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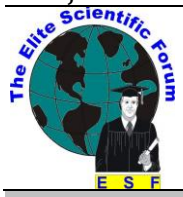

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RESEARCH ARTICLE

A Study on Molecular Prevalence, Intensity and Associated Risk Factors for Ovine and Caprine Theileriosis from Southern Punjab, Pakistan

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ABSTRACT

A molecular survey was conducted to determine the prevalence of *Theileria* spp. infection and risk factors associated with theileriosis in sheep and goats during 2013 in Multan district, south of province Punjab, Pakistan. A total of 250 animals (sheep=156 and goats=94), from randomly selected herds were sampled, for collection of blood from different regions of Multan. Using a questionnaire data regarding animal characteristics including animal species, sex, age, breed and tick infestation and herd characteristics including location, size and composition was collected. Overall incidence of theileriosis in small ruminants was found to be 12.5 and 36% through blood smears and PCR assay, respectively. The frequency of *Theileria* spp. infection was found significantly ($P<0.05$) higher in sheep 72.2% (n=65) compared to goats 37.8% (n=25). Among *Theileria* spp., *T. lestoquardi* was recorded higher (50%) than *T. ovis* (26.7%) while overall mixed infection of both species was recorded 17.6% in small ruminants. Sheep and goat data, analyzed separately which indicated that the infection of *T. lestoquardi* was 57 and 52%, respectively while that of *T. ovis* was 41.7 and 26.6% in sheep and goats, respectively. The species, age, size and tick infestation on studied small ruminants were significant risk factors ($P<0.05$) associated with ovine and caprine theileriosis. In conclusion, small ruminants of Southern Punjab are infected with Theileria species with high prevalence rate of *T. lestoquardi* as compared to *T. ovis*.

INTRODUCTION

Theileriosis is a tick-borne protozoan disease caused due to *Theileria* (*T.*) spp. belonging to phylum apicomlexa and are commonly found in subtropical parts of the world (Altay et al., 2007; Radostits et al., 2000). Tick borne diseases cause economical losses in terms of death of infected animals, abridged productivity, lower health status and high cost for control of such diseases (Makala et al., 2003; Uilenberg, 1997). Theileriosis cause higher morbidity and mortality and thus results in high economical losses in sheep and goats production systems (Aktas et al., 2005). *Theileria* spp. infection in small ruminants is caused by six *Theileria* species, from which *T. lestoquardi, T. uilenbergi* and *T. luwenshuni* are immensely pathogenic while other three species *T. separata, T. ovis* and *T. recondite* are mildly pathogenic in nature (Razmi and Yaghfoori, 2013). The most prevalent tick vectors for ovine and caprine theileriosis are *Hyalomma anatolicum* and *Rhipicephalus sanguineus* (Razmi et al., 2007). Malignant ovine theileriosis (MOT) resulted in 100% mortality in small ruminants (Latif et al., 1994) and caused by *T. lestoquardi* infection (Telmadarraiy et al., 2012). Animals suffering from *T. lestoquardi* exhibit fever, emaciation, lymphadenopathy, anorexia, dyspnea, anemia, icterus, jaundice, pyrexia, intermittent diarrhea, weakness and termination of rumination (Sayin et al., 2009). *Theileria* piroplasms are more detrimental in small ruminants as compared to cattle and are considered a serious animal health around the globe (Schnittger et al., 2000). In Pakistan, studies on the economic impact and production affliction due to MOT have not been undertaken yet. The lesser insight concerning epidemiological aspects of ovine and

caprine theileriosis, vector transmission and its control justifies the need of the present study.

Theileria species infection is generally diagnosed by microscopy or occurrence of clinical symptoms in the infected animals but such techniques are only reliable during acute infection but not valid for carrier animals with low parasitemia rate (Inci et al., 2010). PCR amplification is preferable molecular technique, mostly used for detection of piroplasms during recent years. The current survey was aimed at unveiling a specific, valid and sensitive molecular tool such as PCR for the diagnosis of *Theileria* infection, intensity and to determine risk factors responsible for the spread of *Theileria* spp. infection in and around Multan, south of Punjab, Pakistan.

MATERIALS AND METHODS

Study area

Multan is located between 29'-22' north latitude and 71'-4' east longitude in south of province Punjab, Pakistan. Multan occupies a total area of 5,630 sq. miles with plain fertile land while the climatic conditions are exceptionally warmer in summer season and cooler in winter season. The extreme hotness during summer is anticipated by the monsoon rains in month of August. The temperature drops down to 5ºC during two months i.e. December and January.

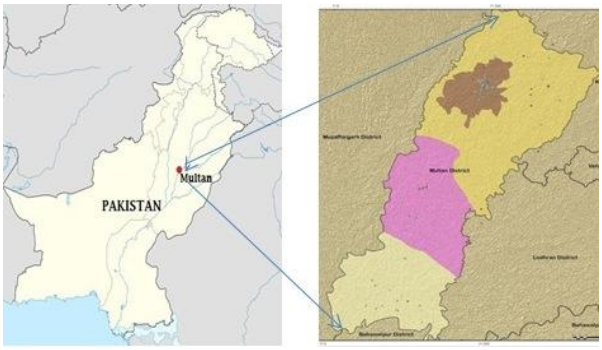

Fig. 1: Study area

Blood sampling

A total of 156 sheep and 94 goats (n=250) were arbitrarily selected for blood sampling without any apparent signs of theileriosis from sampling sites during the year 2013. Blood sample (5 mL) was taken from jugular vein of animals in glass tubes having few drops of 0.5M ethylene diamine tetra acetic acid (EDTA). The glass tubes containing blood samples were properly labeled and information regarding animal species, age group, gender and breed of sampled animals were collected through a survey questionnaire. In all the samples, presence of *Theileria* spp. was confirmed by microscopy and PCR amplification.

Microscopic examination

Thin blood smears were prepared on clean glass slides with the help of a spreader. The slides were air dried and fixed in absolute methanol for 1 minute in the field. The smears were stained in the laboratory with 5% Giemsa solution and examined under oil immersion lens (100X). Parasitemia ratio was evaluated by counting *Theileria* infected RBCs by observing at least 200 microscopic fields and then expressed in percentage ratio as described by Jalali et al. (2014).

PCR amplification

For DNA extraction, inorganic method was used as described by Shaikh et al. (2004). The quality of extracted DNA from collected blood samples was evaluated by spectrophotometer analysis at 260/280 nm density constant and gel electrophoresis technique. The extracted DNA was used for PCR amplification by using three different primers set as shown in Table 1.

The first pair of primers including set F: 5′-AGTTTCTGACCTATCAG-3′ and R: 5′-TTGCCTTA AACTTCCTTG-3′ produced 1098 bp amplified fragment of the *ssu* rRNA gene specific for genus *Theileria.* The final 50 µL PCR mixture contained 5 µL of template DNA, 5 µL of 10X PCR buffer (100 mM Tris–HCl (pH 9) 500 mM KCl, 1% Triton X-100, 5 µL of 50Mm $MgCl_2$, 6 µL of dNTPs, 4 µL of each primer (Penicon) at a conc. of 10 pmol/µL, 2 U of Taq DNA polymerase (Vivantis) and 20.5 µL of distilled water. PCR amplification was performed by using thermal cycler (Biorad, USA). The reaction was incubated for 5 min at 94°C to denature the genomic DNA, followed by 45 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. The reaction was finished with final extension step of 7 min at 72°C. For the documentation of different *Theileria* species, two primers sets Tssr 170F: 5′ TCGAGACCTTCGGGT-3′ and Tssr 670R: 5′-TCCGGACATTGTAAAACAAA-3′ for amplification 785 bp gene fragments of *T. lestoquardi* and 5′-GTGCCGCAAGTGAGTCA-3′ and 5′-GGACTGATG AGAAGACGATGAG-3′ were used for amplification

Table 1: Primers used for the detection of genus *Theileria, T. ovis* and *T. lestoquardi.*

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

of 520 bp gene fragments of *T. ovis* species (Altay et al., 2005). For the amplification of target DNA of both species of *Theileria*, the reaction volume was 25 µL comprising 4 µL of template DNA, 2.5 µL of 10 X PCR buffer (100 mM Tris–HCl (pH 9) 500 mM KCl, 1% Triton X-100), 2 µL of 25Mm $MgCl_2$, 2 µL of 250M each of the four dNTPs, 2 µL of each primer (Penicon) at 10 pmol/µL conc., 2 U of Taq polymerase DNA (Vivantis) and 10 µL of PCR water. For *Theileria lestoquardi* DNA amplification, PCR thermo profile was adjusted as 3 min at 94°C for denaturation of genomic DNA, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min followed by final extension step at 72°C for 7 min. Thermo profile for *T. ovis* was adjusted at 96°C for 3 min, for denaturing genomic DNA, followed by 5 cycles at 94°C for 30 sec, 56°C for 30 sec. and 72°C for 1 min. The 5 cycles were again followed by 30 cycles of 94 °C for 30 sec, at 54 °C for 30 sec and at 72 °C for 1 min. The PCR program was ended with a final extension step of 72°C of 7 min. PCR amplified products were run for separation on 1.5% agarose gel in TBE buffer and visualized by UV-illuminator. The 100-1500 bp ladder (Vivantis) was used as DNA marker. The positive controls of DNA of *T. ovis* and *T. lestoquardi* were obtained from Professor Urike Seitzer from Borstel, Germany.

Statistical analysis

MiniTab, USA (Version 16) was used for data analysis. For analysis age groups were classified i.e. ≤ year, 1-2 years and ≥ 3 years old. Based on size, the herds were divided into three classes i.e. 1-30, 31-60 and > 60 animals as well as three groups of herd composition i.e. only sheep, only goats or mixed herds. Various risk factors i.e. species of animal, gender, age and tick presence on the animal body were evaluated to determine the association of theileriosis. Fisher's exact and chi square test were used for statistical analysis. A *p* value <0.05 was considered as significant during statistical analysis.

RESULTS

The overall prevalence of theileriosis was recorded 12.4% and 36% through microscopic examination and PCR amplification, respectively. All samples positive in microscopy were confirmed through PCR amplification. Based on PCR, higher infection rate was found in Budhla sanatt (62%) and the lower reported in Kothay wala (17.5%). There was significant association (P<0.05) between different sampling sites and theileriosis as shown by chi square analysis described in Table 2.

Theileria infection was found significantly (P<0.05) higher in sheep 41.7% (n=65) compared to goats 26.6% (n=25) indicating sheep were more prone to this parasite as revealed by Fisher's exact test. From the 90 positive samples, 55.6% (n=50) were found positive for *T. lestoquardi* and 26.7% (n=24) for *T. ovis*; while mixed infection was detected in 17.8% (n=16) cases and the correlation among different *Theileria* spp. was significant (P<0.05) as depicted in Table 3. Similar trends of higher prevalence of *T. lestoquardi and T. ovis* were recorded 57% (n=37) and 24.6% (n= 16) in sheep and 52% (n=13) and 32% (n= 8) in goats, respectively.

The animal species, age and tick prevalence shown significant correlation (P<0.05) while gender revealed non-significant correlation (P>0.05) with theileriosis. *Theileria* spp. infection found higher in age group ≥ 3 years (43. 9%) followed by 1-2 years (35.3%) and lower in ≤ 1 year (21.7%) and significant association found among different age categories and theileriosis in overall small ruminants (Table 4). Ovine and caprine

Table 2: Results of microscopic screening and PCR amplification in sheep and goats under study.

Area	No. of samples	Test				
		Micr. Examination		PCR Examination		
		Positive	(%)	Positive	(%)	P value*
Piran Ghaib	30	4	(13.3)	9	(30.0)	
Budhla sanatt	35	6	(17.2)	22	(62.9)	
Kothay wala	40	5	(12.5)	7	(17.5)	
Muti tal	30	3	(10.0)	12	(40.0)	
Mouza Tatypur	65	10	(13.3)	25	(38.5)	
Mouza Azam Hans	50	3	(6.0)	15	(30.0)	0.000 [b]***
Total	250	31	(12.4)	90	(36.0)	0.000[a]***

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

Table 3: The frequency of different *Theileria* species based on PCR in sheep and goats

	N	Overall (%)	*T. ovis* (%)	*T. lestoquardi* (%)	Mixed	P value
Over all	250	90 (36)	24 (26.7)	50 (55.6)	16 (17.8)	0.00[a]
Sheep	156	65 (41.7)	16 (24.6)	37(57)	12 (20)	0.00[a]
Goats	94	25 (26.6)	8 (32)	13(52)	3 (12)	0.01[a]

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

theileriosis found higher in males 38.1% (n=21) as compared to females 35.4% (n=69) in overall small ruminants as stated in Table 4. Fisher Exact test revealed statistically significant correlation with tick infestation and theileriosis (39.5%) in overall small ruminants. When sheep and goat's data analyzed separately, age and tick infestation in goats and only tick infestation in sheep revealed positive correlation with theileriosis (P<0.05). Chi square results indicated that herd size and herd composition were significantly correlated with theileriosis (P<0.05). The smaller herd containing 1-30 animals/herd were more infected (60%) as compared to larger herds and the herds comprising only sheep were more infected with theileriosis (54.5%) than herds comprising only goats (33.3%) or mixed herd containing both sheep and goats (30.3%) (Table 5).

DISCUSSION

Epidemiological investigations on *Theileria* infection revealed that different *Theileria* species co-exist in a single host. These had also been reported to infect small ruminants resulting in 100% mortalities due to MOT (Naz et al., 2012). Few studies are available on ovine and caprine theileriosis in small ruminants from Punjab province, Pakistan and most of them are based on blood smears screening (Naz et al., 2012; Rehman et al., 2010; Irshad et al., 2010). Current study identified 12.4% *Theileria* spp. infection based on microscopy which is in accordance with the findings of Hassan et al. (2013) and Razmi and Yaghfoori (2013) who reported 14 and 18.6% infection rates, respectively through microscopic screening. *Theileria* species

Table 4: Relationship among *Theileria* piroplasms diagnosed by PCR and studied parameters of small ruminants.

Animal type	Parameters		No. of samples	Piroplasms Positive (%)	Piroplasms Negative (%)	P Value*
Sheep and goats (Combined)	Animal type	Sheep	156	65 (41.7)	91 (58.3)	0.02[a]*
		Goats	94	25 (26.6)	69 (73.4)	
	Sex	Male	55	21 (38.1)	34 (61.9)	
		Female	195	69 (35.4)	126 (64.6)	0.75[a]
	Age	≤ 1 year	69	15 (21.7)	54 (78.3)	
		≤ 2 year	51	18 (35.3)	33 (74.7)	
		≥ 3 year	130	57 (43.9)	73 (56.1)	0.00[b]*
	Ticks	Present	190	75 (39.5)	115 (61.5)	
		Absent	60	15 (25)	45 (75)	0.04[a]*
Sheep	Sex	Male	29	13 (45)	16 (55)	
		Female	127	52 (41)	75 (59)	0.84 [a]
	Age	≤ 1 year	39	20 (51.3)	19 (48.7)	
		1- 2 year	46	15 (32.6)	31 (67.4)	
		≥ 3 year	71	30 (46.4)	41 (54.6)	0.21[b]
	Ticks	Present	128	58 (45.3)	70 (50.8)	
		Absent	28	7 (25)	21 (75)	0.04 [a]*
	Breed	Lohi	20	10 (50)	10 (50)	
		Kajli	136	55 (40.4)	81 (59.6)	0.47 [a]
Goats	Sex	Male	26	6 (25)	20 (75)	
		Female	68	19 (25)	49 (75)	0.79 [a]
	Age	≤ 1 year	20	2 (10)	18	
		1- 2 year	25	5 (20)	20 (73.3)	
		≥ 3 year	49	18 (36.8)	31 (63.2)	0.03[b]*
	Ticks	Present	50	20 (40)	30 (60)	
		Absent	44	5 (11.3)	39 (88.7)	0.00 [a]*
	Breed	Nacchi	25	10 (40)	15 (60)	
		Beetal	50	9 (18)	41 (82)	
		Teddy	19	6 (31.6)	13 (68.4)	0.104[b]

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

Table 5: Relationship among *Theileria* piroplasms identified by PCR and different herd variables of sheep and goats.

	Parameters	No. of samples	Piroplasms positive	Piroplasms negative	P* Value
Size of herd	1- 30	25	15 (60)	10 (40)	
	31-60	59	31 (52.6)	28 (47.4)	0.00[b]*
	More than 60	166	44 (26.5)	122 (63.5)	
Herd composition	Sheep only	55	30 (54.5)	25 (45.5)	
	Goats only	30	10 (33.3)	20 (66.7)	
	Sheep and goats	165	50 (30. 3)	115 (69.7)	0.03[b]*

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

cannot be substantially detected and differentiated based on microscopic examination particularly in subclinical infections (Heidarpour et al., 2010). PCR, the specific diagnostic technique is needed to identify the role of different *Theileria* spp. causing ovine and caprine theileriosis (Aktas et al., 2005; Heidarpour et al., 2009). Overall 36% blood samples found positive through PCR using first primer set (Table 1) specific for genus *Theileria*. The microscopic examination is limited to diagnose *Theileria* infection in carrier animals due to low parsitemia compared to PCR technique (Aktas et al., 2007; Altay et al., 2007). The sensitivity of PCR is appraised that only one infected cell in 10^7 RBCs comparable to blood parasitemia level 0.00001% could be detected by using this methodology (Altay et al., 2007).

The infection of theileriosis detected significantly ($P<0.05$) higher in sheep (41.70%) compared to goats (26.6%) indicating that *Theileria* parasite prefer sheep over goats. The results are comparable with researchers around the globe who reported higher prevalence of theileriosis in sheep than goats as 28.9 and 4.1% respectively, as reported by Guo et al. (2002) in China and 27.6 and 13.1% in sheep than goats respectively, reported by Altay et al. (2007). Similar trend of higher infection rate of theileriosis was described by Irshad et al. (2010) and Iqbal et al. (2013) from Pakistan. The higher incidence of *Theileria* spp. infection in sheep compared to goats could be attributed to variation in skin nature of both animal species. The skin of goat is thin and more resistant to tick attachment than skin of sheep. Furthermore, due to sheep wool ticks easily entangled in skin and cause *Theileria* infection (Naz et al., 2012).

From 90 PCR positive samples *T. lestoquardi*, *T. ovis* and mixed infection was detected in 55.6, 26.7 and 17.8% respectively. Similar trends of higher infection of *T. lestoquardi* was reported by Heidarpour et al. (2009) who identified 53.3% *T. lestoquardi* infection as compared to 44.7% of *T. ovis* in small ruminants in Iran using nested PCR technique. Zaeemi et al. (2011) conducted a study on ovine theileriosis in Iran and found 54.8% and 40.2% prevalence of *T. lestoquardi* and *T. ovis* respectively. Contrary to our study Yaghfoori et al. (2013) detected higher infection (43%) of *T. ovis* compared to *T. lestoquardi* (3%) in studied population of small ruminants in Iran. The variation in incidence of theileriosis could be attributed to variation of geoclimatic conditions, tick infestation rate, breed of animals under study, management practices and techniques used for detection of ovine theileriosis. In current study, when sheep and goats data were analyzed separately, higher prevalence of *T. lestoquardi* as compared to that of *T. ovis* was reported both in sheep and goats and the association was also significant.

During current survey we also collected the data of every animal and herd to identify the association of many characteristics of studied animal and herd as risk factors in ovine and caprine theileriosis. Data regarding age of small ruminants indicated that animals with age group less than one year were more infected (51.5%) followed by age group older than three years (46. 4%) and lower in age group 1-2 years (32.6%) and significant association was found among age groups and theileriosis. The results are consistent with Iqbal et al. (2013) who observed higher infection in animals of age less than one year. Guo et al. (2002) also stated higher infection which resulted death in younger small ruminants in Ganan region, China. The higher incidence in younger animals could be due to less immunity level against piroplasmic infections. *Theileria* spp. infection found higher in males 38.1% (n=21) as compared to females 35.4% (n=69) in overall small ruminants but correlation was non-significant. The findings of the present survey indicated that gender of animal is very important factors regarding *Theileria* infection. When sheep and goats data were separately analyzed, similar trends of higher infection of theileriosis were reported in males (68.4%) compared to females (43.2%) in sheep while in goats the prevalence was same in both sexes (25%) but association was not significant. Current study is in covenant with findings of Iqbal et al. (2013) who stated that males were more infected (30.7%) compared to females i.e. 12.7% from Pakistan. The gender of the studied animals not affected the incident of theileriosis reported by Rehman et al. (2010) and Naz et al. (2012) during their study from Pakistan. The present results advocated that during pregnancy, lactation and heat reduced tick infestation in the females which results in lower infection of theileriosis.

The data of sheep and goats also revealed the significant association between theileriosis and tick infestation on animals. Ananda et al. (2009) found that the risk of haemoprotozoan diseases was greater after highest tick infestation in domestic ruminants. The results revealed significant association of theileriosis and tick infestation in overall small ruminants. Both in sheep and goats, tick infested animals had higher infection rate compared to animals without ticks and association was significant which is in accordance to Aktas et al. (2005) and Iqbal et al. (2013) who reported higher infection rate in animals infested with ticks. The results confirmed the role of ticks as vector for transmission of theileriosis. Similar trends of tick infestation and *Theileria* infection have been stated earlier by different authors around the globe (El-Azazy et al., 2001; Ahmed et al., 2002; Razmi et al., 2003; Bishop et al., 2004; Yin et al., 2007). The higher incidence of theileriosis in tick infested animals endorsed that

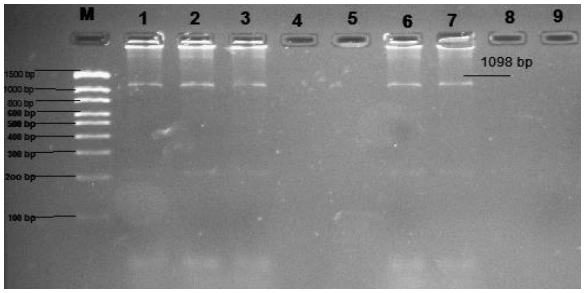

Fig. 2: Agarose gel electrophoresis of amplified PCR products obtained from *Theileria* species genomic DNA using *Theileria* Specific primers Lane, M, 100 -1500 bp DNA marker (Vivantus); Lane, 1, Positive control; 2 and 3:, Parasite positive blood sample; Lane 4, Negative control (distilled water); Lane 5, 8 and 9:, Parasite negative blood samples; Lane 6,7, Parasite positive blood samples.

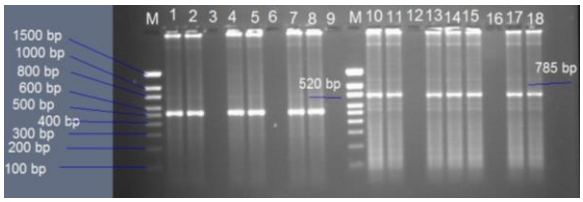

Fig. 3: Agarose gel electrophoresis of amplified PCR products obtained from *Theileria ovis and T. lestoquardi g*enomic DNA using *T. ovis* and *T. lestoquardi* primers. Lane: M, 100 -1500 bp DNA marker (Vivantus); Lane: 1, Positive control; 2, Parasite positive blood sample; 3, Negative control (distilled water); 4,5, 7,8, Parasite positive blood sample; Lane 6,9, Parasite negative blood sample; Lane: M, 100 -1500 bp DNA marker; Lane: 10, Positive control; 12, Negative control (distilled water); 11,13,14,15,17, 18, Parasite positive blood sample; Lane 16, Parasite negative blood sample.

piroplasmosis is related with tick activity (Yeruham et al., 1995). Breed was statistically correlated (P<0.05) with theileriosis in goats and higher infection reported in Naachi breed (40%) followed by Teddy (31.6%) and beetal breed (18%). However in sheep, breed association was found non-significant (P>0.05) and higher frequency of theileriosis was recorded in Lohi breed (50%) compared to Kajli breed (40%). These results are in line with Shahzad et al. (2013) who reported 24% theileriosis in Lohi sheep from Okara, Pakistan.

During present study the herd characteristics were studied in order to identify their role in spread of theileriosis and found that smaller herd containing 1-30 animals more infected (60%) compared to larger herds. The results are in agreement with Madiha et al. (2015) who found higher prevalence in herds containing animals 1-15 while contradicts to Durrani et al. (2012) who stated that incidence of theileriosis not affected by the herd size in small ruminants. Herd composition was significantly associated (P<0.05) with presence of theileriosis as indicated by Chi square analysis. The herds having sheep only were more infected with theileriosis (54.5%) compared with herds having either only goats (33.3%) or both sheep and goats (30.3%). The results are not in accordance with that of Saeed *et al.* (2015) who stated that mixed herds having both sheep and goats increased chances of theileriosis in small ruminants.

In conclusion, the results confirmed that microscopic examination has limited value for diagnosis of theileriosis especially with low parasitemia level in small ruminants. Sheep have been found more infected with *Theileria* spp. infection compared to goats. The prevalence of *T. lestoquardi* was higher as compared to *T. ovis* in small ruminants in the area under study. Further studies on MOT in Pakistan are required before embarking upon large schemes for small ruminants (sheep and goats) breeding.

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Authors' contributions

The field survey, the laboratory work i.e. microscopic examination, DNA extraction & PCR amplification assay was performed by MR. Blood samples were collected by MR, MZU. The results were compiled by MR and the manuscript was analyzed and reviewed by ZT. All authors read and approved the final manuscript.

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