Pak. j. sci. ind. res. Ser. B: biol. sci. 2017 **60**(1) 36-45

A Study on Molecular Diagnosis of *Theileria* Species Infection by PCR Amplification in Sheep and Goats in Multan, Pakistan

Muhammad Riaz* and Zahida Tasawar
Zoology Division, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan-60800, Pakistan

(received November 11, 2015; revised September 26, 2016; accepted October 17, 2016)

Abstract. In this present study polymerase chain reaction (PCR) assay was used for identification and differentiation of *Theileria* species infection in Multan, Pakistan. Out of 220 blood samples collected from sheep and goats, 31.2% (70/220) were found positive for *Theileria* species by PCR amplification compared to only 9.1% (20/220) on blood smear. *Theileria* infection was observed in 39.3% (57/145) of sheep and 18.6% (13/75) of goats sampled. The prevalence of *Theileria ovis* and *Theileria lestoquardi* in the 70 positive samples was found to be 57% (40/70) and 30% (21/70), respectively with only 12.3% (9/70) of blood samples having a mixed infection of both *T. ovis* and *T. lestoquardi.* Overall the prevalence of *T. ovis* infection was higher than *T. lestoquardi* in both sheep and goats. Herds with sheep only had significantly higher parasitic prevalence. The results confirm that PCR is direct, specific and sensitive tool for diagnosis of ovine theileriosis.

Keywords: sheep, goats, DNA extraction, PCR amplification, *Theileria ovis, Theileria lestoquardi*

Introduction

The role of livestock in the national economy of Pakistan is very important. They provide nutrition, energy and organic fertilizers for crops (Eyduran *et al.*, 2009; Ahmad *et al.*, 2007; Manan *et al.*, 2006). This role is most clearly appreciated in the rural community, where thirty million people are involved in subsistence production, with an average each family having 2-3 cattle/buffaloes and 5-6 small ruminants (sheep or goats). From just these few animals each family derives 30 and 40% of their annual income (Bakhsh *et al.*, 2014; Irshad *et al*., 2010; Duranni *et al.*, 2006). According to an economic survey conducted in 2013-14, livestock expansion can improve economic conditions and reduce poverty of rural inhabitants in Pakistan through the sale of surplus animals and their byproducts (Irshad *et al*., 2010). Small ruminants are also an important animal's protein source in the national diet (Nusrullah *et al.*, 2013). Pakistan after China and India, is the more reliant on small ruminants livestock with more than two dozen indigenous breeds of goats and sheep (Khan *et al.*, 2007). Despite the importance of small ruminants to the rural economy the production by these animals is not as efficient as it could be (Shahzad *et al.*, 2013). One of the main causes of this inefficiency is the tick borne haemoparasitic diseases. These diseases are responsible for high morbidity and mortality rates in the affected animals and negatively impacts the economic performance of rural sector in Pakistan (Aktas *et al.*, 2005). The climate of Pakistan is subtropical and highly suitable for the survival of ticks which transmit many ticks borne diseases including theileriosis and babesiosis in cattle and small ruminants (Gosh *et al.*, 2007).

Both domestic and wild small ruminants in tropical and subtropical regions of the world are infected with *Theileria* species. In Pakistan it is believed that two *Theileria* species, *Theileria ovis* and *Theileria lestoquardi* are responsible for ovine theileriosis. However, there are few reports on ovine theileriosis in Pakistan and further research is needed to understand its epidemiology.

Ovine theileriosis is caused by several *Theileria* species which are transmitted by ticks in wild and domestic ruminants (Irshad *et al.*, 2010; Heiderpour *et al.*, 2009). Among the *Theileria* associated with ovine theileriosis *Theileria lestoquardi*, *T. luwenshuni* and *T. uilenbergi* are considered extremely pathogenic (Schnittger *et al.*, 2000) whilst *T. separata*, *T. ovis* and *T. recondite* are less pathogenic or benign and cause subclinical infections in sheep and goats (Ahmad *et al.*, 2006). *Theileria lestoquardi* and *T. ovis* are the main *Theileria* species reported in small ruminants from Pakistan (Rehman *et al.*, 2012; Irshad *et al.*, 2010). *Theileria lestoquardi* (*T. leastquardi*) causes malignant ovine theileriosis (MOT) in sheep and goats (Heiderpour *et al*., 2009),

*Author for correspondence; E-mail: mriaz_sabri@yahoo.com

which is equivalent to tropical theileriosis in cattle (Tageldin *et al.*, 1992). The main signs of MOT are enlarged superficial lymph nodes, pale and icteric mucous membranes, diarrhoea or constipation, high fever, listlessness and emaciation (Naz *et al.*, 2012; Rehman *et al.*, 2010). *Theileria ovis* (*T. ovis*) is the cause of ovine theileriosis with fever, weight loss, reduce production and eventually death of infected animals are the commonly observed symptoms (Shahzad *et al.*, 2013; Durrani *et al.*, 2011).

Several reports on the basis of microscopic screening in Pakistan showed that *Theileria* infection ranges from 7.4-16.5% in sheep and 3.8-8.2% in goats (Shahzad *et al*., 2013; Durrani *et al*., 2012; Naz *et al.*, 2012; Durrani *et al*., 2011; Irshad *et al.*, 2010; Rehman *et al.,*2010). PCR amplification method revealed 35% prevalence of *Theileria* species in sheep during a study at Lahore (Durrani *et al.*, 2011); 6% incidence of *Theileria ovis* observed in sheep and goats from Punjab and Khyber Pukhtoonkhwa, Pakistan (Durrani *et al.*, 2012) and 37% prevalence of *T. ovis* in Lohi sheep breed during a study in Okara, Pakistan (Shahzad *et al.*, 2013). All these reports confirm that ovine theileriosis is endemic in Pakistan and potentially damaging livestock production. Giemsa stained thin blood smears examination and clinical symptoms based detection are the conventional methods used to study theileriosis in small ruminants (Telmadarriy *et al.*, 2012; Inci *et al.*, 2010). However, in some cases, recovered animals frequently sustain subclinical infections which are often not detectable through microscopy. Serological methods such as immunoflurescent antibody test (IFAT) are also used to determine subclinical infections (Sayin *et al.*, 2009). However, these methods are not reliable in recovered animals due to low parasitemia level in these animals (Aktas *et al.*, 2007; Schnittger *et al.*, 2004). In order to implement disease control programmes the exact status of *Theileria* infection in sheep and goat herds need to be established. The use of ploymerase chain reaction (PCR) amplification to diagnose *Theileria* species infection in epidemiological studies is therefore ideal since this technique is more sensitive than microscopy and allows detection of hemoprotozoans at very low parasitemia levels.

No previous epidemiological studies have been conducted on ovine theileriosis in tehsil Jalalpur, district Multan, Punjab, Pakistan. Hence, the aim of this study was to identify the *Theileria* species infecting sheep and goats in tehsil Jalalpur and estimate the prevalence of infection in a sample of sheep and goat flocks.

Materials and Methods

Animals and blood sampling. A total of 220 apparently healthy small ruminants (sheep = 145 and goats = 75) were selected from 12 herds using a multistage sampling method (Thrusfield, 2005) during 2013 from different localities of tehsil Jalalpur, district Multan. Sheep and goats of both sexes were sampled. Approximately 10% animals from each herd were bled from the jugular vein into 5 mL Eppendorf tubes containing few drops of 0.5 M EDTA as a preservative for DNA extraction. Data were collected from each individual sampled animal including species, sex, age, and breed and from each herds, data including its location, size and species of animal. The data was collected using a questionnaires completed by investigator during sample collection. The collected samples were transported to Institute of Pure and Applied Biology at Bahauddin Zakariya University, Multan, Pakistan in a cold pack and stored at -20 °C until DNA extraction. All the experiments were approved by the ethical committee of Institute of Pure and Applied Biology at Bahauddin Zakariya University, Multan, Pakistan.

Microscopic study of blood samples. Thin blood smears were prepared in the field as stated in the manual of Tick Fever Research Center, Australia (TFRC, 1996), dried in air, used methanol as fixative and stained with 10% giemsa solution. For removal of extra stain, the stained smears were rinsed with tap water for 2-3 times and then dried in air. Binocular microscope was used for examining blood smears with an oil immersion lens of 1000× magnification. *Theileria* parasites were recognized as described by Zajac and Canboy (2000).

DNA extraction. DNA was extracted from collected blood samples following inorganic method (Shaikh *et al.*, 2004). The quality of extracted DNA was assessed by spectrophotometer analysis at 260/280 nm density constant and gel electrophoresis method. The extracted DNA was either used for PCR amplification or stored at -20 °C.

PCR amplification. For PCR reaction three primer's sets (Table 1) were used to amplify different gene segments for genus *Theileria* and *Theileria* species during present study. The first primers set was used for the amplification 1098 base pair (bp) region of rRNA (ssu rRNA) gene fragment from *Theileria* genomic

Table 1. Primers used for the detection of genus *Theileria*, *T. ovis* and *T. lestoquardi* in sheep and goats in district Multan, Pakistan

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

DNA. For PCR amplification, the 50 µL reaction volume composed of 5 µL of template DNA, 5 µL of 10 × PCR buffer (100 mMTris–HCl (pH 9) 500 mMKCl, 1% Triton ×-100), 5 µL of 50 Mm $MgCl_2$, 6 µL of 250 M each of the four dNTPs, 4 µL of each primer (Penicon) at a concentration of 10 pmol/µL, 2 U of Taq DNA polymerase (Vivintas) and 20.5 µL of PCR water. PCR was carried out in a BIORAD touchdown thermocycler with following cycling programme for genus *Theleria* at 94 °C for 3 min., followed by 35 cycles, 1 min. for denaturation at 94 °C, 1 min for annealing at 60 °C and 7 min. for extension at 72 °C, with a final extension step of 7 min at 72 °C.

The second primer set was used for amplification 785 bp gene coding for the 30 kDa *T. lestoquardi* merozoite surface antigen. Total reaction volume used for PCR amplification was 25 µL comprising 4 µL of template DNA, 2.5 µL of 10 × PCR buffer (100 mMTris–HCl (pH 9) 500 mMKCl, 1% Triton ×-100), 2 µL of 25 Mm $MgCl_2$, 2 µL of 250 M each of the four dNTPs, 1.5 µL of each primer (Penicon) at 10 pmol/µL concentration, 2 U of Taq DNA (Vivantus) and 12.5 µL of PCR water. For *Theileria lestoquardi* Thermo profile was used 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. The third primers set was used for amplification 520 bp gene fragment of 18SS rRNA gene of *Theileria ovis* (Altay *et al*., 2005). The final reaction volume was 25 µL containing 3 µL of template DNA, 2.5 µL of 10 × PCR buffer (100 mMTris–HCl (pH 9) 500 mMKCl, 1% Triton ×-100), 2 µL of 25 Mm $MgCl_2$, 2 µL of 250 M each of the four dNTPs, 2 µL of each primer (Penicon) at a concentration of 10 pmol/µL, 2 U of Taq DNA (Vivantus) and 11 µL of PCR water. Cycling conditions for *Theileria ovis* was consisting of 3 min at 96 °C for 3 min followed by 5 cycles, 94 °C for 30s, at 56 °C for 30s and 72 °C for 1 min. The 5 cycles were again followed by 30 cycles. Each cycle consisted of 94 °C for 30s at 54 °C for 30s and at 72 °C for 1 min. The PCR programme was ended with a final extension step of at 72 °C for 7 min. 1.5% concentration agarose gel was used for separation of amplified PCR products by using gel electrophoresis. PCR products (5 µL) admixed with 6× loading dye (Vivantus) was loaded on gel with 100-1500 bp (Vivantus) ladder as molecular marker. After running the gel, UV trans illuminator was used for assessment of PCR product. Positive control genomic DNA of *Theileria ovis* and *T. Lestoquardi* was provided by Professor Urike Seitzer (VIIRC, Borstel, Germany).

Statistical analysis. Age of the animals was categorised into three groups <1 year, 1-2 years and >2 years old. Three categories of herd size were made i.e., 1-30, 31-60 and more than 60 animals/herd.

Also, herds were grouped based on composition i.e., herds with sheep only or goats only and mixed herds. The Chi square test or Fisher's exact test was used to test independence between variables and the odds ratio was used to measure the association between variables and risk of ovine theileriosis. MiniTab (Version 16) was used for statistical analysis.

Results and Discussion

The sampling sites, total number of samples collected and the number of *Theileria* positive samples along with prevalence details are presented in Table 2. Microscopy revealed 20 (9.1%) blood samples positive while PCR identified 70 (31.8%) blood samples positive. All samples positive with blood smear examination were also positive with PCR amplification. The highest prevalence of *Theileria* infection based on PCR diagnosis was observed in Shujatpur (54.1%) and the lowest was

Table 2. Sampling sites and total number of samples collected for prevalence of theileriosis in sheep and goats in district Multan, Pakistan

Area	No. of samples	Test				P*value
		Microscopic examination		PCR examination		
		Positive	(%)	Positive	(%)	
Basti Mehwala	21	3	14.3	11	52.4	
Shujatpur	37	4	10.8	20	54.1	
Baileawala	26	2	7.7	3	11.5	
Basti Matam	43	5	11.6	11	25.6	
Basti Boherai	52	2	3.8	14	26.9	
Chalk M 65	41	4	9.8	11	26.8	0.001[a]
Total	220	20	9.1	70	31.8	0.000[b]

[a] = Chi square test; [b] = Fisher exact test.

in Mozza Bailae wala (11.5%). The prevalence of *Theileria* infection between different sampling sites was found statistically significant ($p < 0.05$) (Table 2).

Two strains of *Theileria* species i.e., *T. ovis* and *T. lestoquardi* were identified. From the 70 *Theileria* sp. positive samples, 40 (57.2%) were identified as *T. ovis,* 21(30.0%) as *T. lestoquardi* while mixed infection was diagnosed in 9 (12.8%) blood samples (Table 3). No such PCR amplified fragment was detected in negative control samples.

In the present study, prevalence of theileriosis has been detected by PCR amplification in blood samples collected from different locations of tehsil Jalalpur, district Multan, Pakistan, along with risk factors associated with ovine theileriosis. It has been observed that 31.8% samples were positive for theileriosis through PCR. Based on microscopy, Rehman *et al.* (2010) found 16.5% *Theileria* infection in sheep from Okara, Pakistan while Naz *et al.* (2012) reported 11.2% positive blood samples in small ruminants during a study at Lahore, Pakistan. These results are similar to those based on microscopic examination in the present study. However, Irshad *et al.* (2010) recorded much lower *Theileria* infection rate (5%) in small ruminants at two livestock stations in Pakistan as compared to the present study. The difference in prevalence may be due to variation in the geoclimatic conditions and parasitemia level in the infected animals. Durrani *et al.* (2011) has reported 35% *Theileria* infection in small ruminants in Lahore district, Pakistan which was high as compared to present findings in tehsil Jalalpur, Multan district indicating that climatic conditions and animals distribution affects the prevalence of ovine theileriosis.

From 70 piroplasm positive samples including 57 sheep (39.3%) and 13 goats (17.3%) indicated that sheep are more prone to theileriosis than goats ($p < 0.05$). Naz *et al.* (2012) reported 13.9% and 8.2% prevalence of theileriosis in sheep and goats which is in agreement with the present study that sheep are more susceptible to *Theileria* sp. infection. Similar differences in species susceptibility between goats and sheep have also been reported in China (Guo *et al*., 2002) and Turkey (Altay *et al*., 2012). It is suggested that difference in species susceptibility could be due to goat skins being thinner

Table 3. Molecular prevalence of *Theileria* species in sheep and goats by PCR amplification in district Multan, Pakistan

Specie		*Theileria* sp. primers	*T. ovis*	*T. lestoquardi*	*T. ovis* + *T. lestoquardi*	Total *T. ovis*	Total *T. lestoquardi*
					N		
Sheep	145	57(39.3%)	36(24.8%)	13(8.9%)	8 (5.5%)	44(30.3%)	21(14.4%)
Goats	75	13(17.3%)	4 (5.3%)	8 (10.6)	1 (1.3%)	5 (6.6%)	6(12.0%)
Total	220	70(31.8%)	40(18.1%)	21(9.5%)	9 (4.0%)	49(22.2%)	27(12.2%)
		P=0.008[a]*	P=0.002[a]*	P=0.81[a]	P=0.17[a]	P=0.002[a]*	P=0.1[a]

[a] = fisher exact test; * = statistical significant.

and more easily resistant to attachment of ticks as compared to skin of sheep. Also due to presence of sheep wool, the ticks could easily become entangled in sheep skin and transmit tick borne diseases (Naz, 2012). The low prevalence in goats may also reflect the habitats where they graze which may be less suitable for tick survival or that the steep and inaccessible pastures means that goats have minimal contact with animals infested with ticks (Alessandra and Santo, 2012).

The same *Theileria* species found in this study have been reported by many researchers from different parts of the world (Yaghfoori *et al*., 2013; Heiderpour *et al*., 2010; Heiderpour *et al*., 2009) from Iran and Durrani *et al.* (2011) from Pakistan. The overall prevalence of *T. ovis* infection was higher (30.3%) as compared to *T. lestoquardi* (14.4%) by PCR testing during present study. Iqbal *et al.* (2013) reported 43.7% and 37.5% prevalence of *Theileria* species infection in sheep and goats, respectively during a study in Pakistan. The difference in infection rate may be due to difference of animal breeds under study, genetic resistance against *Theileria* species, difference of immunity level and abundance of tick vectors.

In sheep, the prevalence of *T. ovis* infection was found significantly ($p < 0.05$) higher (24.8%) as compared to *T. lestoquardi* (9 %) in the present study. Shahzad *et al.* (2013) recorded 37.0% infection of *T. ovis* in Lohi sheep in Okara district while Durrani *et al.* (2011) reported 27.50% infection of *T. ovis* through PCR amplification in sheep at Lahore district which was higher than the present study. But contrary to present study, Durrani *et al*. (2012) revealed lower prevalence (6.0%) of *T. ovis* in sheep by using the same primers in two provinces of Pakistan. The variation in prevalence rate may be due to difference of tick infestation, breed of animals, management practices and techniques used for diagnosis.

In goats, the incidence of *T. lestoquardi* was observed higher (10.7%) as compared to *T. ovis* (5.3%) but no significant association was found ($p > 0.05$) (Table 3). Similar reports of higher incidence of *T. lestoquardi* in goats was reported as 40.0% in China by Luo and Yin (1997); 20.8% in Iran by Zangana and Naqid (2011) and 78.3% in Nubian goats in Sudan by Hussein *et al.* (2013). The difference in prevalence of *T. lestoquardi* may be due to higher vector availability for *T. lestoquardi*

Table 4. *Theileria* parasites presence in sheep and goats along with studied parameters of animal characteristics in district Multan, Pakistan

Animal type	Parameters		No. of samples (%)	Piroplasms positive (%)	Piroplasms negative	P* Value
Sheep and goats (Combined)	Sex	Male	40	16 (40.0)	24 (60.0)	
		Female	180	54 (30.0)	126 (70.0)	0.26[a]
	Age	≤ 1 year	53	21 (39.6)	32 (60.4)	
		≤ 2 year	85	25 (29.4)	60 (70.6)	
		≤ 3 year	82	24 (29.2)	58 (70.8)	0.37[b]
Sheep	Sex	Male	21	11 (52.3)	10 (47.7)	
		Female	124	46 (37.0)	78 (63.0)	0.22[a]
	Age	≤ 1 year	33	16 (48.4)	17 (51.6)	
		≤ 2 year	59	20 (33.9)	39 (67.1)	
		≤ 3 year	53	21(39.6)	32 (60.4)	0.49[b]
	Breed	Lohi	132	55 (41.6)	87 (58.4)	
		Kajli	13	2 (15.3)	11 (84.7)	0.13[a]
Goats	Sex	Male	19	5 (26.3)	14 (73.7)	
		Female	56	8 (14.2)	48 (85.8)	0.29[a]
	Age	≤ 1 year	20	5 (25.0)	15 (75.0)	
		≤ 2 year	26	5 (19.2)	21 (80.8)	
		≤ 3 year	29	3 (10.3)	26 (89.7)	0.39[b]
	Breed	Nachi	53	9 (16.9)	44 (83.1)	
		Beetal	14	2 (14.2)	12 (85.8)	
		Teddy	8	2 (25.0)	6 (75.0)	0.89[b]

[a] = fisher exact test; [b] = Chi square test.

and resistance against *T. ovis* in that areas. There was no association of gender in small ruminants with *Theileria* parasitic presence. *Theileria* species infection was found higher in males (40.0%) as compared to females (30.0%) as stated in Table 4. This current study is consistent with findings of Iqbal *et al.* (2013) who reported that male had higher infection (30.7%) as compared to females i.e. (12.7%) during a study in Pakistan. The difference in prevalence of theileriosis between males and females might be endorsed due to influence of hormones in determining hemoprotozoans infections in small ruminants. The effect of age on theileriosis revealed that age group <1 year was more infected (39.6%) followed by age group 1-2 years (29.4%) and lower in >3 years (29.2%) in small ruminants (Table 4) but no significant association was found between different age groups on theileriosis. Iqbal *et al.* (2013) observed higher infection of theileriosis in small ruminants of age less than one year which is in accordance to present study. Similar results have been found by Guo *et al.* (2002), when higher incidence and death was found in young and adult small ruminants during a study in Ganan region, China. The higher prevalence of theileriosis in younger animals may be due to the fact that younger small ruminants have less immunity against this parasite. The breed of sheep and goats has no significant effect on the prevalence of theileriosis in small ruminants.

By comparing the odd ratio there was significant association ($p < 0.05$) of *T.ovis* infection between breeds of sheep whereas *T. lestoquardi* infection was found significant ($p < 0.05$) in overall gender of small ruminants (Table 5). Herd size showed no significant effect ($p > 0.05$) on prevalence of ovine theileriosis but smaller herd size 1-30 animals/herd were more infected (37.5%) as compared to larger herds (Table 6). Present results are in agreement to Durrani *et al.* (2012) who reported that prevalence of theileriosis was not affected by the herd size in small ruminants during a study from Punjab and Khyber Pukhtoonkhwa provinces in Pakistan. Herd composition was significantly associated ($p < 0.05$) with presence of theileriosis as herds composed of sheep only had higher parasitic prevalence (50.0%) than herds composed of goats only (32.3%) or having both sheep and goats kept together (23.7%) as stated in Table 6.

Table 5. Prevalence of *Theileria* species in sheep and goats and association of different parameters describing animal characteristics based on PCR in district Multan, Pakistan

Animals	Parameters		*Theileria ovis*			*Theileria lestoquardi*		
			Pos./exam.	OR(95%CI)	P value	Pos./exam.	or(95%CI)	P value
Sheep	Gender	Male	7/21			2/21		
		Female	29/124	1.63(0.60-4.44)	0.32	11/124	1.08(0.22-6.22)	0.92
	Breed	Lohi	0/13			2/13		
		Kajli	36/132	0.04(0.00-0.68)	0.02*	11/132	2.0(0.39-10.2)	0.40
	Age	<1 year	9/33			5/33		
		1-2 year	12/59	1.46(0.54-3.97)	0.44	3/59	3.3(0.73-14,9)	0.11
		>2 year	15/53	1.00(0.35-2.51)	0.91	5/53	1.7(0.45-6.44)	0.42
Goat	Gender	Male	2/19			3/19		
		Female	2/56	3.14 (0.44-24.2)	0.26	5/56	1.9(0.41-8.8)	0.40
	Breed	Teddy	0/8			1/8		
		Beetal	1/14	0.52(0.01-14.5)	0.70	3/14	0.52(0.04-6.04)	0.60
		Nachi	3/53	0.84(0.04-17.9)	0.91	4/53	1.7(0.17-18.0)	0.63
	Age	<1 year	1/20			2/20		
		1-2 year	1/26	1.2(0.07-21.5)	0.87	2/26	1.33(0.17-10.38)	0.78
		>2 year	2/29	1.4(0.11-16.6)	0.78	4/29	1.4(0.23-8.72)	0.69
Overall	Gender	Male	9/40			7/40		
		Female	31/180	1.4(0.60-3.22)	0.43	14/180	2.5(0.94-6.70)	0.05*
	Age	<1 year	10/53			5/53		
		1-2 year	13/85	1.2(0.52-3.18)	0.50	7/85	0.86(0.25-2.86)	0.80
		>2 year	17/82	0.88(0.37-2.12)	0.79	9/82	1.18(0.37-3.74)	0.77

OR = odd ratio.

Table 6. Association between presence of *Theileria* spp. infection identified by PCR in sheep and goats and the studied parameters describing animal and herd characteristics of Tehsil Jalalpur, Multan, Pakistan

Parameters		No. of samples	PCR positive	PCR negative	P* value
Size of herd	1-30	40	15 (37.5)	25 (62.5)	
	30-60	70	23 (32.8)	47 (68.2)	
	More than 60	110	32 (29.0)	68 (71.0)	0.82
Herd composition	Sheep herd only	60	30 (50.0)	30 (50.0)	
	Goats herd only	30	10 (33.3)	20 (64.7)	0.002*
	Sheep and goats mixed herd	130	30 (23.1)	95 (748.3)	

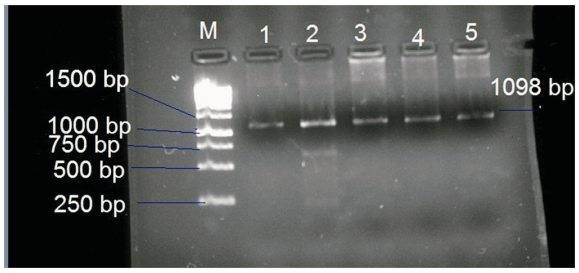

Fig. 1. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria* species genomic DNA using *Theileria* specific primers; Lane: M, 100-1500 bp DNA marker; Lane: 1, positive control; 2 to 5, parasite positive blood sample.

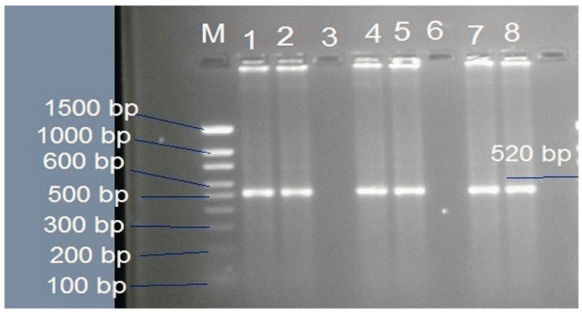

Fig. 2. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria ovis* genomic DNA using *Theileria ovis* primers; Lane: M, 100 bp DNA marker; Lane: 1, positive control; 2, parasite positive blood sample; 3, negative control (distilled water); 4, parasite positive blood sample; 5, parasite negative blood sample; 6, parasite positive blood sample; 7 to 8, parasite positive blood samples.

Earlier studies confirmed that PCR amplification is better technique as compared to microscopic examination to identify carrier small ruminants infected with theileriosis without any obvious signs of the disease (Sayin *et al.*, 2009; Razmi *et al.*, 2006; Aktas *et al.*, 2005). Lower prevalence of *Theileria* infection (9.1%) has been observed by microscopic screening than PCR (31.8%) which was in accordance with previous studies that microscopic screening is only effective during acute infection when parasitemia level is high but during low parasitemia level only PCR amplification is effective for diagnosis of infection. Durrani *et al.* (2011) in Lahore, Pakistan reported 35% prevalence of *Theileria* species infection in small ruminants by PCR amplification and 22% detected by blood smear screening while Durrani *et al.* (2012) found only 1% prevalence of theileriosis by microscopy as compared to 6% by PCR amplification

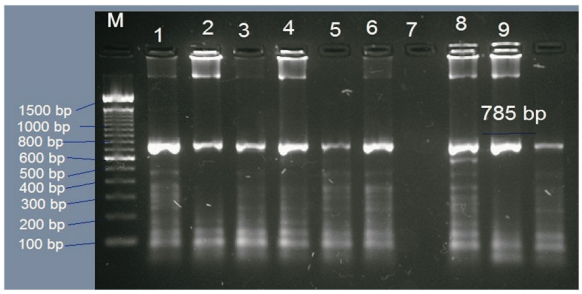

Fig. 3. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria lestoquardi* genomic DNA using *Theileria lestoquardi* primers. Lane: M, 100 bp DNA marker; Lane: 1, positive control; 2 to 6, parasite positive blood sample; 7, negative control (distilled water); 8 to 10, parasite positive blood sample.

in small ruminants during a study from two provinces of Pakistan indicating that PCR is better than microscopy. There are only few reports on prevalence of theileriosis in sheep and goats in Punjab, Pakistan but to our knowledge this is first report describing the status on *Theileria* species infection in tehsil Jalalpur, distric Multan, Pakistan. Presence of *Theileria* species infection indicates that ovine theileriosis is endemic in this region.

Conclusion

PCR amplification method for the detection of ovine *Theileria* infections is sensitive and specific for tracing *Theileria* infection in carrier animals and provides validated measures that are valuable for studying the epidemiology of theileriosis in small ruminants. The present study revealed that sheep as compared to goats are more susceptible to *Theileria* infection. *T. ovis* is more important cause of ovine theileriosis in small ruminants. The herds comprising only sheep have higher risk of *Theileria* infection and prevalence rate of theileriosis in small ruminants in tehsil Jalalpur, district Multan cannot be ignored. In order to enhance livestock production in the studied areas special attention should be given to the control of *Theileria* piroplasms in sheep and goats.

Acknowledgement. This manuscript is a part of Ph.D. dissertation of the corresponding author funded by the Higher Education Commission, Islamabad, Pakistan under the indigenous Ph.D. fellowship scheme (Phase IV). Grateful acknowledgements are also due to all the "Veterinary Assistants" for their help during the sample collection.

References

Ahmad, J., Alp, H., Askin, M., Seitzer, U. 2007. Current status of ticks in Asia. *Parasitology Research*, **102:** 159-162.

Ahmed, J.S., Luo, J., Schnittger, L., Seitzer, U., Jongejan, F., Yin, H. 2006. Phylogenetic position of small ruminant infecting piroplasms. *Annals of New York Academy of Science*, **1081:** 498-504.

Aktas, M., Altay, K., Dumanli, N. 2007. Molecular identification, genetic diversity and distribution of *Theileria* and *Babesia* species infecting small ruminants. *Veterinary Parasitology*, **147:** 161-165.

Aktas, M., Altay, K., Dumanli, N. 2005. Survey of *Theileria* parasites of sheep in eastern Turkey using polymerase chain reaction. *Small Ruminant Research*, **60:** 289-293.

Alessandra, T., Santo, C. 2012. Tick-borne diseases in sheep and goats: clinical and diagnostic aspects. *Small Ruminant Research*, **106:** S6-S11.

Allsopp, B.A., Baylis, H.A., Allsopp, M.T., Cavalier-Smith, T., Bishop, R. P., Carrington, D. M., Sohanpal, B., Spooner, P. 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. *Parasitology*, **107:** 157-165.

Altay, K., Dumanli, N., Aktas, M. 2012. A study on ovine tick-borne hemo-protozoan parasites (*Theileria* and *Babesia*) in the East Black Sea Region of Turkey. *Parasitology Research*, **111:** 149-153.

Altay, K., Aydin, M.F., Uluisik, U., Aktas, M., Dumanli, N. 2008. Use of multiplex PCR for the diagnosis of *Theileria annulata* and *Theileria buffeli. Turkiye Parazitolojii Dergisi*, **32:** 1-3.

Altay, K., Dumanli, N., Patricia, J., Holman, B., Aktas, M. 2005. Detection of *Theileria ovis* in naturally infected sheep by nested PCR. *Veterinary Parasitology*, **127:** 99-104.

Bakhsh, K., Maqsood, A., Hassan, S., Kamran, M.A. 2014. The economic role of livestock assets in cotton productivity in Punjab, Pakistan. *Pakistan Journal of Agriculture Science*, **4:** 1091-1094.

Durrani, S., Khan, Z., Khattack, R.M., Andleeb, M., Ali, M., Hameed, M., Taqddas, A., Faryal, M., Kiran, S., Anwar, H., Riaz, M., Sajjid, M., Sheikh, R.S., Ali, M., Iqbal, F. 2012. A comparison the presence of *Theileria ovis* by PCR amplification of their SSU RNA gene in small ruminants from two provinces of Pakistan. *Asian Pacific Journal of Tropical Disease*, **2:** 43-47.

Durrani, A.Z., Younus, M., Kamal, N., Mehmood, N., Shakoori, A.R. 2011. Prevalence of Ovine *Theileria* Species in district Lahore. *Pakistan Journal of Zoology*, **43:** 57-60.

Durrani, A.Z., Kamal, N., Khan, M.S. 2006. Incidence of theiloriosis and estimation of packed cell volume, Total erythrocyte count and Haemoglobin in buffaloes. *Journal of Animal and Plant Science*, **16:** 3-4.

Eyduran, E.K., Karakus, S. 2009. Usage of factor scores for determining relationships among body weight and body measurements. *Bulgarian Journal of Agriculture Science*, **15:** 374-378.

Gebrekidan, H., Hailu, A., Kassahun, A., Rohousova, I., Maia, C., Talmi-Frank, D., Warburg, A., Baneth, G. 2014. *Theileria* infection in domestic ruminants in northern Ethiopia. *Veterinary Parasitology*, **200:** 31-38.

Ghosh, S., Bansal, G.C., Gupta, C., Ray, D., Khan, M.Q., Irshad, H., Shahiduzzaman, M., Seitzer, U. Ahmed, J.S. 2007. Status of tick distribution in Bangladesh, India and Pakistan. *Parasitology Research*, **101:** 207-216.

Guo, S., Yuan, Z.,Wang, G.W.W., Denglu, M., Hongde, D. 2002. Epidemiology of ovine theileriosis in Ganan region, Gansu Province, China. *Parasitology Research*, **88:** 36-37.

Heidarpour, B.M., Khazraiinia, P., Haddadzadeh, H.R.B. 2010. Identification of *Theileria* species in sheep in the eastern half of Iran using nested PCR-RFLP and microscopic techniques. *Iranian Journal of Veterinary Research*, **11:** 262-265.

Heidarpour, B.M., Haddadzadeh, H.R., Kazemi, B., Khazraiinia, P., Bandehpour, Aktas, M. 2009. Molecular identification of ovine *Theileria* species by a new PCR RFLP method. *Veterinary Parasitology*, **161:** 171-177.

Inci, A., Ica, A., Yildirim, A., Duzlu, O. 2010. Identification of *Babesia* and *Theileria* species in small ruminants in Central Anatolia (Turkey) via reverse line blotting. *Turkish Journal of Veterinary and Animal Science*, **34:** 205-210.

Iqbal, F., Khattak, R.M., Ozubek, S., Khattak, M.N.K., Rasul, A., Aktas, M. 2013. Application of the reverse line blot assay for the molecular detection of *Theileria* and *Babesia* species in sheep and goats blood samples from Pakistan. *Iranian Journal of Parasitology*, **8:** 289-295.

Irshad, N., Qayyum, M., Hussain, M., Khan, Q.M. 2010. Prevalence of tick infestation and theileriosis in sheep and goats. *Pakistan Veterinary Journal*, **30:** 178-180.

Khan, M.S., Khan, M.A., Ahmad, S., Mahmood, S. 2007. Genetic resources and diversity in Pakistani sheep. *International Journal of Agriculture Biology*, **9:** 941-944.

Kirvar, E., BIlhan, T., Katzer, F., Wilkie, G., Hooshmand-Rad, P., Brown, D. 1998. Detection of *Theileria lestoquardi* (hirci) in ticks, sheep, and goats using polymerase chain reaction. *Annals of New York Academy of Science*, **849:** 52-62.

Manan, A., Khan, Z., Ahmad, Abdullah, B. 2006. Prevalence and identification of ixodid tick genera in frontier region Peshawar. *Journal of Agriculture and Biological Science*, **2:** 21-25.

Nagore, D., Josune Garcýa-Sanmartý, N., Ana, L., Garcýa-Perez, Ramon, Juste, A., Hurtado, N. 2004. Identification, genetic diversity and prevalence of *Theileria* and *Babesia* species in a sheep population from Northern Spain. *International Journal of Parasitology*, **34:** 1059-1067.

Nasrullah, M., Abdullah Baber, M.E., Jabbar, A., Javed, K., Nasir, M. 2013. Performance of Beetal goats and Lohi sheep under different feeding management systems. *Pakistan Journal of Zoology*, **45:** 107-111.

Naz, S., Maqbool, A., Ahmed, S., Ashraf, K., Ahmed, N., Saeed, K., Latif, M., Iqbal, J., Ali, Z., Shafi, K., Nagra, A. 2012. Prevalence of theleriosis in small ruminants in Lahore, Pakistan. *Journal of Veterinary and Animal Science*, **2:** 216-220.

Razmi, G.R., Eshrati, H., Rashtibaf, M. 2006. Prevalence of *Theileria* species infection in sheep in South Khorasan province. *Iranian Journal of Veterinary Parasitology*, **140:** 239-243.

Razmi, G.R., Hosseini, M., Aslani, M.R. 2003. Identification of tick vectors of ovine theileriosis in an endemic region of Iran. *Veterinary Parasitology*, **116:** 1-6.

Rehman, Z.U., Khan, M.S., Awais, M., Aleem, M., Shabbir, M.Z., Khan, J.A. 2010. Prevalence of theileriosis in sheep in Okara district, Pakistan. *Pakistan Journal of Zoology*, **42:** 639-643.

Sayin, F., Nalbantoglu, S., Bayram, B.A., Çkakmak, A., Karer, Z. 2009. Epidemiological studies on sheep and goat *Theilåria* infection. *Ankara Universitesi Veteriner Fakultesi Dergisi*, **56:** 127-129.

Schnittger, L., Yin, H., Qi, B., Gubbels, M.J., Beyer, D., Niemann, S., Jongejan, F., Ahmed, J.S. 2004. Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting. *Parasitology Research*, **92:** 189-196.

Schnittger, L., Yin, H., Jianxun, L., Ludwig, W., Shayan, P., Rahbari, S., Voss-Holtmann, A., Ahmed, J.S. 2000. Ribosomal small-subunit RNA gene sequence analysis of *Theileria lestoquardi* and a *Theileria* species highly pathogenic for small ruminants in China. *Parasitology Research*, **86:** 352-358.

Shahzad, W., Noor, H., Ahmad, M.D., Munir, R., Saghar, M.S., Mushtaq, M.H., Ahmad, N., Ghulam Akbar, G., Mehmood, F. 2013. Prevalence and molecular diagnosis of *Babesia ovis* and *Theileria ovis* in Lohi sheep at Livestock Experiment Station (LES), Bahadurnagar, Okara, Pakistan. *Iranian Journal of Parasitology*, **8:** 570-578.

Shaikh, R.S., Ramzan, K., Nazil, S., Khan, S.N., Riazuddin, S. 2004. A new locus for non syndromic

deafness DFNB maps to chromosomes 11 p 13-p 12. *American Journal of Medical Genetics Part A*, **138:** 392-395.

Tageldin, M.H., Zakia, A.M., Nagwa, E.G., ElSawi, S.A.S. 1992. An outbreak of theileriosis in sheep in Sudan. *Tropical Animal Health and Production,* **24:** 15-16.

Telmadarraiy, Z., Oshaghi, M.A., Hosseini Vasoukolaei, N., Yaghoobi Ershadi, M.R., Babamahmoudi, F., Mohtarami, F. 2012. First molecular detection of *Theileria ovis* in *Rhipicephalus sanguineus* ticks in Iran. *Asian Pacific Journal of Tropical Medicine*, **5:** 29-32.

Thrusfield, M. 2005. *Determinants of Disease.* 76 pp., 3rd edition. Blackwell Publishing Limited, UK.

Yaghfoori, S., Razmi, G.R., Heidarpour, M. 2013. Molecular detection of *Theileria* species in sheep and vector ticks in Fasa and Kazeroun areas, Fars Province. *Iranian Archive of Razi Institute*, **68:** 159-164.

Zaeemi, M., Haddadzadeh, H., Khazaiinia, P., Kazemi, R., Bandehpour, M. 2011. Identification of different *Theileria* species (*Theileria lestoquardi, Theileria ovis* and *Theileria anuluta*) in naturally infected sheep using nested PCR-RFLP. *Parasitology Research*, **108:** 837-843.

Zajac, A.M., Conboy, G.A. 2000. *Veterinary Clinical Parasitology*, 172 pp., 7th edition, Blackwell Publishing Limited, UK.

Zangana, I.K., Naqid, I.A. 2011. Prevalence of piroplasmosis (*Theileriosis* and *Babesiosis*) among goats in Duhok Governorate. AL-Anbar, Iraq. *Journal of Veterinary Science*, **4:** 50-57.