INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 9, No. 4, p. 61-71, 2016

RESEARCH PAPER OPEN ACCESS

Detection and discrimination of *Theileria* species infection by using PCR amplification in small ruminants in and around Multan, Pakistan

Muhammad Riaz*, ZahidaTasawar

Department of Zoology, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan , Pakistan

Key words: DNA Extraction, PCR, *Theileria lestoquardi*, *Theileria ovis*, Sheep and goats.

http://dx.doi.org/10.12692/ijb/9.4.61-71 Article published on October 11, 2016

Abstract

The present study was carried out to determine prevalence of *Theilera* species infection and risk factors involved in spread of theileriosis in sheep and goats in and around Multan, Southern Punjab, Pakistan. A total of 200 blood samples were collected from apparently healthy small ruminants comprising sheep (n=161) and goats (n=39) from different sampling sites of Multan, Pakistan, from randomly selected herds. Data on animal characteristics i.e. species, age, gender as well as herd characteristics was collected from through questionnaires. Microscopic examination revealed 7.0% blood samples positive while PCR DNA amplification revealed 22.5% samples positive for *Theileria* species infection which produced 1098 base pairs DNA fragment of 18S ssu rRNA. Sheep were found significantly ($P < 0.05$) more infected with theileriosis (17.5%) than goats i.e. (5.0%). 12.5% blood samples produced 785 base pairs DNA fragment considered positive for *T. lestoquardi* while 6.0% samples produced 520 base pairs DNA fragment considered positive for *T. ovis* . *Theileria lestoquardi* infection was found 11.8% and 15.3% while *T. ovis* infection was found 5.6% and 7.7% in sheep and goats respectively ($P < 0.05$). Mixed herds (containing both sheep and goats) and herds having animals with age group ≤ 1 year are at more risk of *Theileria* piroplasmic infection. The present study revealed PCR is more reliable diagnostic tool for theileriosis in small ruminants and can be used for screening of *Theileria infection* in order to improve the livestock production in Pakistan.

* **Corresponding Author:** Muhammad Riaz and ZahidaTasawar ✉ mriaz_sabri@yahoo.com

Introduction

Pakistan is an agricultural country, has heavy livestock population which play ample share in national economy of the country as it adds national and agricultural GDP (20.9 % and 56.3% respectively, GOP 2014-15). Approximately 50 million rural populations of the country rear small ruminants (sheep and goats) as an additional income source having 5-6 sheep/goats by a single family which helps them to drive 30 - 40% of their income (Durrani *et al.* 2012). Current livestock-population (2014-2015) of the country includes 35.6 million buffaloes, 41.2 million cattle, 29.4 million sheep, 68.4 million goats and 1.0 million camels (Anonymous, 2015). These produce 52.632million tones of milk, 1.951 million tons of beef, 0.671 million tons of mutton and 44.6 thousand tons of wool and 77.04 million skins and hides (Anonymous, 2015). Small ruminants have high adoptability to adjust in extreme climatic conditions. Pakistan, next to China and India is the dominant goat generating country throughout the world. The outcome of raising such a heavy livestock population in Pakistan is not as much as it should be (Shahzad *et al.* 2013). The main constraint to livestock production in Pakistan is parasitic infestation (both ectoparasites and endoparasites) (Sajid *et al.,* 2008). Ticks due to rigorous irritation and toxicosis are documented as tarnished threats to livestock production (Niyonzema and Kiltz, 1986). Due to transmition of wide range pathogenic agents in vertebrate hosts ranging from viruses to helminthes, ticks are potent source of economic losses in livestock all over the world (Fuente *et al.,* 2008). Tropical and subtropical part of the world, like Pakistan, due to encouraging climatic conditions for tick growth, is more prevalent to tick-borne diseases (Gosh *et al.*2007). In Pakistan *ixodid* ticks are involved in the spread of various hemoprotozoans diseases to wild and domestic ruminants including theileriosis and bebesiosis. (Durrani *et al.,* 2012.).

Theileriosis in small ruminants, causes clinical illness and high mortalities, is economically important disease of Southern Europe, Middle East, China and Indian subcontinent (Uilenberg, 1997).

Ovine theileriosis is widely distributed in areas where sheep and goats are reared. *Theileria lestoquardi, T. luwenshuni* and *T. uilenbergi* are deliberated extremely pathogenic while *T. separata, T. ovis* and *T. recondite* are in general less pathogenic species in small ruminants (Yin *et al.,* 2007). During acute infection of malignant ovine theileriosis, small ruminants have the same symptoms as present in cattle during tropical theileriosis (El Hussein *et al.,* 1993).*Theileria lestoquardi* causes fever, emaciation, lymphadenopathy, wasting, malaise anorexia, rapid heartbeat, dyspnea, listlessness, anemia, icterus, jaundice, pyrexia, intermittent diarrhea or constipation, weakness, termination of rumination and transitory haemoglobinurea during malignant ovine theileriosis (Sayin*et al.,* 2009). During theileriosis, obvious drop in WBCs and RBCs counts is observed which result due to leukopenia which persists for several days, the reduced value of PCV is also reported in the infected animals. Recovery from ovine theileriosis is often slow and sub-acute signs of theileriosis, fever and anemia are repeated (Woldehiwet, 2007).

The diagnosis of theileriosis based on blood smear screening method and presence of clinical symptoms which are reliable only in acute cases but deficient in carrier animals due to morphological resemblance among piroplasmosis (Telmadarriy *et al.,* 2012). In some cases, the recovered animals sustain subclinical infections which are identified by serological methods such as IFAT and ELISA (Leemans *et al.,* 1999)but these methods are not impeccable due to low parasitemia level and cross-reactions of related pathogens (Altay *et al.,* 2012). Therefore molecular techniques such as PCR amplification methods are used for detection of hemoparasites and this technique offers higher sensitivity and specificity than common microscopic method (Altay *et al.,* 2008). The objectives of present study were to diagnose the prevalence of *Theileria* species infection in blood from sheep and goats in and around Multan district, Southern Punjab, by polymerase chain reaction (PCR), compare this with conventional microscopic method and studied the possible risk factors liable for the spread of theileriosis in Southern Punjab, Pakistan.

Materials and methods

Field study

The study was carried out in Multan district which is located in south of the Punjab Province, Pakistan. Multan district is situated between 29'-22'north latitude and 71'-4' east longitude with an extreme temperature 49°C during summer and 1°C in winter with an average precipitation of 127 mm. Sheep and goats husbandry is one of the most economically important occupations in this area. Mostly small ruminants (sheep and goats) are kept together and the average herd size ranges between 30-100 animals.

Blood samples

Blood samples were collected from 200 from apparently healthy small ruminants (161 sheep and 39 goats) from randomly selected herds located in important livestock production regions of five sampling sites of shershah, district Multan, Pakistan during 2013. 10% animals from each herd was selected for blood collection from jugular vein and immediately preserved in 5 ml eppendorf tubes containing few drops of 0.5 M EDTA as a preservative for DNA extraction. Data was collected regarding animal characteristic including species, age, gender and tick presence and herd characteristics comprising location, size and herd composition was collected through questionnaires completed during sampling in order to determine the risk factors involved in the spread of *Theileria* species infection. All the experiments were approved by the ethical committee of Institute of Pure and Applied Biology at Bahauddin Zakariya University Multan, Pakistan.

Blood smears

Blood smears (thin and thick) were prepared in field as stated by Tick Fever Research Center (TFRC, 1996). The smears were air dried, fixed in absolute methanol in field and then brought to the laboratory of Institute of Pure and Applied Biology, Bahhudin Zakaryia University, Multan, for further processing. Afterwards, they were stained by 5% Giemsa (acidity of 7.2) for 45 min and then, analyzed by light microscopy regarding piroplasmic forms. Morphological characteristics of *Theileria* were identified according to key described by (Soulsby, 1982; Urquhart *et al.,* 1988).

DNA isolation

DNA extraction was carried out by following inorganicmethod previously described by Sheikh *et al.* (2005). 5µl of extracted DNA was analyzed on a 0.8% agarose gel at 80 V for 45 min and then visualized under UV illuminator after staining with ethidiumbromide. The extracted DNA was stored at –20 °C for PCR amplification.

Oligonucleotide design and PCR amplification

For the amplification of 18SSU r RNA gene of *Theileria* species three primers set was used during the present study. The first primers was [fwd5'-AGTTTCTGACCTATCAG -3' and rev 5'-TTGCCTTAAACTTCCTTG-3'], formerly used for production of fragment of a small subunit ribosomal RNA for genus *Theileria* by Allsopp *et al.* (1993). A total of 50 µl reaction volume was used for *Theileria* specific primers. The reaction volume comprised 5 µl of template DNA, 5 µl of 10 X PCR buffer (100 mMTris–HCl (pH 9), 500 mMKCl, 1% Triton X-100), 10 pM of primers, 250M each of the four DNA bases and 2 U *Taq*. DNA polymerase (Vivantus, U.K.). The thermoprofile used for amplification was containing at 94°C for 3 min. for initial denaturation, followed by 35 cycles at 94°C for 1 min. for denaturation, 60°C for 1 min. for annealing and 72°C for 1 min. for extention with a final extension step of 72°C for 7 min.

The second primers set was [fwd5'-GTGCCGCAAGTGAGTCA-3' and rev 5'-GGACTGATGAGAAGACGATGAG-3'] used for gene coding for the 30 kDa *T. lestoquardi* merozoite surface antigen by Kirvar *et al.*(1998). The third set of primers was [fwd5-TCGAGACCTTCGGGT-3' and rev 5'-TCCGGACATTGTAAAACAAA-3'] was used for *T. ovis* (Altay *et al.*, 2005). The PCR For *T. ovis* and *T. lestoquardi* was performed in a reaction volume of 25 µl containing 3 µl of template DNA, 3 µl of 10 X PCR buffer (100 mMTris–HCl (pH 9), 500 mMKCl, 1% Triton X-100), the primers (10 pM of each as described in Table 1), 250M each of the four deoxynucleotide triphosphates and 2 U *Taq*. DNA polymerase.

The rmoprofile for *Theileria lestoquardi* was consisting 35 cycles, 94°C for 3 min. for initial denaturation, each cycle involved denaturation at 94°C for 1 min., annealing at 56°C for 1 min. and extension at 72°C for 1 min. with a final extension step of 72°C for 7 min. Cycling conditions for *Theileria* ovis were 3 min. at 96°C was followed by 5 cycles, 30 sec. at 94°C, 30 sec. at 56°C and of 1 min. at 72°C. These 5 cycles were followed by 30 cycles. Each cycle consisted of 30 sec. at 94°C, 30 sec. at 54°C and 1 min. at 72°C with a final extension step of 7 min. at 72°C. The positive DNA samples of *T. lestoquardi* and *T. ovis* was provided by Urike Seitzer (Veterinary Infectiology and Immunology Research Center, Borstel, Germany). The polymerase chain reaction products were separated on 1.5% agarose gel. 5µl of each PCR product and 1.5µl loading buffer was loaded on agarose gel. Following gel electrophoresis the detection of 1098bp fragment was considered positive for *Theileria* species infection. 520 bp amplified DNA fragment and 785 bp amplified DNA fragment was considered positive for *T. ovis* and *T. lestoquardi* respectively.

Statistical analysis

For statistical analysis three age categories of animals were made i.e. ≤ 1 years, 1- 2 years and ≥ 3 years old. Herds were also grouped into three categories i.e., 1-30, 31-60 and more than 60 animals as well as three categories of herds composition were made i.e., only sheep, only goats or mixed herds. Fisher's exact test (for 2 ×2 tables) was used to evaluate the association of theileriosis and various parameters i. e. species, gender, breed and presence of ticks were studied of the animal. The association of *Theileria* species prevalence and different age groups and herd composition was determined by one way analysis of variance (ANOVA). MiniTab, USA (Version 16) was used for statistical analysis.

Results

Theileria species infection was recorded significantly higher through PCR (22.5%; n=45) as compared to blood smear screening method (7.0%; n=14). The higher infection rate was found in Ambwala (40.0%) and the lowest was found in Sultanpur (10.0%) as shown in Table 1.

Table 1. Thin blood smears and PCR DNA amplification results from Sher Shah Town, Multan District, Punjab, Pakistan. 2013.

Study Area	N	Mic. Positive	(%)	PCR Positive	(%)	P * value
Gagarkachr	46	4	8.6	12	26.0	
Sultan pur	50	3	6.0	5	10.0	
Anmbala	25	1	4.0	10	40.0	
Kohmaqbolwala	24	2	16.6	5	20.8	ANOVA
Bastikhuda dad	55	4	7.2	13	23.6	0.05[a]
Total	200	14	7.0	45	22.5	0.00 [b]

a= ANOVA

b= Fisher Exact Test.

Table 2. Distribution and frequency of *Theileria* by PCR and association of presence of infection in sheep and goats from Sher shah Town, Multan District, Punjab, Pakistan.

Infection ststus	Theileria species	Piroplasms Positive (%)		
		Sheep	Goats	Overall
Single infection	*Theileria* spp.	35 (17.5)	10 (5.0)	45 (22.5)
	Theileria ovis	9 (5.6)	3 (7.7)	12 (6.0)
	Theileria lestoquardi	19 (11.8)	6 (15.3)	25 (12.5)
Mixed infection	*T. ovis* + *T. lestoquardi*	7 (4.34)	1 (2.5)	8 (4.0)
Total samples	200	161	39	

ANOVA results indicated significant association of theileriosis and sampling sites of the studied region ($p < 0.05$). The *Theileria* infection in sheep was found 17.5% in sheep while in goats as 5.0% indicating that sheep are prone to *Theileria* species infection. The prevalence of *Theileria* species infection in small ruminants retrieved through PCR amplification represented in Table 2. *Theileria lestoquardi* infection was (12.5%) most prevalent followed by *Theileria ovis* (6.0%), 4.0% blood samples revealed mixed infection of both species. The prevalence of *T. lestoquardi* was found 11.8% and 15.3% While those of *Theileria ovis* was found 5.6% and 7.7% in sheep and goats respectively.

Table 3. *Theileria* parasites presence in sheep and goats along with studied parameters of animals characteristics in Sher Shah, Multan District, Punjab, Pakistan.

Animal Type	Parameters		N	Piroplasms positive	Piroplasms negative	P * Value
Sheep and goats (Mix)	Sex	Male	55	15 (27.2)	55 (22.8)	
		Female	145	30 (20.6)	152 (79.4)	0.657[a]
	Age	≤ 1 year	78	18 (25.6)	76 (24.4)	ANOVA
		≤ 2 year	80	18 (23.0)	81 (27.0)	
		≥ 3 year	42	9 (21.4)	50 (78.6)	0.769 [a]
	Ticks	Present	40	15 (37.5)	25 (62.5)	
		Absent	160	30 (18.7)	130 (82.3)	0.01 [a]
Sheep	Sex	Male	44	13 (29.5)	21 (79.5)	
		Female	117	22 (18.8)	88 (81.2)	0.19 [a]
	Age	≤ 1 year	61	6 (9.8)	55 (90.2)	ANOVA
		≤ 2 year	68	20 (29.4)	48 (70.6)	
		≥ 3 year	32	9 (28.1)	23 (71.9)	0.01*
	Ticks	Present	30	15 (50.0)	15 (50.0)	
		Absent	131	20 (15.2)	111 (84.8)	0.01 [a]
	Breed	Lohi	146	32 (21.9)	114 (78.4)	
		Kajli	15	3 (20.0)	12 (80.0)	1.00 [a]
Goats	Sex	Male	13	2 (15.3)	11 (84.7)	
		Female	26	8 (30.7)	18 (69.3)	0.68 [a]
	Age	≤ 1 year	17	5 (29.4)	12 (70.6)	ANOVA
		≤ 2 year	12	4 (33.3)	8 (66.7)	
		≥ 3 year	11	1 (9.0)	10 (91.0)	0.36
	Ticks	Present	10	5 (50.0)	5 (50.0)	
		Absent	29	5 (17.2)	24 (82.8)	0.01 [a]
	Breed	Teddy	25	4 (16.0)	21 (84.0)	ANOVA
		Nacchi	11	5 (45.0)	6 (55.0)	
		Beetal	5	1 (20.0)	4 (80.0)	1.17

a= Fisher Exact Test.

The animal characteristics with relation to the *Theileria* species infection were depicted in Table 3. The sheep with age ≤ 1year were found more prone to parasitic infection and there was significant association between theileriosis and different age groups ($p < 0.05$). Fisher exact test was applied to correlate with the prevalence of theileriosis and presence of ticks on animals and a highly significant correlation ($p < 0.01$) was observed indicating that ticks are involved in the spread of *Theileria* species infection. Sheep and goats data was also separately analyzed.

Table 4. Herd characteristics and Theileria prevelance based on PCR in Sher shah, Multan District, Punjab, Pakistan.

	Parameters	N	Pirsms positive	Piroplasms negative	P* Value
Size of herd	1- 30	15	3 (20.0)	12 (80.0)	
	31-60	50	10 (20.0)	40 (80.0)	ANOVA
	More than 60	135	32 (23.7)	115 (66.3)	0.70
Herd	Goats only	25	7 (28.0)	18 (72.0)	
	Sheep only	40	16 (40.0)	24 (60.0)	ANOVA
composition	Sheep and goats	135	22 (16.2)	13 (83.8)	0.05*

ANOVA results in sheep indicated that prevalence of theileriosis was significantly associated (P =0.05) in different age groups. Fisher exact test revealed the prevalence of theileriosis and ticks presence in sheep was also significant ($p < 0.01$). In goats the presence of ticks on animals was the only parameter which was significantly associated ($p < 0.01$).with the prevalence of theileriosis in small ruminants.

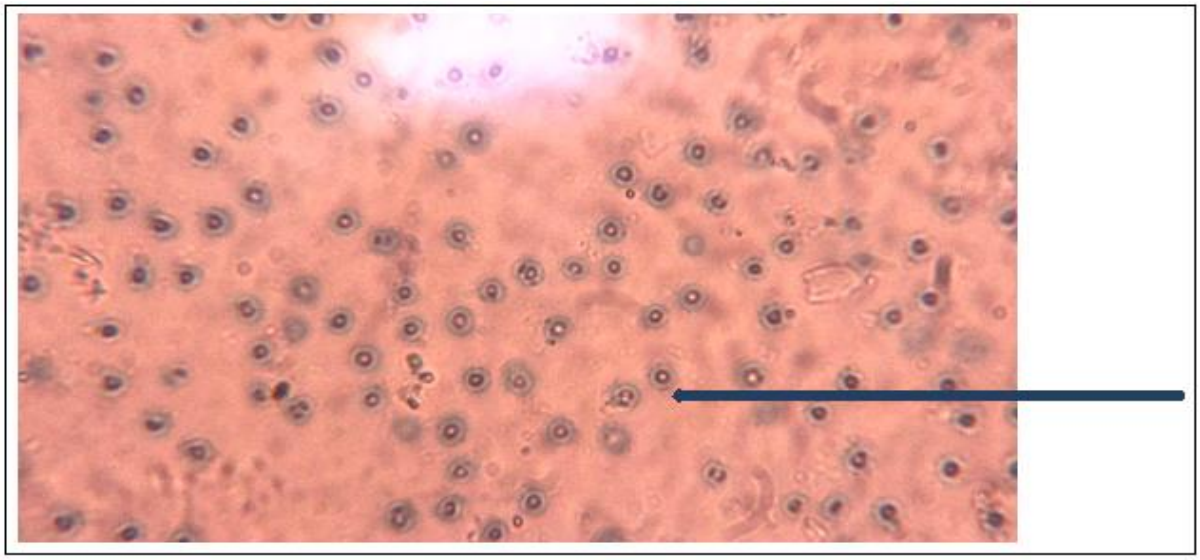

Fig. 1. *Theileria* piroplasms in stained blood smears.

The herds with sheep only showed higher *T. lestoquardi* infectionas compared to the herds having goats only or having both sheep and goats ($p< 0.05$) (Table 4). For the confirmation of PCR specificity, the amplified DNA fragment of *Theileria ovis* and *T. lestoquardi* were sequenced. The identification of PCR product was confirmed through BLAST. Gene bank accession number K723613, KP019206 for *Theileria ovis* while EF092916, EF092917 was for *T. lestoquardi* revealed 99% similarity.

Discussion

PCR amplification method and blood smear screening method used in order to determine *Theileria* species infection in Shershsah, district Multan, Pakistan. PCR revealed 22.5% (N=45) prevalence of *Theileria* species infection in sheep and goats. The higher infection was found in Ambwala (40.0%) and the lowest was reported in Sultanpur (10.0%). Due to climatic difference of different parts of Shershah Multan, prevalence of theileriosis was found significant between sampling sites as indicated by by ANOVA ($P < 0.05$). Durrani *et al.* (2011) reported 35% prevalence in sheep at district Lahore, Pakistan higher than current study while lower prevalence *Theileria* species infection (6.0 %) reported from two provinces of Pakistan by Durrani *et al.* (2012).

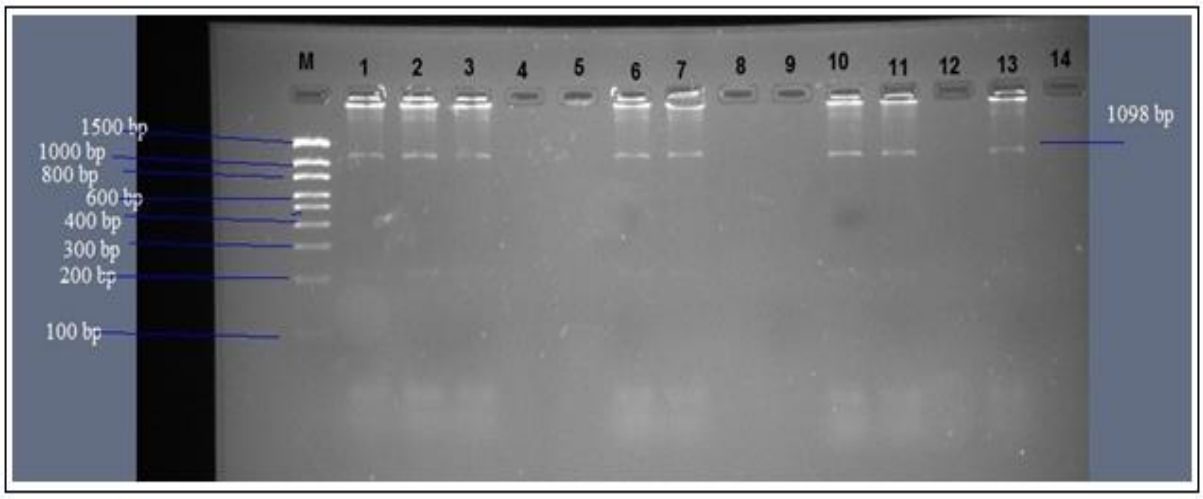

Fig. 2. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria* species genomic DNA using *Theileria* Specific primers. Lane M. DNA size marker of 1000.1500 bp.; Lane 1.2.3. 6.7.10.11. 13. *Theileria* spp. DNA positive control; 4.5. 8.9.12. 14. Negative control.

The difference of theileriosis from present study might be due do difference of sample size and breeds variation. Aktas *et al.* (2005) reported 41.2% blood samples positive for *Theileria* infection through PCR in sheep of Eastern Turkey. Gebrekidan *et al.* (2014) found 62.35% positive blood samples for theileriosis in domestic ruminants in Ethiopia. The higher prevalence of *Theileria* species infection could be due to different geographical location and season of sampling when vector population was very high.

Theileria species infection found significantly ($P < 0.05$) higher in sheep (17.5%) than in goats (5.0%) as indicated by Fisher exact test. Similar results of higher prevalence have been reported by many authors in Pakistan and other authors. Irshad *et al.* (2010) reported higher infection in sheep (7.36%) as compared to goats (3.8) during a study in Attock and Islamabad, Pakistan. Naz *et al.* (2012) from Lahore, Pakistan also reported higher *Theileria* species infection in sheep (13.9%) than in goats (8.2%), both studies are in agreement to the current study. Similar results of higher infection in sheep were reported by many authors around the globe confirmed that sheep are more prone to the *Theileria* infection.

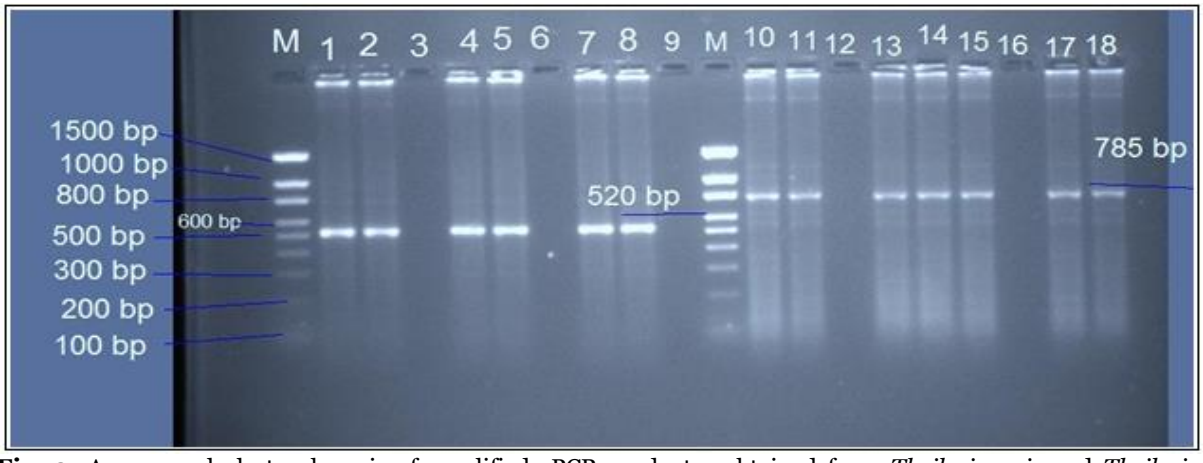

Fig. 3. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria ovis* and *Theileria lestoquardi* genomic DNA. Lane M. DNA size marker 100-1500 bp.; Lane 1.2. 4.5.7.8. *Theileria ovis* DNA positive control; Lane 3. 6.9. *Theileria ovis* Negative control; Lane 10.11.13.14.15.17.18. *Theileria lestoquardi* DNA positive control; Lane 12.16. *Theileria lestoquardi* Negative control.

The higher infection in sheep may be due to difference of skin nature, the skin of goats is thick and more resistant for attachment to ticks compared sheep skin. The presence of sheep wool, in which ticks could easily entangled and caused tick borne diseases (Naz *et al.*,2012). In goats the lower prevalence might be due to goat' s ability to pasture isolated and steep areas which have lower chance of tick infestation than sheep.

Theileria ovis and *T. lestoquardi* are suspected to cause ovine theileriosis in Pakistan (Durrani *et al.* 2011; Durrani *et al.,* 2012). During present study, the prevalence of *T. lestoquardi* found higher (12.5%) than *T. ovis* (6.0%) and the association was found significant ($P < 0.05$). Heiderpour *et al.* (2009) reported 87.5% and 12.5% prevalence of *T. lestoquardi* and *T. ovis* infection respectively during a study in Iran. A similar study revealed 55.3% and 44.7% positive blood samples were positive for *T. lestoquardi* and *T. ovis* respectively by Heiderpour *et al.* (2010). Both studies were in agreement with current study that *T. lestoquardi* was more prevalent *Theileria* species in sheep than in goats. Highest prevalence of *T. lestoquardi* compared to *T. ovis* might be endorsed due to more vector availability for *T. lestoquardi* infection and lower resistance breeds for *T. lestoquardi* infection. Contrary to current study, higher prevalence (16.5%) of *T. ovis* in blood samples were reported by Rehman *et al.* (2010). The difference might be endorsed due to different geoclimatic conditions and vector availability to ovine theileriosis in the studied area.

The significantly ($P < 0.05$) higher prevalence of *T. lestoquardi* (11.8%) as compared to *T. ovis* (5.6%) observed in sheep during present study. Similar findings of higher prevalence *T. lestoquardi* (54.8%) than *T. ovis* (40.2%) in sheep reported from Iran by Zaeemi *et al.* (2011). In goats higher prevalence of *T. lestoquardi* (15.3%) as compared to *T. ovis* (7.7%) observed during present study. The higher incidence of *T. lestoquardi* reported by many authors: (40.0 %) in China by Luo and Yin (1997) ; 20.8% in Iran by Zangana and Naqid (2011); 78.3% in Nubian goats in Sudan by Hussein *et al.* (1998).

The difference of infection rate may be endorsed due to higher availability of tick vector for *T. lestoquardi* and breed resistance against *T. ovis* infection among the studied areas. The results of present study indicated that gender of small ruminants (sheep and goats) is very important regarding *Theileria* species infection. It was found that male animals had higher infection rate (27.2%) as compared to females (20.6%) but the association was found non significant ($P > 0.657$) (Table 3). Naz *et al.* (2012) also reported that the gender not affect the occurrence of ovine theileriosisduring a study in Pakistan. When the age of small ruminants compared between *Theileria* piroplasms positive and negative, the results indicating that age group ≤ 1 in sheep was more infected with *Theileria* infection and the association was found significant ($p < 0.05$). Razmi *et al.* (2003) and Iqbal *et al.* (2013) also reported higher incidence of theileriosis in animals having age less than one year which is in accordance to the present study. But contrary to our study the adult sheep and goats were found more infected by *Theileria lestoquardi* infection by Guo *et al.* (2002). Similar results were obtained by Zangana and Naqid (2011) that in age group above three years had higher infection rate of piroplasms when compared with other groups. The difference of theileriosis might be due to variation in immunity level and genetic resistance in small ruminants against theileriosis.

Significantly higher prevalence of theileriosis was found ($p < 0.05$) in herds comprising sheep only than herds having either goats only or mixed herds of both sheep and goats. The results of present study are in agreement with Saeed *et al.* (2015) who also reported that mixed herds aggregates the *Theileria* species infection in small ruminants during a study in Khyber Pakhtoon Khaw, Pakistan.

The blood smear screening method revealed 7.0% (N=14) blood samples positive for *Theileria* infection. Earlier studies based on blood smears and clinical symptoms reported 5.01% *Theileria* species infection from two different research farms at Okara and Islamabad (Irshad *et al.* 2010), 11.9% found in Lahore, Pakistan (Naz *et al.,* 2012). The difference might be due to low parasitemia level which was not detected by light microscopy.

Several studies accepted that PCR is preferable diagnostic method than blood smear to examine carrier small ruminants with lower parasitemia level and without apparent clinical symptoms of theileriosis. The diagnosis and detection of parasitic species is often difficult in the carrier stage and in animals having mixed infection animals. Polymerase chain reaction (PCR) methodologies are preferable in the field of parasitology because for the amplification parasitic DNA in vitro only small amount of DNA material is required rather than larger amount of DNA which is impossible to acquire from parasites during different phases of life cycles in hosts (Gasser *et al.* 1997). For epidemiological studies the molecular diagnosis approaches such as PCR are more applicable and helpful in order to understand the behavior of disease in a studied population since such studies contain different aspects such as genetic diversity of populations, vulnerability to infections as well as environmental distribution of parasitic infections and probable mutations. There are only limited reports on the *Theileria* species prevalence in small ruminants in Southern Punjab and this may be the first study for investigation of *Theileria* species infection through polymerase chain reaction (PCR) in small ruminants in Multan, Southern Punjab, Pakistan.

Conclusion

PCR amplification method is sensitive and specific for tracing *Theileria* species infection in carrier animals and provides validated measures that are valuable to study epidemiology and treatment of theileriosis in small ruminants. The present study revealed that goats compared to sheep are more prone to *Theileria* species infection. *T. lestoquardi* is the more prevalent *Theileria* species in small ruminants to cause ovine theileriosis. The herds comprising only sheep have higher risk of *Theileria* species infection. The prevalence rate of theileriosis in small ruminants in district Multan cannot be ignored. For control of *Theileria* piroplasms in sheep and goats special attention should be given in order to enhance livestock production in the studied areas.

Acknowledgement

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). Grateful acknowledgements are due to all the "Veterinary Assistants" for their help during the sample collection.

Conflict of interest

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

References

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.

Allsopp BA, Baylis HA, Allsopp MT, Cavalier-Smith T, Bishop RP, Carrington DM, 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, Aydin MF, Uluisik U, Aktas M, Dumanli N. 2008. Use of multiplex PCR for the diagnosis of *Theileria annulata* and *Theileria buffeli.* Türkiye Parazitoloji Dergisi **32,** 1–3.

Altay K, Dumanli N, Aktas M. 2012. Molecular identification, genetic diversity and distribution of *Theileria* and *Babesia* species infecting small ruminants. Veterinary Parasitology **47,** 161-165. http://dx.doi.org/10.1016/vetper.2007.04.001

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.

Anonymous. 2015. *Economic survey of Pakistan*. Finance Division, Economic Advisors Wing, Ministry of Finance, Government of Pakistan, Islamabad. 377-382 P.

Durrani S, Khan Z, Khattack RM, Andleeb M, Ali M, Hameed M, Taqddas A, Faryal M, Kiran S, Anwar H, Riaz M, Sajjid M, Sheikh RS, 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 biomedicine **2,** 43-47.

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

El Hussein AM, ElGhali A, Mohammed SA. 1993. Efficacy of buparvaquone in the treatment of malignant theileriosis of sheep in Ed-Damer Province, N. State, Sudan: a field trial. Sudan Journal of Veterinary Research **12,** 51–57.

Fuente DL, Estrada-Pen JA, Venzal JM, Kocan KM, Sonenshine DE. 2008. Ticks as vectors of pathogens that cause diseases in human and animals. Frontier Bioscience **13,** 6938-6946. http://dx.doi.org/10.2741/fbs.3200

Gasser RB, Monti JR, Zhu XQ, Chilton N B, Hung, GC, Guldberg P. 1997. Polymerase chain reaction-linked single-strand conformation polymorphism of ribosomal DNA to fingerprint parasites. Electrophoresis **18,** 1564-1566.

Ghosh S, Bansal GC, Gupta C, Ray D, Khan, MQ, Irshad H, Shahiduzzaman M, Seitzer U, Ahmed JS. 2007. Status of tick distribution in Bangladesh, India and Pakistan. Parasitology Research **101,** 207- 216. http://dx.doi.org/10.1007/s0436-007-684-7

Guo S, Yuan Z, Wang GWW, Denglu M, Hongde D. 2002. Epidemiology of ovine theileriosis in Ganan region, Gansu Province, China. Parasitolology Research **88,** 36-37. http://dx.doi.org/10.1007/s0436-001-0568-1

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

Heidarpour BM, Khazraiinia P, Had-Dadzadeh HR, Kazemi B. 2010. Identification of *Theileria* species in sheep in the eastern half of Iran using nested PCR-RFLP and mi- croscopic techniques. Iranian Journal of Veterinary Research **11,** 262-26.

Iqbal F, Khattak RM, Ozubek S, Khattak MNK, Rasul A, Aktas M. 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. Iranian Journal of Parasitology **8,** 289-295.

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

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 Acadamy of Sciences **849,** 52-62.

Leemans I, Brown D, Fossum C, Hooshmand-Rad P, Kirvar E, Wilkie G, Uggla A. 1999**.** Infectivity and cross-immunity studies of *Theileria lestoquardi* and *Theileria annulata* in sheep and cattle: II. *In vitro* studies. Veterinary Parasitology **82,** 193-204. http://dx.doi.org/10.1016/S0304-4017(99)-00014-X

Luo J, Yin H. 1997. Theileriosis of sheep and goats in China. Tropical Animal Health Production **29,** 8S-10S.

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 Sciences **2,** 216-220.

Niyonzema A, Kiltz HH. 1986. Control of ticks and tick-borne diseases in Burundi. Australion Centre for International Agriculture Research Proceedings. **17,** 16–7.

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

Rehman ZU, Khan MS, Awais M, Aleem M, Shabbir MZ, Khan JA. 2010. Prevalence of theileriosis in sheep in Okara district, Pakistan. Pakistan Journal of Zoology **42,** 639-643.

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

Saeed S, Jahangir M, Fatima M, Shaikh RS, Khattak RM, Ali M, Iqbal F. 2015. PCR based detection of *Theileria lestoquardi* in apparently healthy sheep and goats from two districts in Khyber Pukhtoon Khwa (Pakistan). Tropical Biomedicine**32,** 225–232 .

Sajid MS, Iqbal Z, Khan MN, Muhammad G. 2008. Point Prevalence of hard ticks infesting domestic ruminants of lower Punjab, Pakistan. International Journal of Agriculture Biology **10,** 349-51.

Shahzad W, Noor H, Ahmad MD, Munir R, Saghar MS, Mushtaq MH, Ahmad N, 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. Iranianian Journal of Parasitology **8,** 570-578.

Shaikh RS, Ramzan K, Nazil S, Khan SN, Riazuddin S. 2004. A new locus for non syndromic deafness DFNB maps to chromosomes 11p13-p12. American Journal of Medicine and Genetics Annals **138.** 392-395.

Soulsby EJ. 1982. Helminths arthropods and Protozoa of domesticated animals. 7th Ed., Bailliere Tindalland Cassel Ltd., London, UK. 579-581.

Telmadarraiy Z, Oshaghi MA, Hosseini Vasoukolaei N, Yaghoobi Ershadi MR, 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. http://dx.doi.org/10.1016/S1995-7645(11)-60240-X

Uilenberg G. 1997. General review of tick-borne diseases of sheep and goats worldwide. Parassitologia **39,** 161-165.

Urquhart GM, Armour J, Duncan JL, Dunn AM. Jennings FW. 1996. Veterinary Parasitilogy. 2nd ed. Blackwell Science Ltd, Oxford, London, 246-2 P.

Woldehiwet Z. 2007.Tick-bornediseases .In: Aitken, I. D.(eds.) Diseases of Sheep,4thed., Blackwell publishingLtd, Oxford, 353-354 P.

Yin H, Schnittger L, Luo J, Seitzer U, Ahmad JS. 2007. Ovine theileriosis in China: a new look at an old story. Parasitology Research **101,** 191- 195. http://dx.doi.org/10.1007/s00436-007-0689-2

Zaeemi M, Haddadzadeh H, Khazaiinia P, Kazemi R, Bandehpour M. 2011. Identification of different *Theileria* species (*Theileria lestoquardi, Theileria ovis* and *Theileria annuluta*) in naturally infected sheep using nested PCR-RFLP. Parasitology Research **108,** 837-843. http://dx.doi.org/10.1007/s00436-010-2119-0

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