The Journal of Animal & Plant Sciences, 27(3): 2017, Page: 809-818
ISSN: 1018-7081

IDENTIFICATION OF THEILERIA SPECIES (*Theileria ovis* and *Theileria lestoquardi)* BY PCR IN APPEARENTLY HEALTHY SMALL RUMINANTS IN AND AROUND MULTAN, SOUTHERN PUNJAB, PAKISTAN

M. Riaz and Z. Tasawar

Zoology Division, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan 60800, Pakistan
Correspondence Author Email: mriaz_sabri@yahoo.com

ABSTRACT

Small ruminants (sheep and goats) in Asian agriculture system maintain a valuable biological and economical role. Theileriosis, a tick borne disease considered major deterrents in health and productive performance of small ruminants. Both domestic and wild small ruminants of tropical world are infected with *Theileria* species infection. Ticks and Tick-borne diseases caused annual loss of USS 7000million throughout the world.Two *Theileria* species *Theileria ovis* and *Theileria lestoquardi* are responsible for ovine theileriosis in Pakistan. Information about ovine theileriosis is limited and further research is needed to understand its epidemiology.In present study PCR assay was used to diagnose the prevalence of theileriosis and potential risk factors involvedin the spread of theileriosis in small ruminants from district Multan, Pakistan. Out of 300 collected blood samples of sheep and goats from different herds,PCR amplification revealed 17.0% samples positive or *Theileria* species infection while microscopy revealed only 3.7 % samples positive. Out of 51 positive samples,*T. ovis*and*T. lestoquardi* wasdiagnosed 60.8% and 35.2 % respectively while two animals (4.0%) had mixed infection of both *Theilria* species based on PCR. *Theileria*species infection observed significantly ($p < 0.05$)higherin sheep (24.66%) than in goats (9.33%).The prevalence of *T. ovis*and *T. lestoquardi*was higher in sheep (14% and 9.4%) compared to goats (5.3% and 4.0%) while mixed infection of both species was found (1.33%) only in blood samples collected from sheep. The herds consisting sheep only and age of the studied animalswas found statistical significant ($p < 0.05$) for the spread of ovine theileriosis.The results revealed that PCR is sensitive and specific molecular tool for the investigation of ovine theileriosis and can be used for screening of piroplasms in small ruminants.

Key words: Theileriosis, PCR, blood smears, sheep, goats, *Theileria lestoquardi, Theileria ovis.*

INTRODUCTION

*Theileria*belongs toorder Piroplasmida, are protozoans, causing *Theileria* spp. infection in wild and domestic ruminants (Heiderpour *et al*., 2010). In Asian agricultural system small ruminants sustain an important role in ecological niche and are of great economic importance (Irshad *et al.,* 2010). Low production and death due to parasitic infection are major causes of extensive economic losses in livestock sector (Youquan *et al.,* 2011). Piroplasmosis due to tick borne hemoprotozoan of genus *Theileria* imposes a key saddle in small ruminant production in tropical environments (Gebrekidan *et al.*, 2013; Altay *et al.*, 2007). Theileriosis is caused by six species of *Theileria*in small ruminants (sheep and goats) (Salih *et al.,* 2003; Altay *et al.,* 2008; Razmi and Yghfoori *et al.,* 2013). Three species of *Theileria, T. lestoquardi, T. luwenshuni* and *T. uilenbergi* are extremely pathogenic and main cause of higher mortality while *T. separata, T. ovis* and *T. recondite* are less pathogenic in small ruminants (Yin *et al.,* 2011; Schnittger *et al.,* 2000). Sheep and goats rearing sites have major risks of ovine theileriosis (Iqbal*et al.,* 2013).*T. lestoquardi* is the main causal agent of ovine malignant theileriosis inIndian subcontinent, West Asia and Mediterranean Basin (Altay *et al.,* 2007; Gao *et al*., 2002). Ovine theileriosis caused due to *T. lestoquardi, T. ovis*and *T. separate* had been reported by many researchers (Ahmad *et al.,* 2006), however, *T. lestoquardi* and *T. ovis* are the main causal agents of ovine theileriosis in Pakistan, reported from different parts of the country (Irshad *et al.,* 2010; Rehman *et al.,* 2010; Durrani *et al.,* 2011). During malignant ovine theileriosis, the host's leukocytes are infected by macroschizonts, where they multiply and invade lymphoid organs of host accompanied by severe clinical signs and may lead to death in preacute form of the disease (Sayin *et al.,* 2009; Rehman*et al.,* 2010). Susceptible animals usually die within 3-4 weeks as a result of widespread lymphocytolysis (Ahmed *et al.,* 2011).

The conventional methods for diagnosis of ovine theileriosis based on giemsa stained blood smears and identification through clinical symptoms, but these methods are consistent only in acute cases, but not applicable in carrier animals due to morphological resemblances of *Theileria* piroplasms (Inci *et al.,* 2010; Telmadarriy *et al.,* 2012). Subclinical infections can be diagnosed by serological methods in epidemiological studiesbut these methods are not reliable due to their

limitations. *Theileria* piroplasms may occasionally be present in the erythrocytes of longterm carriers where antibodies have tendency to disappear (Dumanli *et al.,* 2005). These animals may still be infected despite of negative serological test. Presently, the DNA based molecular diagnosis method;polymerase chain reaction (PCR) is moresensitive, specific and rapid for the identification of different *Theileria* piroplasms (Altay *et al.,* 2008; Schnittger *et al.,* 2004). Dehkordi *et al.,* (2012) reported the efficacy of PCR is higher (75%) than blood smear examination (25%) during a study in Iran.Bovine theileriosis had been extensively studied, however very limited information exists regarding the epidemiological aspects of ovine theileriosis and further investigations are required in Pakistan. The piroplasms information reported earlier is typically based on clinical signs and morphological assessment in Pakistan. The current study was designed to identify *Theileria* species infectionby PCR prevalentin small ruminants around district Multan, Southern Punjab, Pakistan.

MATERIALS AND METHODS

Blood Sampling: Blood was collected from jugular vein of 300 small ruminants, apparently healthy sheep (N = 150) and goats (N = 150) at random from various herds during 2013 located in Shujaabad Tehsil, Multan district, Pakistan. The collected 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 characteristics were collected during sampling. All the experiments were approved by the ethical committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Pakistan.

Blood smear examination: Thin blood smears prepared in field during sampling, and then air dried, fixed in methanol which used as preservative. For staining blood smears, 10% giemsa solution was used Durrani*et al.,* (2008). Binocular microscope was used to examine stained blood smears under oil immersion lens at 1000 xmagnification. The *Theileria* parasites were recognized as described by Zajac and Canboy, (2006).

DNA extraction: For DNA extraction from blood samples, inorganic method was used (Shaikh*et al.,*2005).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. TE-blood mixture was centrifuged at 13,000 rpm for 5 min. The supernatant was discarded 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 mMTrisHCl, 2 mM EDTA, 400 mMNaCl) and 20 µl of 10% sodium dodecyl sulfate and 1 µl Proteinase K and 1 µl Proteinase K for protein digestion in resuspended pellets and incubated overnight in a shaker at 37°C. The digested proteins were precipitated by adding 1 ml of 5 M NaCl, followed by vigorous shaking and chilling on ice for 15 min. The precipitated proteins were pelleted by centrifugation at 13,000 rpm for 5 min. and removed and supernatant was shifted to other new tubes. Chilled isopropanol in equal volume was added into the eppendorf containing supernatant; the DNA in the form of fiber was appeared in the eppendorf tubes and pelleted by centrifugation at 13,000 rpm for 5 min. After this the DNA pellet was washed with 70% ethanol and dissolved in TE buffer. The extracted DNA quantification was carried out by using 1.0%agarose gel.

PCR amplification: A touchdown thermocycler of 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 UrikeSeitzer (VIIRC, Borstel, Germany) provided positive control DNA of *T. ovis* and *T. lestoquardi.* Thermo profile for PCR of *Theileria* specific was containing at 94^{O}C for 3 min., followed by 35 cycles, 1 min. at 94^{O}C for denaturation , 1 min. at 60^{O}C for annealing and 1 min. at 72^{O}C for extension, with a final extension step of 72^{O}C for 7 min. Thermo profile for *T. lestoquardi* was comprising at 94^{O}C for 3 min., followed by 35 cycles at 94^{O}C for 1 min., 56^{O}C for 1 min. and 72^{O}C for 1 min. with a final extension step of 72^{O}C for 7 min. Thermo profile of *T. ovis* was consisting for 3 min. at 96^{O}C, was followed by 5 cycles, 30s at 94^{O}C, 30s at 56^{O}C and of 1 min. at 72^{O}C. These 5 cycles were followed by 30 cycles. Each cycle consisted of 30s at 94^{O}C, 30s at 54^{O}C and 1 min. at 72^{O}C. The PCR program was ended with a final extension step of 7 min. at 72^{O}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 were visualized on 1.5% gel stained with ethidium bromide and observed under UV illuminator for valuation and record.

Table 1. Primers used for the detection of *Theileria* and its species*T.ovis* and *T. lestoquardi* in small ruminants.

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)

Statistical analysis: For statistical analysis animals were divided into three age groups: ≤ 1 year, 1-2 years and > 2 years old. Herds were divided into three different size categories containing animals 1-30, 31-60 and > 60 animals. Also herds were divided due to their composition i.e. having sheep only, goats only or mixed containing both sheep and goats.Chi square and Fisher's exact test was used to statistically analyze the data regarding *Theileria* infection and possible risk factors responsible for the spread of *Theileria* species infection in studied population of sheep and goats. MiniTab (Version 16) used to statistically analyze the recorded data of small ruminants.

RESULTS

Microscopic examination of 300 stained blood smears revealed 3.7% (11/300) small ruminants were found infected with *Theileria*pirolpasms which were observed ribbon or curved shaped under binocular microscope (Figure 1).

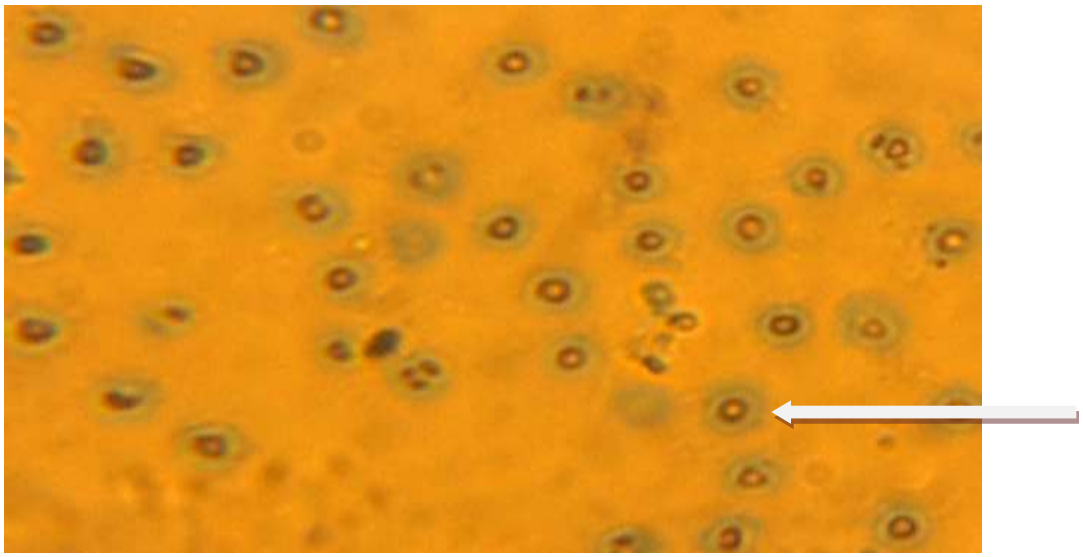

Fig. 1.Blood smears showing infection of *Theileria* pirolplasms in blood cells

To evaluate the true status of theileriosis in small ruminants, the blood samples were investigated by PCR amplification which revealed significantly higher prevalence 17% (51/300) of *Theileria* species by the amplification of a 1098-bp DNA fragment of 18SSU ribosomal r RNA (Fig. 2) compared to microscopic examination (3.7%). All the positive blood smears were confirmed by PCR. The prevalence of *Theileria*piroplasms in different sampling sites were significantly associated ($p < 0.05$) as indicated by chi square analysis ($p < 0.05$) shown in Table 2. The PCR amplification showed higher infection of theileriosis in MozaDairapur (30%) while lower in Bastijhakhar (7.50%). Table 3 indicated higher *Theileria* infection in sheep (24.66%) than in goats (9.33%) and the association was significant ($p < 0.05$) between theileriosis and animal species.

Table 2. Microscopic examination and PCR amplification results of sheep and goats obtained from Shujaabad Town, Multan district, Southern Punjab, Pakistan during 2013.

Area	No. of samples	Test				
		Microscopic examination		PCR Examination		
		Positive	%	Positive	%	P*value
Shah Purbha	30	1	3.33	8	26.66	
BastiJhakhar	80	1	1.13	6	7.5	
Dairapur	50	4	8	15	30	
MozaBasti dad	90	2	2.22	8	8.88	
GnawaKhoti	50	3	6	14	28	0.000^{b***}
Total	300	11	3.66	51	17.00	0.000^{a***}

a = Fisher's exact test b = Chi square test;
P < 0.01 = Significant (**); P < 0.001 = Highly significant (***).

Table 3.The incidence of *Theileria*species infection detected through PCR in sheep and goats from Shujaabad Town, Multan district, Punjab, Pakistanduring 2013.

Infection status	Identified *Theileria* species	No and frequency (%) of positive samples			P*value
		Sheep	Goats	Total	
Single infection	*Theileria* spp.	37(24.6)	14(9.4)	51 (17.0)	0.000^{a***}
	Theileria ovis	21(14.00)	8(5.33)	29(9.66)	0.01^{a**}
	Theileria lestoquardi	14(9.33)	6(4.00)	20 (6.66)	0.06^{a}
Mixed infection	*Theileria ovis + T. lestoquardi*	2(1.33)		2(0.66)	
Total samples		150	150	51	

a = Fisher's exact test
P < 0.01 = Significant (**); P < 0.001 = Highly significant (***).

During PCR, the amplified DNA fragment of 520 and 785 base pairs considered positive for *T. ovis* and *T. lestoquardi* respectively in the studied blood samples of small ruminants (Figure 3, 4).The infection rate of*T. ovis*was reported higher in sheep(14%) as compared to goats (5.3%)and *T. lestoquardi*was also higher (9.33%) in sheep than in goats (4%).The mixed infection of both species was found (1.3%) and recorded onlyin blood samplesof sheep.

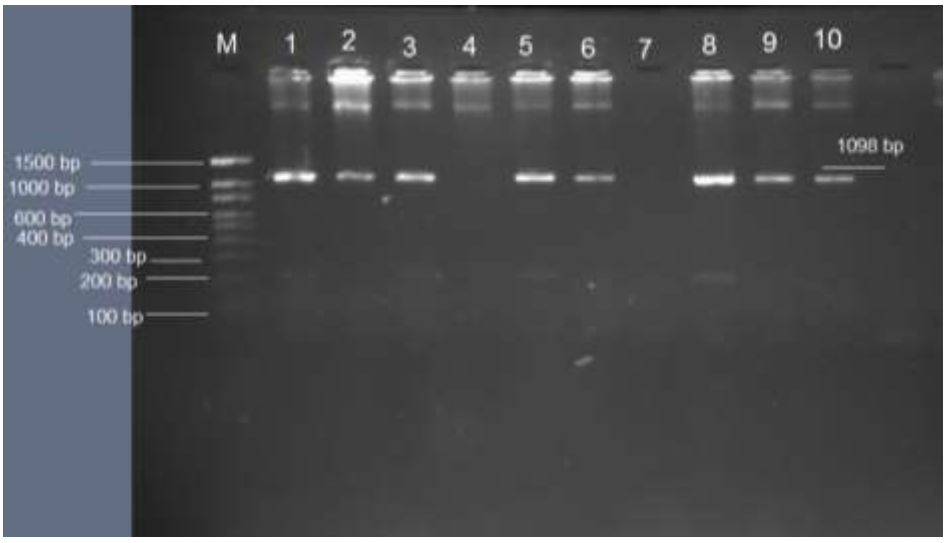

Fig. 2. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria* genomic DNA using *Theileria*specific primers. Lane M. DNA marker100-1500 bp; Lane 1. *Theileria* specific DNA positive control; 4 Negative control (Distilled water); 3.5.6.8.9.10 *Theileria* species DNA positive sample . Lane 4.7.11. *Theileria*species DNA negative sample

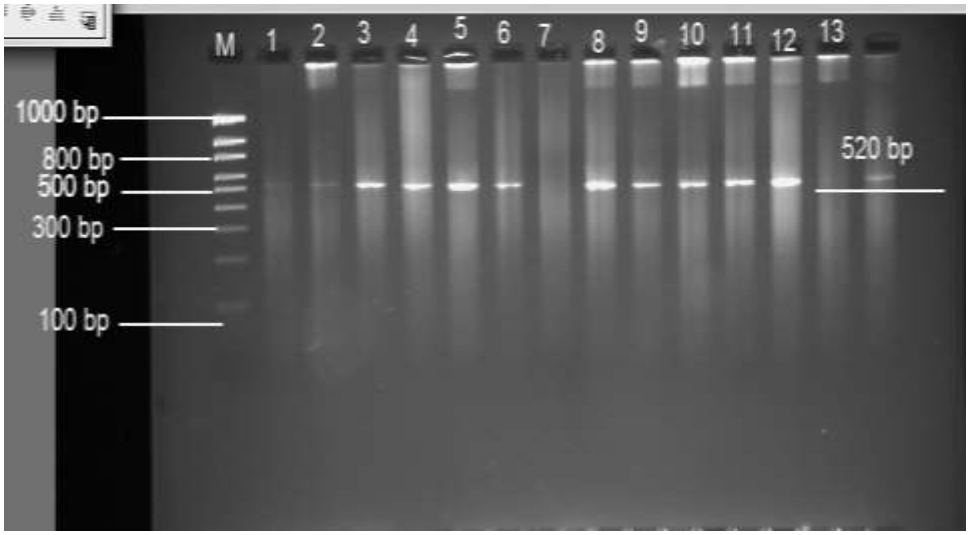

Fig. 3. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria ovis* genomic DNA using *Theileria ovis* primers.Lane: M, 100 bp DNA marker; Lane: 1, Negative control (distilled water); 2, Parasite Negative blood sample; 3, Positive control; 4, 5. 6. 8, 9, 10, 11, 12 Parasite positive blood sample; 7, 13, Parasite negative blood sample.

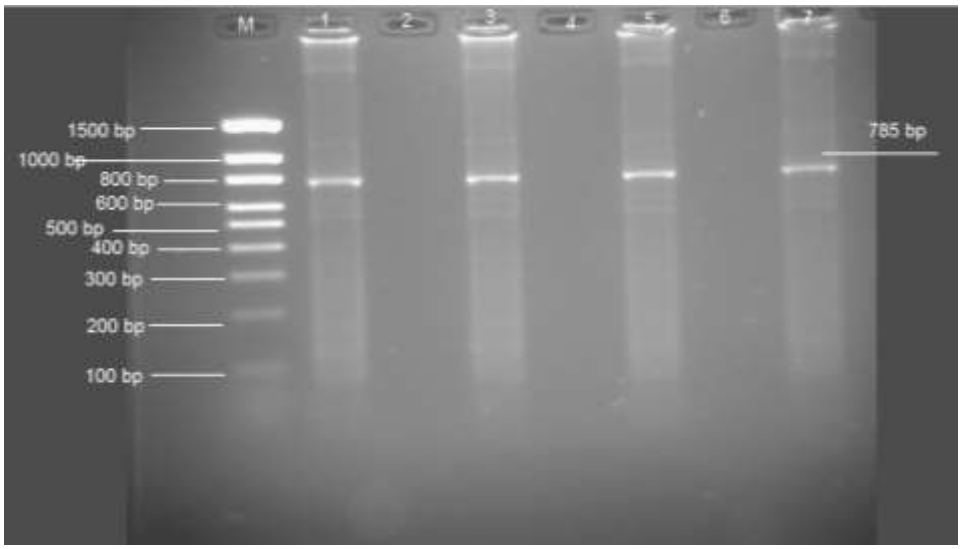

Fig.4. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria lestoquardi* genomic DNA using *Theilerialestoquardi* primers. Lane M.DNA marker of 100 bp. Lane 1. *Theileria lestoquardi* DNA positive control; Lane 2. Negative control (Distilled water); Lane 3, 5, 7, Parasite positive blood sample; 4, 5, Parasite negative blood sample.

For the evaluation of risk factors associated with theileriosis, the pooled data was analyzed. Fisher exact test revealed a highly significant association ($p < 0.05$) of the prevalence of *Theileria* spp. infection with animal species but non significant association with gender of the animal ($p > 0.05$). Chi square results indicated the prevalence of *Theileria* infection was significantly associated with age of the small ruminants ($p < 0.05$)as depicted in Table 4. The collected data of sheep and goats was also separately analyzed which reproduced that gender and breed of animals revealed non significant association ($p > 0.05$) with the prevalence of theileriosis while age of the goats was the only parameter which depicted a highly significantly association ($p < 0.05$) with theileriosis as indicated in Table 4. Chi square results indicated that herd size had non significantly associated($p > 0.05$) with *Theileria* infection while herd composition had significant association ($p < 0.05$) with the ovine theileriosis in small ruminants represented in Table5.*Theileria ovis*and *T. lestoquardi*infection further

confirmed through univariate analysis by odds ratio. Based on odds ratio, the *T. ovis* infection was reported higher than *T. lestoquardi*in malesand females of sheep. The significant association ($p < 0.05$) of *Theileria* species infection was identified in age group of > 2 years in goats as shown in Table 6. *Theileria ovis* infection was identified significant ($p < 0.05$) than*T. lestoquardi* ($p > 0.05$) in different age groups of sheep.

DISCUSSION

PCR amplification is more sensitive and consistent than microscopy to examine carrier animals with low parasitemia level (Aktas *et al.,* 2002; Altay *et al.,* 2007). The current study provides novel information about prevalence of different *Theileria* species in small ruminants in Multan, Southern Punjab, Pakistan. The present study revealed higher *Theileria* species infection by PCR amplification (17%) than blood smears examination (3.7%) in small ruminants. Statistical significant association ($p < 0.00$) perceived between PCR and blood smear screening tests. Durrani *et al.* (2011) reported 35% *Theileria* infection by PCR and 22% by microscopy in sheep from Lahore, Pakistan which was higher compared to present study. Altay *et al.* (2005) identified higher prevalence of theileriosis by PCR (54%) than microscopic screening (19.4%) in Turkey. Similarly Jalali *et al.* (2014) revealed 89.00% infection rate of *Theileria* by PCR in Iran. The difference in prevalence of *Theileria* infection might be validated due to geoclimatic conditions and breeds of animal under investigation (sheep and goats).

The prevalence of theileriosis in sheep and goats was outstanding which share the same pasture. Molecular examination indicated significantly ($p< 0.05$) higher prevalence of theileriosis in sheep (24.7%) than in goats (9.3%) during present study. Irshad *et al.* (2010) also reported 7.3% and 3.8% prevalence in sheep than goats respectively by microscopy in Okara and Islamabad, Pakistan but the prevalence rate was lowered compared to present study. The difference of prevalence might be endorsed that microscopic method cannot identified carrier animals due to low parasitemia level as compared to PCR. Altay *et al.,* (2011) reported *Theileria* species infection in sheep (28.90%) and in goats (4.10%) in Turkey. Iqbal *et al.* (2013) revealed the higher prevalence of theileriosis in sheep (23.00%) than in goats (11.00%) based on RLB during a study in Pakistan. The higher infection rate in sheep compared to goats might be due to difference of skin nature of sheep and goats. The skin of goats is thin and more resistant for attachment of ticks as compared to skin of sheep. The ticks could easily entangle in sheep skin and cause tick borne diseases due to sheep wool.

Theileria infection in small ruminants has been reported due to infection of *Theileria ovis* and *T.lestoquardi* by Heiderpour *et al.* (2009); Heiderpour *et al.*(2010); Yaghfoori *et al.* (2013) from Iran and Durrani *et al.* (2011) from Pakistan. During the present study, similar *Theileria* species reported but the prevalence of *T. ovis*was significantly ($p< 0.05$) higher 9.7% than *T. lestoquardi* 6.7% and mixed infection of both species recorded 0.7% in small ruminants. Significantly ($p< 0.05$) higher prevalence of *T. ovis*(14%) was recorded in sheep than in goats (5.3%) during present study. Similarlyhigher prevalence of*T. ovis*infection has been reported by many authors whoare in agreement to the present study. Sayin *et al.* (2009) revealed higher *T. ovis* infection 64.2% and 12.4% in sheep and goats in Turkey. Rjeibi*et al.* (2014) reported higher rate of *T. ovis* in sheep as compared to goats during a study in Africa. The difference of infection rate could be due to difference of climatic conditions and genetic resistance in different breeds against theileriosis.

In sheep, the infection rate of *T. ovis* was significantly higher (16.7%) compared to *T. lestoquardi* (9.4%) during current study. Altay *et al.* (2011) revealed higher prevalence of *T. ovis* (18.9%) compared to other *Theileria* species infection in Turkey. Yaghfoori *et al.* (2012) reported higher prevalence of *T. ovis* (43%) than *T. lestoquardi* (3%) in sheep in Iran which is in agreement to present study. Contrary to present study, Heiderpour *et al.* (2009) reported 87.5% *T. lestoquardi*and 12.5% *T. ovis* infection in Iran. 54.8% and 40.2% prevalence of *T. lestoquardi* and*T. ovis* respectively revealed in sheepin Iran by Zaeemi *et al.* (2011). Ingoats, the infection of *T.ovis* and*T. Lestoquardi* was found 5.4% and 4.0% respectively during the present study.

For assessment of animals and herds characteristics as possible risk factor for the spread of *Theileria* species infection in small ruminants, some basic data of every animal and herd was collected during the survey. The females had higher infection rate (17.9%) than that of males (9.4%).Our results indicated a non significant association ($p > 0.05$) of ovine theileriosis and gender in small ruminants. Similar trends of higher theileriosis recorded when only goat data was analyzed. In sheep, the infection rate of both *Theileria* species was same in both sexes and the association was non significant ($p > 0.05$). The computed odds ratio revealed that males are at higher risk of *T. ovis* infection than the females, while females are at higher risk of *T. lestoquardi* infection than males in sheep population. In goats, the odd ratios indicated, that the females are at higher risk of getting *T. ovis* and *T. lestoquardi* infection than males.

Age barrier in small ruminants (sheep and goats) against theileriosis was examined during the present study. *Theileria*species infection reported higher in age group greater than two years (23.5%) in sheep and goats than age group less than one year (9.7%) and 1-2 year

(13.4%) but statistically this association was non significant ($p > 0.05$). The present findings are in accordance with Razmi *et al.* (2003) reported non-significant correlation of *Theileria* positivity in different age groups of sheep population. Iqbal *et al.* (2013) found the *Theileria* species infection higher in small ruminants having age less than one year which is dissimilar to the present findings may be due to difference of breeds of small ruminants under investigation. The higher incidence of theileriosis in sheep and goats in older age animals compared to lower age animals might be endorsed due to inverse age resistance.

Table 4. Association between *Theileria*species infection identified through PCR in sheep and goats and studied parameters of animal characters from Shujaabad Town, Multan district, Southern Punjab, Pakistan during 2013.

Animal type	Parameters		No. of samples	Piroplasms Positive	Piroplasms Negative	P* Value
Sheep and goats	Animal type	Sheep	150	37	113	0.000[a***]
		Goats	150	14	136	
(Combined)	Sex	Male	32	3 (9.4%)	29 (90.6%)	
		Female	268	48 (17.9%)	220 (82.1%)	0.319[a]
	Age	≤ 1 year	41	3 (9.7%)	38 (90.3)	
		≤ 2 year	127	17 (13.4%)	109 (86.6%)	
		≥ 3 year	132	31 (23.5%)	101 (76.5%)	0.02[b**]
Sheep	Sex	Male	13	3(23.0%)	9(77.0%)	
		Female	137	34 (24.8%)	104(75.2%)	1.000[a]
	Age	≤ 1 year	17	3 (17.6%)	14 (82.4%)	
		≤ 2 year	60	17 (28.4%)	43 (71.6)	
		≥ 3 year	73	17 (23.3%)	56 (76.7%)	0.61[b]
	Breed	Lohi	135	35 (26.0%)	105(74.0%)	
		Kajli	15	2 (13.4%)	13 (86.6)	0.317[a]
Goats	Sex	Male	19	0	19 (100%)	
		Female	131	14 (10.7%)	116 (89.3%)	0.217[a]
	Age	≤ 1 year	25	-	25 (100.0%)	
		≤ 2 year	67	1(156%)	66(98.5%)	
		≥ 3 year	58	13 (22.5%)	45 (67.5%)	0.001[b***]
	Breed	Nacchi	90	10 (11.2%)	80(88.8%)	0.549[b]
		Beetal	35	3 (8.6%)	32 (93.4%)	
		Teddy	25	1 (4.0%)	24 (96.0%)	

a = Fisher's exact test b = Chi square test;
$P < 0.01$ = Significant (**); $P < 0.001$ = Highly significant (***).

Breed wise prevalence indicated significantly ($p< 0.05$)highest infection of *T. ovis* in Lohi than Kajli breed in sheep but in goats not significant association was found between goat breeds and theileriosis ($p> 0.05$). The difference of *Theileria* infection in different breeds of sheep and goats might be due to variance of genetic resistance against theileriosis, host management and ecological factors which are involved in the spread of ticks and tick borne diseases.

Herd size is an important risk factor in the spread of ovine theileriosis. In the present study, the highest prevalence of *Theileria* species infection was in herds having greater size (17.5%) than smaller size but chi square indicated non significant association ($p> 0.05$) of theileriosis and different herd size. The higher infection in larger size might be an indication that piroplasms spreads more rapidly in places where animals are kept in overcrowded conditions. The prevalence of *Theileria* species infection reported higher (28.6%) in herds having sheep only than in herds having either only goats (7.3%) or both sheep and goats (16.0%). Statistical analysis chi square revealed a significant association ($p< 0.05$) between *Theileria* spp. infection and herd composition.

For the confirmation of PCR specificity, the amplified DNA fragments of four selected samples were sequenced. To perform sequence comparison of *Theileria* species sequence and other rDNA sequences, were searched the NCBI Gen Bank® with BLAST by using the entire sequence as query.The amplified PCR product of*T. Ovis* and *T. lestoquardi i* was confirmed by Gene bank accession number K723613, Kp019206 for *T. ovis* and EF092916, EF092917 for *T. lestoquardi.* The detectable sequences confirmed 99% identity of registered sequence of *T. ovis* and *T. lestoquardi.*

Table 5.Association between *Theileria* species infection detected through PCR in sheep and goats and the studied parameters of herd characteristics from Shujaabad Town, Multan district, Southern Punjab, Pakistan during 2013.

	Parameters	No.of samples	Piroplasms positive	Piroplasms negative	P* Value
Size of herd	1- 30	5	0	5 (100.0%)	
	31-60	121	21 (17.5%)	100 (82.5%)	
	More than 60	174	30 (17.0%)	144 (83.0%)	0.595[a]
]Herd composition	Sheep only	63	18 (28.6%)	45 (71.4%)	
	Goats only	56	4 (7.2%)	52 (92.8%)	
	Sheep and goats	181	29 (16.0%)	152 (84.0%)	0.007[a***]

a = Chi square test;
P < 0.01 = Significant (**); P < 0.001 = Highly significant (***).

Table 6. Association of *Theileria* species infection detected through PCR in sheep and goats and studied parameters from Shujaabad Town, Multan district, Southern Punjab, Pakistan during 2013.

Species	Parameters		Theileria ovis			Theileria lestoquardi		
			Pos./exam	OR(95%CI)	P value	Pos./exam	OR(95%CI)	P value
Sheep	Gender	Male	2/13			1/12		
		Female	19/137	1.12 (0.23-5.49)	0.88	13/137	0.86(0.10-7.62)	0.89
	Breed	Lohi	19/135			14/135		
		Kajli	2/15	1,06 (0.22-5.06)	1.00	0/15	0.11(0.00-6.02)	0.29
	Age	<1 year	1/17			2/17		
		1-2 year	10/60	0.02(0.00-0.22)	0.00**	7/60	0.47 (0.04-5.1)	0.50
		>2 year	10/73	0.07(0.03-0.15)	0.00**	5/73	0.55(0.16-1.84)	0.33
Goat	Gender	Male	0/20			0/20		
		Female	7/131	NA	0.54	7/131	NA	0.54
	Breed	Nachi	6/90			4/90		
		Beetal	2/35	1.17(0.22-6.13)	0.84	1/35	0.93(0.12-8.35)	0.934
		Teddy	0/25	NA	0.39	1/25	1.58(0.17-16.7)	0.68
	Age	<1 year	0/25	NA		0/25		
		1-2 year	1/67		0.93	1/67	NA	0.93
		>2 year	7/58	9.05 (1.07-75.0)	0.04*	6/58	9.06(1.06-70)	0.04*
Overall	Gender	Male	2/32			1/32		
		Female	26/268	0.36 (0.08-1.6)	0.61	20/268	0.23 (0.03- 1.8)	0.16
	Age	<1 year	1/42			2/42		
		1-2 year	10/127	0.28(0.03-2.2)	0.23	8/127	0.74(0.15-3.06)	0.71
		>2 year	17/131	1.74(0.76-3.9)	0.19	11/131	2.2 (092-5.4)	0.07

P= value (P > 0.05 = Non significant; P < 0.01 = Significant (*); P < 0.001 = Highly significant (**).

Conclusions: The results of the present study have demonstrated that PCR is more specific and sensitive molecular tool for the detection of *Theileria* species infection in small ruminants. PCR diagnosis method is appropriate for tracing ovine theileriosis in carrier animals during epidemiological studies and designing control programs in small ruminants in endemic areas. Our study also indicated that microscopic examination of stained blood smears has limited value for differentiation between Theileria species especially with low parasitemia level. The present study publicizedthat small ruminants are very much susceptible to *Theileria* species infection. Molecular examination indicated that *T. ovis* has high prevalence when compared to *T. lestoquardi*. The infection rate of *Theileria ovis* and *T. lestoquardi* in small ruminants in Shujaabad, district Multan, Punjab, Pakistan cannot be ignored and special attention should be paid for the control of ovine theileriosis and tick infestation in order to enrich the small ruminants production.

Acknowledgements: This manuscript is a part of my Ph. D. dissertation funded by the Higher Education Commission, Islamabad, Pakistan under the indigenous Ph.D. fellowship scheme (Phase IV). Authors would like to thank all the veterinarians for theirhelp during sample collection.

Author's contribution: Survey, blood sample collection, the experimental work in the laboratory i.e., microscopic examination, DNA extraction & PCR amplification and the compilation of results were performed by Muhammad

Riaz while the manuscript was critically analyzed and reviewed by Prof. Dr. Zahida Tasawar.

Conflict of interest: It is declared that there is no conflict of interest between the authors.

REFERENCES

Ahmed, J.S., J. Luo., L. Schnittger., U. Seitzer., F. Jongejan, and H. Yin (2006). Phylogenetic position of small ruminant infecting piroplasms. Ann. N. Y. Aca. Sci. 1081: 498–504.

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

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

Altay, k.,N. Dumanli., J. Patricia., B. Holman, and M. Aktas (2005). Detection of *Theileria ovis* in naturally infected sheep by nested PCR. Vet. Parasit.127: 99-104.

Altay, K., M. Aktas, and N. Dumanli (2007). *Theileria*infections in small ruminants in the Eastand Southeast Anatolia. Turk. Paraz. Derg. 31: 268–271.

Altay, K., M. Aktas., N. Dumanli, andM. A. Fatih (2008). Evaluation of PCR and comparison with RLB for detection and differentiation of *Theileria* sp. MK and other *Theileria* and Babesia species of small ruminants. Parasit. Res. 103: 319-323.

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

Dehkordi, F.S., P. Parsaei, S. Saberian, S. Moshkelani, P. Hajshafiei, S. R. Hoseini, M. Babaei, and M. N. Ghorbani (2012). Prevalence study of Theileria annulata by comparison of four diagnostic techniques in southwest Iran. Bulgarian J. Veter. Med. 15: 123-130.

Durrani, A. Z., M. Ahmad, M. Ashraf, M. S. Khan, J. A. Khan, N. Kamal, and N. Mumtaz (2008). Prevalence of theileriosis in buffaloes and detection through blood smear examination and Polymerase chain reaction test in district Lahore. The J. Anim. Pl. Sci. 18: 59- 62

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

Dumanli, N., K. Altay,P. J. Holman, and M. Aktas (2005). Detection of *Theileria ovis* in naturally in-fected sheep by nested PCR. Vet. Parasitol. 127: 99–104.

Gao, Y.L., H.Yin., J. X. Luo., W. Q. Ouyang., H. M. Bao., G. Q. Guan., Q.C. Zhang., W.S. Lu, and M.A. Ma (2002). Development of an enzyme linked immunosorbent assay for the diagnosis of*Theileria* spp. infection in sheep. Parasit. Res. 88: 8-10.

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

Heidarpour, B.M., P. Khazraiinia, and H. R. B. Haddadzadeh (2010). Identification of *Theileria* species in sheep in the eastern half of Iran using nested PCR- RFLP and microscopic techniques. Iran. J. Vet. Res. 11: 262 -265.

Inci, A.,A. Ica., A, Yildirim, and O. Duzlu (2010). Identification of Bebsia and *Theileria* species in small ruminants in Central Anatolia (Turkey) via reverseline blotting. Turk. J. Vet. Anim. Sc. 34: 205-210.

Iqbal, F., R. M. Khattak., S. Ozubek., M. N. K. Khattak., A. Rasul, and M. Aktas (2013). Application of the Reverse Line Blot Assay for the molecular detection of *Theileria* and *Babesia* spp. in sheep and goats blood samples from Pakistan. Iran. J. Parasit.8: 289-295.

Irshad, N., M. Qayyum., M. Hussain, and Q. Khan (2010).Prevalence of tick infestation and theileriosis in sheep and goats. Pakistan Vet. J. 30:178-180.

Jalali, S.M.,Z. Khaki, B.D. Kazemi., S. Rahbari., P. Shayan., M. Bandehpour, and S.P.Yasini (2014).Molecular Detection and Identification of *Theileria* Species by PCR-RFLP Method in Sheep from Ahvaz, Southern Iran. Iran. J. Parasit. 9: 99-106.

Kirvar, E., T. BIlhan., F. Katzer., G. Wilkie., P. Hooshmand-Rad, and D. Brown (1998). Detection of *Theileria* lestoquardi (hirci) in ticks, sheep, and goats using polymerase chain reaction. Ann. N. Y. Acad. Sci. 849: 52-62.

Nagore, D., G. S. Josune., A. J. Ramon, and H. Ana (2004).Identification, genetic diversity and prevalence of *Theileria* and Babesia species in a sheep population from Northern Spain.Int. J. Parasit. 34: 1059-1067.

Razmi, G, and S. Yaghfoori (2013). Molecular surveillance of *Theileria ovis*, *Theileria* lestoquardi and *Theileria* annulata infection in

sheep and ixodid ticks in Iran. Onderste. J. Vet. Res. 80: 635-639.

Razmi, G. R., M. Hosseini, R.and R. Aslani (2003). Identification of tick vectors ov ovine theileriosis in an endemic region of Iran. Vet. Parasitol. 116: 1-6.

Rehman, Z. U., M. S. Khan., M. Awais., M. Aleem., M. Z. Shabbir,and J. A. Khan (2010). Prevalence of theileriosis in sheep in Okara district, Pakistan. Pakistan J. Zool. 42: 639-643.

Rjeibi, M. R., M. Gharbi.,M. Mhadhbi., W. Mabrouk.,B. Ayari., I. Nasfi.,M. Jedidi., L. Sassi.,M. Rekik, and M. A. Darghouth (2014). Prevalence of piroplasms in small ruminants in North-West Tunisia and the first genetic characterization of Babesia ovis in Africa. Parasite. 21: 23-30.

Salih, D. A., E. L. Huwsein., M. Layat,and M. K. Taha (2003). Survey of *Theileria* lestoquardi antibodies among Sudanese sheep.Parasitol. Res. 111:361-367.

Sayin, F., S. Nalbantoglu., B. A. Yukari., A. Çakmak, and Z. Karaer (2009). Epidemiological studies on sheep and goat *Theileria* infection. Ank. Uni. Vet. Fak.Derg. 56:127-129.

Schnittger, L., H. Yin., B. Qi., M. J. Gubbels., D. Beyer., S. Niemann., F. Jongejan, andJ. S. Ahmed (2004). Simultaneously detection and differentiation of *Theileria* and *Babesia* parasite infecting small ruminants by reverse line blotting. Parasit. Res. 92:189-196.

Shaikh, R. S., K. Ramzan., S. Nazil., S. N. Khan, and S. Riazuddin (2005). A new locus for non syndromic deafness DFNB maps to chromosomes 11p13-p12. Am. J. Med. Genet. Ana. 138: 392-395.

Telmadarraiy, Z., O. M. Ali., H. V. Nasibeh., Y.E. M. Reza., F. Babamahmoudi, andM. Fatemeh (2012).First molecular detection of *Theileria ovis* in *Rhipicephalus sanguineus*ticks in Iran. Asi. Pac. J. Trop. Med. 34: 29-32.

Yaghfoori, S., G. R. Razmi, and M. Heidarpour (2013). Molecular detection of *Theileria* spp.in sheep and vector ticks in Fasa and Kazeroun areas, Fars Province. Iran. Arch. RaziInstit. 68: 159-164.

Yin, H., L. Schnittger, J. Luo., U. Seitzer, and J. S. Ahmed (2011). Ovine Theileriosis in China: a new look at an old story. Parasit. Res. 101:191–195

Youquan, Li., G. Guiquan, M. Miling., L. Junlong., R. Qiaoyun., L. Jianxun, and Y. Hong (2011). *Theileria ovis* discovered in China. Exp. Parasit. 127: 304-307.

Zajac, A. M., and G. A. Conboy (2006).Veterinary clinical parasitology.7th Ed. Blackwell Publishing, Ames, Iowa, USA. pp: 52–55.