5%) in sheep as compared to goats (9.5%). All the positive samples identified through blood smears were confirmed by PCR. The incidence of *Theileria* piroplasms among different sampling sites was significantly associated ($p < 0.05$) as indicated by chi square analysis as shown in Table 2. The PCR amplification showed higher infection of theileriosis in Basti Jhanday wala (60%) while lower in Botay wala (6.7%). The current investigation is the foremost report on ovine and caprine theileriosis in Musapak Shaheed, Multan district, Pakistan. PCR analysis indicated the incidence of theileriosis was more rampant in area under study.

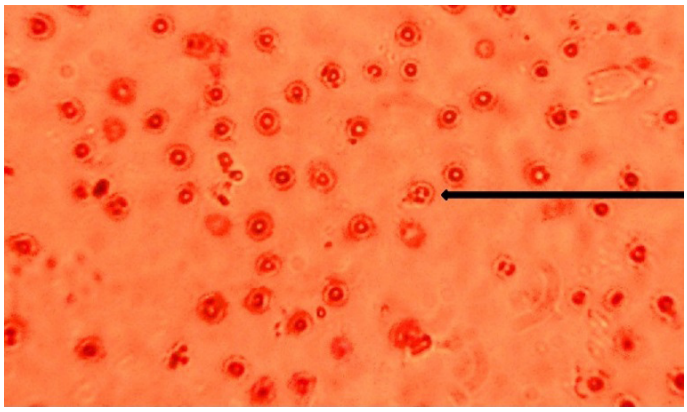

Figure 2: *Theielria piroplasms in infected RBCs.*

Table 1: *Primers used for the detection of genus Theileria infection and T.ovis and T. lestoquardi in small ruminants in Musapak shaheed, Multan, Southern Punjab, Pakistan during 2013.*

Primer specificity	Target gene		Product size (bp)	Reference
Theileria specific	*18SSU rRNA*	F. 5′-AGTTTCTGACCTATCAG-3′	1098	(Allsopp *et al.*, 1993)
		R. 5′-TTGCCTTAAACTTCCTTG-3′		
Theileria ovis	*18SSU rRNA*	F. 5′-TCGAGACCTTCGGGT-3′,	520	(Altay *et al.*, 2005)
		R. 5′-TCCGGACATTGTAAAACAAA-3′		
Theileria lestoquardi	*18SSU rRNA*	F. 5′-GTGCCGCAAGTGAGTCA-3′	785	(Kirvar *et al.*, 1998)
		R.5′GGACTGATGAGAAGACGATGAG3′		

Table 2: *Microscopic examination of thin blood smears and PCR amplification results of sheep and goats in Musapak Shaheed, Multan, Southern Punjab, Pakistan during 2013.*

Area	No. of samples	Test				
		Micr. examination		PCR examination		
		Positive	%	Positive	%	P*value
Basti Garrialla	40	2	(5)	10	(25)	
Botay wala	30	1	(3.3)	2	(6.7)	
19 Kasi	10	-	-	1	(10)	
Basti Jhaday wala	25	3	(12)	15	(60)	
Makhdoom Rashid	20	2	(10)	3	(15)	
Jalal abad	30	3	(10)	8	(26.7)	
Dera Budahu	20	5	(25)	6	(30)	0.000 [b]***
Punjnand	25	2	(8)	3	(12)	
Total	200	17	(8.5)	48	(24)	0.000[a]***

a: Fisher's exact test; b: Chi square test; P < 0.01 = Significant (*).

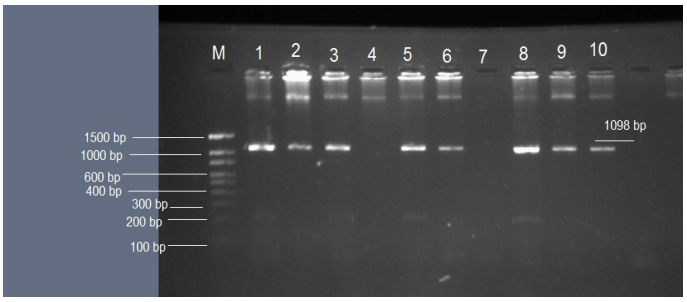

Figure 3: *Agarose gel electrophoresis of amplified PCR products obtained from Theileria species genomic DNA using Theileria specific primers. Lane M. DNA marker 100-1500 bp; Lane 1. Theileria specific DNA positive control; 4. Negative control (Distilled water); 2.3.5.6.79.10. 11. 13. Theileria species DNA positive sample. Lane .5. 8. 9. 12 Theileria species DNA negative sample.*

The infection rate of Theilaeria lestoquardi and T. ovis

Theileria spp. identified through PCR was *T. lestoquardi* and *T. ovis* in small ruminants during current study. The blood samples detected positive by microscopy verified through PCR amplification. *Theileria* piroplasms did not discern in stained blood smears that were found negative during PCR amplification. The diagnosis of hemoprotozoans is often onerous in the carrier animals due to low parasitemia and mixed infection of piroplasms (Altay *et al*., 2005) and specific and sensitive molecular techniques are needed for diagnosis of *Theileria* piroplasms (Naz *et al*., 2012). PCR amplification enables the detection and discernment of *Theileria* spp. at low parasitemia rates in carrier small ruminants. The present study revealed higher incidence of theileriosis by PCR amplification (24%) compared to blood smears examination (8.5%) in studied small ruminants. Statistical significant association ($p < 0.00$) perceived between PCR and blood smear screening test. Our results are consistent to previous findings reported in Turkey by Altay *et al*. (2005); in Pakistan by Durrani *et al*. (2011) and in Iran by Jalali *et al*. (2014) who confirmed that PCR amplification revealed higher prevalence of theileriosis in small ruminants compared to microscopy. The lower prevalence reported by microscopy during present study was expected because this technique does not detect carrier small ruminants with low parasitemia rate. During PCR, the amplified DNA fragment 520 bp considered positive for *T. ovis* (Altey *et al*., 2005) and 785 bp for *T. lestoquardi* (Kirver *et al*., 1998) respectively in the studied blood samples of small ruminants as indicated in Figure 4. In overall small ruminants theileriosis found higher in sheep (14.5%) than goats (9.5%) and the association was statistically significant ($p < 0.05$) between theileriosis and animal species as stated in Table 3. The prevalence of *T. lestoquardi*, *T. ovis* and mixed infection (both spp.) was found 16.5%, 5% and 3% respectively in overall small ruminants. Both in sheep and goats the infection rate of T. lestoquardi was reported higher than *T. ovis* during present study. Among *Theileria* positive samples in sheep the *T. lestoquardi* infection was significantly higher (65.5%) compared to *T. ovis* (20.7%) while mixed infection of both species was reported in 13.8% blood samples. Similar trend of higher infection of *T. lestoquardi* was found 73.7% compared to *T. ovis* 21% and mixed infection was found in 5.3% among positive blood samples of goats as described in Table 3.

Table 3: *The frequency of Theileria species based on PCR in sheep and goats in Musa Pak Shaheed Town, Multan District, Southern Punjab, Pakistan during 2013.*

***Theileria* species**	**Overall 200**	**Sheep 93**	**Goats 107**
Genus *Theileria*	48 (24 %)	29 (14.5 %)	19 (9.5 %)
Theileria lestoquardi	33 (16.5 %)	19 (65.5 %)	14 (73.7 %)
Theileria ovis	10 (5 %)	6 (20.7 %)	4 (21 %)
Mixed	6 (3 %)	4 (13.8 %)	1 (5.3 %)
P value	0.00[a]	0.00[a]	0.01[a]

a: Chi square test P < 0.01= Significant (*).

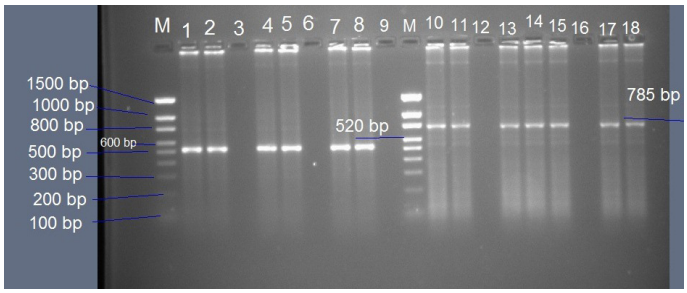

Figure 4: *Agarose gel electrophoresis of amplified PCR products obtained from Theileria ovis and Theileria lestoquardi genomic DNA Lane: M, 100 bp DNA marker; Lane: 1. T. ovis positive control. 3. Negative control (distilled water): 2, 4, 5, 7, 8, Parasite positive blood sample: 6,9, Parasite negative blood sample. Lane: 10. T. lestoquardi positive control. 12 Negative control (distilled water): 11, 13,14,15, 17, 18 Parasite positive blood sample; 16 Parasite negative blood sample.*

The frequency of theileriosis in sheep and goats during current study was outstanding which share the same pasture. PCR identified statistically significantly ($p < 0.05$) higher prevalence of theileriosis in sheep (14.5%) than in goats (9.4%) during present study. The findings are in consistent with the findings of Guo *et al*. (2002) who reported 27.63% sheep and 13.12% goats infected with theileriosis in Ganan region, China. Similar results were found by Irshad *et al*. (2010) during a study in Okara and Islamabad,

Pakistan, higher prevalence in sheep (7.36%) compared to goats (3.8%). Similar trend was found by Altay *et al*. (2012) who reported higher *Theileria* infection in sheep (28.90%) than in goats (4.10%) in Turkey. Iqbal *et al*. (2013) also reported higher prevalence of theileriosis in sheep (23.00%) than goats (11.00%) based on RLB during a study in Pakistan. Statistically significantly higher prevalence ($p < 0.05$) in sheep compared to goats could be due to difference of skin nature of the two livestock species. The skin of goats is thin and more resistant for attachment of ticks as compared to skin of sheep (Naz *et al*., 2012) and variation of resistance in various breeds and due to the fact that goats used marginal areas for breeding where is lower chance for ticks to contact with animal body reported by Alessandra and Santo (2012).

Through PCR amplification, *Theileria lestoquardi* and *T.* ovis were diagnosed in studied small ruminants. Similar *Theileria* spp. was reported by Heiderpour *et al*. (2009, 2010) and by Durrani *et al*. (2011) in sheep and goats. The infection rate of *T. lestoquardi* was significantly ($p< 0.05$) higher (16. 5 %) than *T. ovis* (5%) while mixed infection of both species was recorded 3% in overall small ruminants. Significantly ($p< 0.05$) higher frequency of *T. lestoquardi* 65.5% and 73.3% compared to *T. ovis* 27.7 and 21% was found in sheep and goats respectively during present study. But our results are contradicts to Sayen *et al*. (2009) and Rjeibi *et al*. (2014) who reported higher prevalence of *T. ovis* compared to *T. lestoquardi* in small ruminants in Turkey. This discrepancy may be due to difference of geoclimatic conditions, tick infestation and genetic resistance among different species of small ruminants against theileriosis.

The results revealed *T. lestoquardi* infection was significantly higher (65.5%) compared to *T.* ovis (20.7%) in sheep. The findings are in line with Heiderpour *et al*. (2010) who reported higher prevalence (87.5%) of *T. lestoquardi* compared to (12.5%) of *T. ovis* in small ruminants in Iran. Similar trends of higher prevalence of *T. lestoquardi* (54.8%) compared to *T. ovis* (40.2%) revealed in sheep by Zeemi *et al*. (2011) in Iran. The results of present study disagree with the previous findings of Altay *et al*. (2012) who reported higher prevalence of *T. ovis* (18.9%) compared to other *Theileria* species infection in Turkey. Yaghfoori *et al*. (2013) reported higher prevalence of *T. ovis* (43%) than *T. lestoquardi* (3%) in sheep in Iran which is contradicts to present study. In goats, the infection of *T. lestoquardi* and *T. ovis* was found 73.7% and 4.0% respectively during the present study. The similar results of higher prevalence of *T. lestoquardi* in goats has been reported by many authors in different regions of the world, 40.0 % by Luo and Yin (1997); 20.8% by Zangana and Naqid (2011); 78.3% in Nubian goats by Hussein *et al*. (1993); 14.0% by Hassan *et al*. (2013). The findings of current survey denies by Sayin *et al*. (2009) who reported higher prevalence of *T. ovis* (12.36%) than *T. lestoquardi* in sheep and goats in Turkey. The difference in infection rate of *Theileria* species in sheep and goats might be validated to genetic difference of animal species, tick species acting as vector and geoclimatic conditions of the studied areas.

Risk factors involved in the spread of theileriosis

For the evaluation of risk factors associated with theileriosis, the pooled data was analyzed. Fisher exact test revealed a significant correlation ($p <0.05$) of *Theileria* spp. infection with animal species and presence of ticks but non-significant association with gender of the animal ($p > 0.05$) in overall small ruminants. For assessment of animals and herds characteristics as possible risk factor for the spread of theileriosis in sheep and goats, some basic data of every animal and herd was collected during the survey. The males had higher infection rate (27.7%) than that of females (23.2%). Our results indicated a non-significant correlation ($p>0.05$) between theileriosis and gender in small ruminants. Similar trends of higher theileriosis in male animals recorded when only goat data was analyzed and the correlation with theileriosis was significant ($p < 0.05$). In sheep, the infection rate of both *Theileria* species was same in both sexes and the association was non-significant ($p > 0.05$). The results are in consistent with Hussein *et al*. (2013) who reported that males had higher infection of ovine theileriosis (30.64%) as compared to females (12.74%) in Sudan. The probable reason of lower prevalence in females could be due to fact that during pregnancy lactation, heat reduced tick resistance which in turn led to the lower transmission of disease, but contradicts to Naz *et al*. (2012) who found higher *Theileria* spp. infection in females than males in sheep and goats in Lahore, Pakistan. Chi square results indicated the prevalence of *Theileria* infection was significantly correlated with age of animals ($p < 0.05$) as depicted in Table 4. The collected data of sheep and goats was also separately analyzed which indicated non-significant correlation of theileriosis

with gender and breed in sheep ($p > 0.05$) while age and tick infestation was significantly correlated with theileriosis ($p < 0.05$) as indicated in Table 4. Among goats age of the animal and tick infestation was found non-significant ($p > 0.05$) while gender and breed of goats was found significant ($p < 0.05$) as described in Table 4.

Table 4: *Association between occurrence of Theileria parasites identified by PCR in sheep and goats and the studied parameters describing animal characters in Musa Pak Shaheed, Multan, Southern Punjab, Pakistan during 2013.*

Animal type	Parameters		No. of samples	Piroplasms Positive	Piroplasms Negative	P* value
Sheep and goats (Combined)	Animal type	Sheep	93	29 (41.7)	64 (58.3)	0.03[a*]
		Goats	107	19 (26.6)	88(73.4)	
	Sex	Male	36	10 (27.7)	26 (72.3)	0.52[a]
		Female	164	38 (23.2)	126(76.8)	
	Age	≤ 1 year	57	19 (33.3)	38 (66.7)	0.02[b*]
		≤ 2 year	66	18 (27.3)	48 (72.7)	
		≥ 3 year	77	11 (14.3)	66 (85.7)	
	Ticks	Present	140	41 (39.5)	99 (61.5)	
		Absent	60	7 (25)	53 (75)	0.00[a*]
Sheep	Sex	Male	13	4 (30.8)	9 (69.2)	0.97[a]
		Female	80	25 (31.3)	55 (68.7)	
	Age	≤ 1 year	23	12 (52.2)	11 (47.8)	0.03[b]
		1-2 year	35	10 (28.6)	25 (71.4)	
		≥ 3 year	35	7 (20)	28 (80)	
	Ticks	Present	67	25 (45.3)	42 (50.8)	
		Absent	26	4 (25)	22 (75)	0.04[a*]
	Breed	Lohi	81	27 (50)	54 (50)	0.25[a]
		Kajli	12	2 (40.4)	10 (59.6)	
Goats	Sex	Male	23	8 (32)	15 (68)	0.01[a]
		Female	84	11 (13.1)	73 (86.9)	
	Age	≤ 1 year	34	7 (20.6)	27 (79.4)	0.17[b*]
		1-2 year	31	8 (25.8)	23 (74.2)	
		≥ 3 year	42	4 (9.6)	38 (90.4)	
	Ticks	Present	73	16 (40)	57 (60)	
		Absent	34	3 (11.3)	31 (88.7)	0.11[a*]
	Breed	Nacchi	28	8 (40)	20 (60)	0.05[b]
		Beetal	54	5 (18)	49 (82)	
		Teddy	25	6 (31.6)	19 (68.4)	

a: Fisher's exact test; b: Chi square test; P < 0.01= Significant (*).

Age barrier in small ruminants (sheep and goats) against theileriosis was examined during the present study. *Theileria* species infection reported higher in age group one year (33.3%) in overall small ruminants than age group one to two year (27.3%) and lower in ≥ 3 year (14.3%) and statistically this association was significant ($p < 0.05$). Similar trend of higher *Theileria* spp. infection was found in sheep and the association was significant. The results are in line with Iqbal *et al.* (2013) who reported higher infection rate of theileriosis animals having age less than one year in small ruminants in Pakistan. Guo *et al.* (2002) also found higher incidence and death rate due to *Theileria* spp. infection both in young and adults of sheep and goats during a study in Ganan region, China. The current results are contradicts with Razmi *et al.* (2003) who stated non-significant correlation of *Theileria* positivity in different age groups of sheep population. The discrepancy may be due to difference of resistance against theileriosis in different breeds of small ruminants.

Breed wise prevalence indicated non-significant ($p < 0.05$) association of theileriosis in overall small ruminants but among sheep Lohi breed more infected with theileriosis than Kajli while in goats significant association was found between breeds and theileriosis ($p > 0.05$). Nacchi breed revealed higher infection of theileriosis than Beetal and Teddy breed. The difference of *Theileria* infection among different breeds of small ruminants might be due to variance of genetic resistance against theileriosis, host management and ecological factors which are tangled in spread of ticks and Tick borne diseases. Chi square results indicated that both herd size and herd composition had significant correlation ($p < 0.05$) with theileriosis in small ruminants represented in Table 5. Herd size was found the significant risk factor involved in disseminate of ovine and caprine theileriosis. The higher infection rate of theileriosis reported in herds having smaller size (53.3%) than larger size during present study and chi square indicated a significant correlation ($p < 0.05$) of theileriosis and different herd size. These results are contradicts to Durrani *et al.* (2012) who stated ovine theileriosis was not affected by the herd size during a study from Pakistan. The prevalence of *Theileria* spp. infection reported higher (44%) in herds having sheep only compared to herds having either only goats (10%) or both sheep and goats (20.0%). Chi square indicated a significant corelation ($p< 0.05$) between *Theileria* spp. infection and herd composition. Our results are opposes to Saeed *et al.* (2015) who found that mixed herds aggregates the *Theileria* spp. infection.

Table 5: *Association between occurrence of Theileria parasites identified by PCR in sheep and goats and the studied parameters describing animal and herd characteristics in Multan, Southern Punjab, Pakistan during 2013.*

	Parameters	No. of samples	Piroplasms positive	Piroplasms negative	P* value
Size of herd	1- 30	15	8 (53.3)	7 (46.7)	0.01[b*]
	31-60	50	14 (28)	36 (72)	
	More than 60	135	26 (19.3)	109(80.2)	
Herd composition	Sheep only	25	11 (44)	14 (56)	0.03[b*]
	Goats only	40	10 (25)	30 (75)	
	Sheep and goats	135	27 (20)	108 (80)	

b: Chi square test; P < 0.01 = Significant (*).

Conclusions and Recommendations

Ovine and caprine theileriosis has not been extensively studied in sheep and goats in south of Punjab, Pakistan and to our knowledge this is first study based on PCR assay in Multan district (Pakistan). The results of the current investigation concluded that ovine and caprine theileriosis is endemic in the studied area. Poor hygienic conditions and poverty might be the contributing factor for theileriosis in area under study. The risk of ovine and caprine theileriosis can be significantly reduced by generating public awareness about the disease.

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Novelty Statement

Ovine and Caprine theileriosis caused by Theileria species resulted in higher economic losses and mortalities upto 100% in case of outbreak and there is paucity of information regarding theileriosis in sheep and goats, therefore it is necessary to address this issue in southern Punjab where livestock is an integral part of agricultural system.

Author's Contribution

Survey, blood sample collection, the experimental work in the laboratory i.e., microscopic examination, DNA extraction and PCR amplification and the compilation of results were performed by MR. Statistical analysis was carried out by MZU and ZH. The manuscript was drafted by MR while critically analyzed and reviewed by ZT.

Conflict of interest

The authors have declared no conflict of interest.

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