Pakistan J. Zool., vol. 47(2), pp. 441-446, 2015.

A Study on Molecular Detection of *Theileria lestoquardi* by PCR Amplification in Apparently Healthy Small Ruminants from Five Districts of Southern Punjab

Madiha Fatima,[1] Sadaf Saeed,[1] Rehan Sadiq Shaikh,[2] Muhammad Ali,[2] and Furhan Iqbal[1] *

[1]*Institute of Pure and Applied Biology, Zoology Division. Bahauddin Zakariya University Multan 60800*
[2]*Institute of Molecular Biology and Biotechnology. Bahauddin Zakariya University Multan 60800*
Madiha Fatima and Sadaf Saeed have contributed equally to the manuscript.

Abstract.- The present study was designed to detect the prevalence of *Theileria lestoquardi* in small ruminants of Southern Punjab and to determine the risk factors associated with the spread of theileriosis, if any. A total of 115 blood samples from small ruminants including goat (n=66) and sheep (n=49) were collected from five sampling sites (Bahawalnagar, Dera Ghazi Khan, Layyah, Multan and Muzaffargarh districts) in Southern Punjab, Pakistan from randomly selected herds. Data on the characteristics of the animals (species, gender, age, tick presence or absence and health status) and various herd characters (location, size, composition, dog presence or absence and tick association with the dog if present) was collected on sampling sites through questionnaire. Prevalence of the *T. lestoquardi* was recorded to be 3.47% (n=4) through PCR amplification of their *18S rRNA* gene that produced a 730 base pairs DNA fragment. All positive samples were sheep blood samples collected from Multan indicating that sheep were more prone to this parasite (P = 0.03). It was observed that tick association with the animal was a highly significant (P = 0.00003) risk factor associated with the theileriosis in small ruminants.

Key words: Theileriosis, small ruminants, PCR, 18S SSU rRNA gene, *Theileria lestoquardi*.

INTRODUCTION

Livestock is the backbone of Pakistan's economy as it contributes about 46.0 per cent of agricultural value added and 10.6 per cent to the GDP. Foreign earnings of the livestock sector are more than 35 billion rupees every year (Bilal *et al.*, 2006). Global economic significance of small ruminants can be estimated from the fact that two out of three billion estimated ruminants on the planet are sheep and goats (Bishop *et al.*, 2009).Pakistan being the third largest goat producing country has more than 25 breeds of goat and at least two dozen breeds of sheep (Khan *et al.*, 2007). Many factors, including health problems caused by nutrient deficiencies as well as parasitic infestations and contagious infections, adversely affect the ruminants cause considerable economic losses in terms of low productivity and mortality (Irshad *et al.*, 2010; Namavari *et al.*, 2011; Zulfiqar *et al.*, 2012).

Ovine theileriosis is a tick-borne hemoprotozoan disease in sheep and goats caused by several *Theileria* species and among them *T. lestoquardi* is considered as the most pathogenic one (Durrani *et al.*, 2011). Ticks of the genus *Hyalomma* act as vectors for this parasite (Salih *et al.*, 2003). Ovine theileriosis is characterized by pyrexia, leucopenia, edema of the throat, nasal and ocular discharge, salivation, reduced appetite, paleness of mucus membranes and rough body coat (Naz *et al.*, 2012). Liver and spleen enlargement, ulcer in abomasum and hemoglobinurea can also be observed (Hussein *et al.*, 2007). Susceptible animals usually die within 3-4 weeks as a result of widespread lymphocytolysis (Minjauw and Mcleod, 2003; Ahmed *et al.*, 2011).

The objectives of this study were to detect the prevalence of *Theileria lestoquardi* in the blood samples from sheep and goats of Southern Punjab by the PCR and to provide baseline data regarding *T. lestoquardi* prevalence and risk factors involved in the spread of ovine theileriosis in small ruminants of Southern Punjab.

MATERIALS AND METHODS

Samples and data collection

Blood samples from 115 small ruminants (66 goats and 49 sheep) were collected from randomly

* Corresponding author: furhan.iqbal@bzu.edu.pk
0030-9923/2015/0002-0441 $ 8.00/0

selected herds located in the important livestock production regions of 5 districts of Punjab, Pakistan. No diseased animal was intentionally included in the study. Blood was collected from the jugular vein of the animals by using disposable syringes. The collected blood was immediately preserved in 10 ml Eppendorf tubes by adding a few drops of 0.5M EDTA as anti-coagulant. Data regarding the characteristics of animals and herd was collected through questionnaires completed by investigators on sampling sites in order to calculate the risk factors involved in the spread of ovine theileriosis.

DNA isolation

DNA extraction was carried out by inorganic method following a previous study (Saeed *et al.,* 2015).

Oligonucleotide design and PCR amplification

A set of oligonucleotide primer [fwd 5'-GTGCCGCAAGTGAGTCA-3' and rev 5'-GGACTGATGAGAAGACGATGAG-3'] was used to amplify the 730 bp sequence from *18S rRNA* gene of *T. lestoquardi* as described earlier (Taha *et al.*, 2011).

PCR was performed in a reaction volume of 25 µl comprising 10X buffer A [500 mMKCl, 100 mMTris-HCl, (pH 9.1 at 20) and 0.1% TritonTMX-100], 250 ng genomic DNA, 20 pM of each primer, 0.16 mM of dNTPs, 2.5 U Taq DNA polymerase (Vivantus, UK) and 2.5 mM $MgCl_2$.

T. lestoquardi positive DNA sample provided by Dr. Munir Aktas of University of Firat, Turkey and negative control of PCR mixture without DNA were amplified during every PCR.

DNA amplification was carried out in a DNA thermal cycler (Gene Amp® PCR system 2700 Applied Biosystems Inc., UK). The thermo-profile comprised initial denaturation for 3 min at 94°C, followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. The final extension was carried out at 72° C for 7 min. Samples were kept at 4°C until the amplified PCR products were visualized on 1.5% agarose gel containing ethidium bromide.

Statistical analysis

Statistical package Mini Tab (Mini Tab, USA) was used for statistical analysis. All the values were expressed as mean±Standard deviation. For statistical analysis, animals were grouped into two age categories: less than 1 year to 1 year (young) and more than 1 year old (adult). Herds were divided into two size categories: herds having 1-15 animals and herds with more than 15 animals. Also, herds were divided according to their composition into three categories: herds with sheep only, herds with goat only and mixed herds. The absence or presence of ticks on sheep, goat, cattle and dogs associated with the herds was also recorded. Association between the presence (positive and negative blood samples) of *T. lestoquardi* and the various parameters, i.e. herd location, herd size, species, gender age of animal and absence or presence of ticks on sheep, goat, cattle, calf and dogs in the herd was assessed by contingency table analysis using the Fisher's exact test (for 2 x 2 tables) and/or Mantel-Haenszel test (for multiple 2 x 2 tables). Association of parasite prevalence with herd composition and herd location was determined by one way analysis of variance (ANOVA).

RESULTS

PCR prevalence of T. lestoquardi

Prevalence of the parasite *T. lestoquardi* was recorded to be 3.47% (n=4) through PCR amplification (Table I). Multan was the only sampling site from where *T. lestoquardi* was detected while the samples from Layyah, Muzaffar Garh, Dera Ghazi Khan and Bahawalnagar were found negative for *T. lestoquardi*. All the infected samples were sheep and none of the goat blood sample from any location was found infected with *T. lestoquardi*. ANOVA results indicated that presence of this parasite was not associated with the sampling sites under study (Table I).

Risk factors involved in the spread of T. lestoquardi

When pooled data (from both sheep and goats) was analyzed, it was observed that the age, gender, physical condition of animal were not leucopaenia associated with the prevalence of *T. lestoquardi.* A highly significant correlation (P = 0.00003) was observed between the presence of

ticks on animal and the prevalence of *T. lestoquardi* indicating that ticks are involved in the spread of *T. lestoquardi*. When Fisher exact test was applied to correlate prevalence of parasite with species of animals included in studies and a significant correlation (P = 0.03) was observed indicating that sheep were more prone to this parasite (Table II).

Table I.- Sampling districts along with the total number of samples collected (n) from each site. Prevalence of *Theileria lestoquardi* is given in parenthesis. ANOVA showed a non-significant association between the sampling sites and prevalence of parasite (P = 0.54).

Sampling site	n	*T. lestouardi* +ive	*T. lestoquardi* –ive
Multan	65	4 (6.25%)	61 (93.75%)
Layyah	25	0 (0%)	25 (100%)
DG Khan	16	0 (0%)	16 (100%)
Bahawalnagar	6	0 (0%)	6 (100%)
Muzaffar Garh	3	0 (0%)	3 (100%)
Total animals	**115**	**4 (3.47%)**	**111 (96.53%)**
Sheep	49	4 (8.16%)	45 (91.84%)
Goat	66	0 (0%)	66 (100%)

Sheep data was also analyzed separately and again presence of tick on animals was the only parameter which was highly significantly associated (P = 0.00002) with spread of theileriosis, while all other studied parameters had no effect on spread of *T. lestoquardi* (Table II)

Association between prevalence of parasite in sheep and goat and different characteristics of herds including herd size, herd composition, association of dogs with herds and presence or absence of ticks on the dog was also established. Data analysis showed that all these parameters were statistically not associated with the spread of *T. lestoquardi* (Table III).

DISCUSSION

Ovine theileriosis is an economically important disease of small ruminants in tropics and subtropics (Criado *et al.,* 2009). Among the various species causing ovine theileriosis, *T. lestoquardi* is the most pathogenic and causes malignant theileriosis in sheep and goats, a severe lymphoproliferative disease with high mortality and morbidity (Naz *et al.,* 2012). In present study, *T. lestoquardi* was detected in 3.5% of the samples by amplification of 18S rRNA gene. Multan (n=65) was the only district where the prevalence of parasite was recorded and 6.3% of blood samples were found positive for *T. lestoquardi*. Statistical results (ANOVA) indicated a non-significant association between sampling districts and prevalence of parasite (P = 0.538) (Table I) demonstrating that distribution of *T. lestoquardi* is not limited to any specific site. Earlier studies had reported 16.5% and 21% prevalence of *T. lestoquardi* in Okara and Lahore district respectively which is far more than present study (Durrani *et al.,* 2011; Rehman *et al.,* 2010). Probably the reason for low parasite prevalence in present study was relatively small sample size. Ovine theileriosis was reported in Southern Punjab and 6% incident of parasite in small ruminants was found which is comparable to our study (Durrani *et al.,* 2012). This indicates that different geographical and climatic conditions affect the distribution and prevalence of theileriosis.

Data regarding risk factors involved in the spread of theileriosis was collected. The association between animal type (sheep or goat) and parasite prevalence was statistically significant (P = 0.03) indicating that parasite prefers sheep over goat (Table II). The higher prevalence rate of *T. lestoquardi* in sheep is may be due to their different nature of skin than goats. The goat skin is thin and is more resistant for the tick attachment as compared to sheep's skin. The ticks can easily get entwined in wool of sheep and cause infection (Naz *et al.,* 2012). This hypothesis was supported by the fact that no *Theileria* sp. was found in animals on which ticks were not present (Table II). Other studies are in agreement with those reported by Naz *et al.,* 2012; Ahmed *et al.,* 2011 and Durrani *et al.,* 2012.

When the age of *T. lestoquardi* positive and negative animals was compared, the results indicated that the animals more than 1 year old were more prone to *T. lestoquardi* (4.3%) than younger animals (less than 1 year old) but statistically this association was non-significant (P = 1) (Table II). Similar trend was observed when only sheep data was analyzed but results were again statistically

Table II.- Association between parasite prevalence in sheep and goat and the studied parameters describing animal characters.

Animal type	Parameters		No. of samples	*T. lestoquardi* +ive	*T. lestoquardi* -ive	P-value
Sheep and goat	Animal type	Goat	66	0 (0 %)	66 (100 %)	0.03*
		Sheep	49	4 (8.2 %)	45 (91.8 %)	
	Sex	Male	34	1 (2.9 %)	33 (97.1 %)	1
		Female	81	3 (3.7 %)	78 (96.3 %)	
	Age	> 1 Year	94	4 (4.3 %)	90 (95.7 %)	1
		< 1 Year	21	0 (0 %)	21 (100 %)	
	Ticks	Absent	105	0 (0 %)	105 (100 %)	0.00003
		Present	10	4 (40 %)	6 (60 %)	***
	Health condition	Normal	112	3 (2.6 %)	109 (97.4 %)	0.10
		Diseased	3	1 (33.3%)	2 (66.7%)	
Sheep	Sex	Male	18	1 (5.5%)	17 (94.5%)	1
		Female	31	3 (9.7%)	28 (90.3%)	
	Age	> 1 Year	34	4 (11.7%)	30 (88.3%)	0.29
		< 1 Year	15	0 (0%)	15 (100%)	
	Ticks	Absent	44	0 (0%)	44 (100%)	0.00002
		Present	5	4 (80%)	1 (20%)	***
	Health condition	Normal	48	3 (6.25%)	45 (93.75%)	0.08
		Below normal	1	1 (100%)	0 (0%)	

Probability of Fisher's exact test is mentioned for each parameter. (P > 0.05 = Non significant; P < 0.001 = Highly significant (***).

Table III.- Association between parasite prevalence in sheep and goat and the studied parameters describing animal and herd characters.

Parameters		No. of samples	*T. lestoquardi* +ive	*T. lestoquardi* -ive	P-value
Size of herd	1-15	43	3 (7 %)	40 (93 %)	0.2
	15-30	72	1 (1. 4 %)	71 (98.6 %)	
Herd composition	Goat only	41	0 (0 %)	41 (100 %)	ANOVA
	Sheep only	24	0 (0 %)	24 (100%)	0.2
	Goat and Sheep	50	4 (8%)	46 (92 %)	
Association of dog with herd	Dog absent	20	0 (0 %)	20 (100 %)	0.1
	Dog present	95	4 (4.2 %)	91 (95.8 %)	
Ticks on dog	Absent	17	1 (5.8 %)	16 (94.2 %)	1
	Present	98	3 (3.1 %)	95 (96.9 %)	

Probability of Fisher's exact test is mentioned for each parameter except herd composition where one way ANOVA was calculated.
P > 0.05 = Non-significant

non-significant (P = 0.2) (Table II). A similar observation was reported previously (Naz *et al.*, 2012; Durrani *et al.*, 2012) during studies in small ruminants from Multan and Lahore districts in Pakistan, respectively.

Results indicated that gender of ruminants is a very important factor regarding *T. lestoquardi* infestation. It was observed that females (3.7%) were more prone to parasite as compared to males (2.9%) but statistically this difference was non-significant (P = 1) (Table II). When sheep data was analyzed separately, similar results were observed that eve were more inclined to parasite infestation but again results were not significant statistically (P =1) (Table II). Rehman *et al*. (2010) and Naz *et al*. (2012) had also found in their studies that gender

does not affect the incident of ovine theileriosis.

In present study tick infestation was observed to be the most important risk factor in the spread of ovine theileriosis. All *T. lestoquardi* positive animals had ticks present on their body. Highly significant association (P = 0.00003) was observed between tick infestation and *T lestoquardi* prevalence. When sheep data was analyzed separately it was seen that 80% *T lestoquardi* positive sheep had ticks present on their bodies and these results were statistically highly significant (P = 0.00002) (Table II). These results confirmed that ticks act as a vector for transmission of *Theileria* sp. Similar trend of tick infestation and its association with theileriosis has previously been reported in many studies (Durrani *et al.,* 2011; El-Azazy *et al.,* 2001; Ahmed *et al.,* 2002; Razmi *et al.,* 2003; Bishop *et al.,* 2004; Yin *et al.,*2007).

To determine their importance in the spread of ovine theileriosis various herd characters were also studied. Prevalence of *T. lestoquardi* was higher in small herds (16.7%) than in large herds (3.6%) but statistically this difference was non-significant (P = 0.2) (Table III). Our results are contradictory to Durrani *et al.* (2012) as they had reported that the prevalence of *T. ovis* was not affected by the herd size in small ruminants from Punjab and Khyber Pukhtoon Khwa provinces in Pakistan.

ACKNOWLEDGEMENTS

This project was financed by the Directorate of Research and External Linkages, Bahauddin Zakariya University, Multan (Pakistan) through grant No. DR & EL/D-40 dated 05-04-2012. Authors would like to thank all the veterinarians for their kind help during sample collection.

REFERENCES

AHMED, J., YIN, H., BAKHEIT, M., LIU, Z., MEHLHORN, H. AND SEITZER, U., 2011. Small ruminant theileriosis. *Small Rumin. Res.*,**2**: 135-153.

AHMED, J., YIN, H., SCHNITTGER, L.AND JONGEJAN, F., 2002. Ticks and tick-borne diseases in Asia with special emphasis on China. *Parasitol. Res*., **88**: 51-55.

BILAL, M.Q., SULEMAN, M. AND RAZAQ, A., 2006. *Buffalo: Black gold of Pakistan. Livestock Res Rural Dev.*, 18.

BISHOP, R., MUSOKE, A., MORZARIA, S., GARDENER, M. AND NENE, V., 2004. *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by *ixodid* ticks. *Parasitololoy*, **129**: 271–283.

BISHOP, R.P., ODONGO, D.O., MANN, D.J., PEARSON, T.W., SUGIMOTO, C., HAINE, L.R., GLASS, E., JENSEN, K., SEITZER, U., AHMED, J.S., GRAHAM, S.P. AND DE-VILLIERS, E.T., 2009. Theileria. In. *Genome mapping and genomics in animal-associated microbes* (eds. V. Nene and C. Kole). Springer-Verlag, Berlin, Heidelberg.

CRIADO, A., BULING, A., PINGRET, J.L., ETIEVANT, M., BARALON, C.B., ALONGI, A., AGNONE, A. AND TORINA, A., 2009. Hemoprotozoa of domestic animals in France: Prevalence and molecular characterization. *Vet. Parasitol*., **159**: 73–76.

DURRANI, A.Z., YOUNNS, M., KAMAL, N., MEHMOOD, N. AND SHAKOORI, A.R., 2011. Prevalence of ovine *Theileria* species in district Lahore, Pakistan. *Pakistan J. Zool*., **43**: 57-60.

DURRANI, S., KHAN, Z., KHATTAK, R.M., ANDLEEB, M., ALI, M., HAMEED, H., TAQDDAS, A., FARYAL, M., KIRAN, S., ANWAR, H., RIAZ, M., SAJID, M., SHEIKH, R.S., ALI, M. AND IQBAL, F., 2012. A comparison of the presence of *Theileria ovis* by PCR amplification of their SSU rRNA gene in small ruminants from two provinces of Pakistan. *Asia-Pacif. J. trop. Dis*., **2**: 43-47.

EL-AZAZY, O.M.E., EL-METENAWY, T.M. AND WASSEF, H.Y., 2001. *Hyalommaimpeltatum* (Acari: Ixodidae) as a potential vector of malignant theileriosis in sheep in Saudi Arabia. *Vet. Parasitol.*, **99**: 305–309.

HUSSEIN, H.A., SALAM, M.N.A. AND KARRAM, M.H., 2007. *Theileriosis and babesiosis in catle: heamogram and some biochemical parameters*. XIII Int Cong ISAH Tartu, Estonia.

IRSHAD, N., QAYYUM, M., HUSSAIN, M. AND KHAN, M.Q., 2010. Prevalence of tick infestation and theileriosis in sheep and goats. *Pak. Vet. J.*,**30**: 178-180.

KHAN, M.S., KHAN, M.A., AHMAD, S. AND MAHMOOD, S., 2007. Genetic resources and diversity in Pakistani Sheep. *Int. J. Agric. Biol.*, **9**: 941-944.

MINJAUW, B. AND MCLEOD, A., 2003. *Tick-borne diseases and poverty. The impact of ticks and tick borne diseases on the livelihood of small-scale and marginal livestock owners in India and eastern and southern Africa.* Research report, DFID Animal Health Programme, Centre for Tropical Veterinary Medicine, University of Edinburgh, UK.

NAMAVARI, M., AMRABADI, O., TAHAMTAN, Y. AND MANROURIAN, M., 2011. Molecular diagnosis of tick-borne heamoprotozoan disease agents in ticks collected from sheep. *Proc. Int. Conf. Agric. Anim. Sci*.,

22: 188-190.

NAZ, S., MAQBOOL, A., AHME, S., ASHRA, K., AHMED, N., SAEED, K., LATIF, M., IQBAL, J., ALI, Z., SHAFI, K. AND NAGRA, I.A., 2012. Prevalence of theileriosis in small ruminants in Lahore, Pakistan. *J. Vet. Anim. Sci*., **2**: 16-20.

RAZMI, G.R., HOSSEINI, M. AND ASLANI, M.R., 2003. Identification of tick vectors of ovine theileriosis in an endemic region of Iran. *Vet. Parasitol.,* **116**: 1-6.

REHMAN, Z., KHAN, M.S., AVAIS, M., ALEEM, M., SHABBIR, M.Z. AND KHAN, J.A., 2010. Prevalence of theileriosis in sheep in Okara district, Pakistan. *Pakistan J. Zool.,* **42**: 639-643.

SAEED, S., JAHANGIR, M., FATIMA, M., KHATTAK, R.M., SHAIKH, R.S., ALI, M. AND IQBAL, F., 2015. PCR based detection of *Theileria lestoquardi* in apparently healthy sheep and goats from two districts in Khyber Pukhtoon Khwa (Pakistan). *Trop. Biomed.,* In Press.

SALIH, D.A., ELHUSSEIN, A.M., HAYAT, M. AND TAHA, K.M., 2003. Survey of *Theileria lestoquardi* antibodies among Sudanese sheep. *Vet. Parasitol*., **111**: 361-367.

TAHA, K.M., SALIH, D.A., AHMED, B.M., ENAN, K.A., ALI, A.M. AND ELHUSSEIN, A.M., 2011. First confirmed report of outbreak of malignant ovine theileriosis among goats in Sudan. *Parasitol. Res*., **109**: 1525-1527.

YIN, H., SCHNITTGER, L., LUO, J., SEITZER, U. AND AHMED, J.S., 2007. Ovine theileriosis in China: a new look at an old story. *Parasitol. Res*., **101**: 191–195.

ZULFIQAR, S., SHAHNAWAZ, S., ALI, M., BHUTTA, A.M., HAYAT, S., QADIR, S., LATIF, M., KIRAN, N., SAEED, A., IQBAL, S., ALI, M. AND IQBAL, F., 2012. Detection of *Babesia bovis* in blood samples and its effect on the hematological and serum biochemical profile in large ruminants from Southern Punjab. *Asian-Pacif. J. trop. Biomed.,* **2**: 104-108.

(Received 3 September 2014, revised 15 January 2015)