PREVALENCE AND MOLECULAR DIAGNOSIS OF *THEILERIA ANNULATA*IN BOVINE OF THREE DISTINCT ZONES OF KHYBER PAKHTUNKHWA PROVINCE, PAKISTAN

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ABSTRACT

Tropical theileriosis is a tick-borne hemoparasitic disease and is responsible for huge economic losses in livestock sector of Pakistan. Bovine of three distinct zones of Khyber Pakhtunkhwa (KPK) province were examined to determine the molecular prevalence of *T. annulata* along with associated risk factors. A total of 900 blood samples (n=479 cows; n=421 buffaloes) were collected and examined; 170 (18.88%) were found positive for *T. annulata*. The central zone showed greater prevalence 65/300 (21.66%), followed by southern zone 56/300 (18.66%) and northern zone 49/300 (16.33%). Significant difference ($P< 0.05$) was observed in cows as compared to buffalo population ($P > 0.05$). Univariate analysis of risk factors including temporal zone, specie, breed, sex, age, management system, tick infestation, previous tick history, tick control, types of acaricides used and interval of acaricides usages revealed a significant ($P< 0.05$) association with prevalence of *T. annulata* in bovine. This study will help in developing more effective control of *T. annulata* in bovine of Pakistan. The results revealed here will help in developing more effective control strategies in future for dairy farmers in Pakistan.

Key words: *Theileria annulata,* Prevalence, Risk factors, Temporal Zones.

INTRODUCTION

Tropical theileriosis is caused by *Theileria annulata* (*T. annulata*) a tick-borne hemoparasite of bovine, transmitted by ticks *of Hyalomma spps.* (*Ixodidae* family). The disease has been widely reported around the world including Pakistan (d'Oliveira *et al.,* 1995; Durrani *et al.,* 2010, Tavassoli *et al.,* 2011). Tropical theileriosis is characterized by pyrexia 40-41.5ºC, oculo-nasal discharge and enlargement of superficial lymphnodes and anemia, resultantly there is lethargy and hemoglobinuria may also develop in later stages (d'Oliveira *et al.,* 1995, Gubbels *et al.,* 2000). Tropical theileriosis has been reported as one of the major economically important diseases affecting the health and productivity of bovine (Minjauw and McLeod, 2003). Water buffaloes (*Bubalus bubalis*) act as carrier to *T. annulata* and show very less clinical signs of tropical theileriosis as compared to indigenous and exotic breeds of cattle (Panigrahi *et al.,* 2016). To date the diagnosis of theileriosis mainly rely on clinical findings and microscopic examination (ME) of the Giemsa's stained blood smears in acute cases while immunological assays are used in subclinical cases. In microscopic examination the drawback is difficulty in differentiation between various *Theileria spps.* While with immono-diagnostics the cross reactions to other pathogens is the main problem (Burridge *et al.,* 1974; Pipano, 1974; Leemans *et al.,* 1999; Gubbels *et al.,* 2000). Recently Polymerase Chain Reaction (PCR) has been recommended as the best diagnostic tool for the detection of *T. annulata* in epidemiological studies due to its high sensitivity and specificity than any other technique in practice to date (Almeria *et al.,* 2001; Bhoora *et al.,* 2009; Shahnawaz *et al.,* 2011). In the present study prevalence of *T. annulata* has been studied using PCR in three temporal zones of Khyber Pakhtunkhwa Province, Pakistan.

MATERIALS AND METHODS

A survey was conducted in year 2015 (April to September) and total of 900 blood samples were collected from cows and buffaloes in three distinct temporal zones of KPK (Khyber Pakhtunkhwa Province), Pakistan. These temporal zones were made based on agro-ecological conditions. Apparently healthy animals were randomly selected from geographically distinct areas from six districts of the KPK, namely; Buner, Lower Dir, Mardan, Peshawar, Bannu and Lakki two from each temporal zones (Table 1). Samples were collected after initial screening by microscopic examination of the stained smears as described by (Moretti *et al.,* 2010).

PCR amplification for the detection of *T. annulata:* Genomic DNA was extracted from the samples which showed intraerythrocytic inclusion bodies. The DNA extraction was carried out using DNA extraction kit

(GeneAll®, Exgene™, 105-101) following the manufacturer's directions. DNA concentration was checked by spectrophotometry.

The DNA isolated from smear positive samples were subjected to PCR (Polymeras Chain Reaction) which amplified *T. annulata* cytochrome b gene using specific primers designed through NCBI Primer BLAST tools (forward primer; 5'-ACTTTGGCCGTAATGTTAAAC -3', and reverse primer: 5'- CTCTGGACCAACTGTTTGG-3'). PCR reaction mixture was prepared in a final volume of 20 µl consisting of 10 µl of TOPreal™ qPCR 2x PreMIX (containing 0.2 U of Taq/µl), 2 µl of DNA sample and 0.5 µmol of each primer. Reaction was cycled 35 times after initial denaturation at 95°C for 5 minutes with denaturation at 95°C followed by annealing at 64°C and finally an extension step at 72°C, each step was given 30 seconds, a final elongation step at 72°C for 10 min was performed. A positive control (*T. annulata* DNA), and a negative control (sterile distilled water), were included in each PCR run. The PCR was already optimized for molecular detection of *T. annulata*.The PCR products including the control positive and control negative were observed for positive bands on 2% agarose gel at 120 volt, 200 Amperes for 30 minutes (Fig. 1).

Statistical analysis: The data was analyzed through SPSS (Statistical Pakage for Social Sciences) version 20.00. Chi square test was used to find the association at a statistical significance of 95% Confidence interval. Binary logistic regression of forward Wald statistical model was used to determine the association and odd ratios for exp (B) at a significance level of 95% and cut off value of 0.5. For the goodness of fit of model used Hosmer and Lameshow test was performed. The value of Hosmer and Lameshow test proved the model applied to be highly fit with a value of 0.999.

RESULTS

The samples were diagnosed through microscopic observation and were confirmed through PCR. A binary logistic regression forward Wald statistical analysis was performed on two different sets of data on prevalence of *T. annulata* in Khyber Pakhtunkhwa province in Northern tropical areas of Pakistan and to evaluate the studied Predicted variables as potential risk factors that might be responsible in distribution of the infection in that region. The regression model was found fit with the tested variables studied to analyze as risk factors.

In the present study the overall prevalence of *T. annulata* was 18.88% comprising 23.79% in cow population and 13.30 % in buffaloes. Statistically significant difference was found in point prevalence between the study zones and sampled districts as depicted in Table 1. The central zone had highest prevalence of 21.66% followed by southern zone (18.66%) and northern zone with a prevalence of 16.33%.

Table 2 represents the statistical analysis of predicted variables. Results showed significant ($P< 0.05$) association of animal's sex with prevalence of *T. annulata* (OR = 0.305; CI =0.157-0.593). The prevalence was higher in males as compared to females. Results revealed no significant impact of temporal zones on the prevalence of *T. annulata.* In this study cows were found having twice the risk of getting infected (OR = 2.036; CI = 1.433-2.892) as compared to buffaloes. Breed of the animal was also found significantly associated ($P < 0.05$) with prevalence of *T. annulata*. Among cows the crossbred, while in buffaloes the non-descript buffaloes had the highest prevalence (OR = 2.047; CI = 0.660-6.351). Age of animal also showed significant ($P< 0.05$) association with prevalence as < 6 years aged animals were found at higher risk of getting infection than 1-2 months, 3-12 months and 1-6 years (OR = 1.198; CI = 0.443-3.236). The tick infestation status was also found associated with the occurrence of infection as a potential risk factor (OR = 3.052; CI = 2.116-4.402). The odds of developing infections were 2.5 times more in tick infested animals as compared to non-infested animals. The animals with previous tick history record were significantly ($P< 0.05$) associated with prevalence of *T. annulata* (OR = 209.39; CI = 51.465-851.98) a highly potent risk factor. The animals with no previous tick's infestation history showed a very low prevalence as compared to animals with previous tick history as given in Table 2. Tick control measure also showed significant ($P< 0.05$) association with prevalence of *T. annulata* (OR = 0.280; CI = 0.198-0.396). Cases with no tick control showed high prevalence than those with proper tick control measures. The type of acaricide was found statistically ($P< 0.05$) associated with occurrence of infection as a risk factor. (OR = 1.895; CI = 0.644-5.579). The last but not the least interval of acaricide used also showed a significant impact on the prevalence of *T. annulata* (OR = 1.120; CI = 0.388-3.227). Animals with no repetition of acaricide in tick's abundance period had the highest prevalence as compared to animals with repeated use of acaricides at varying intervals.

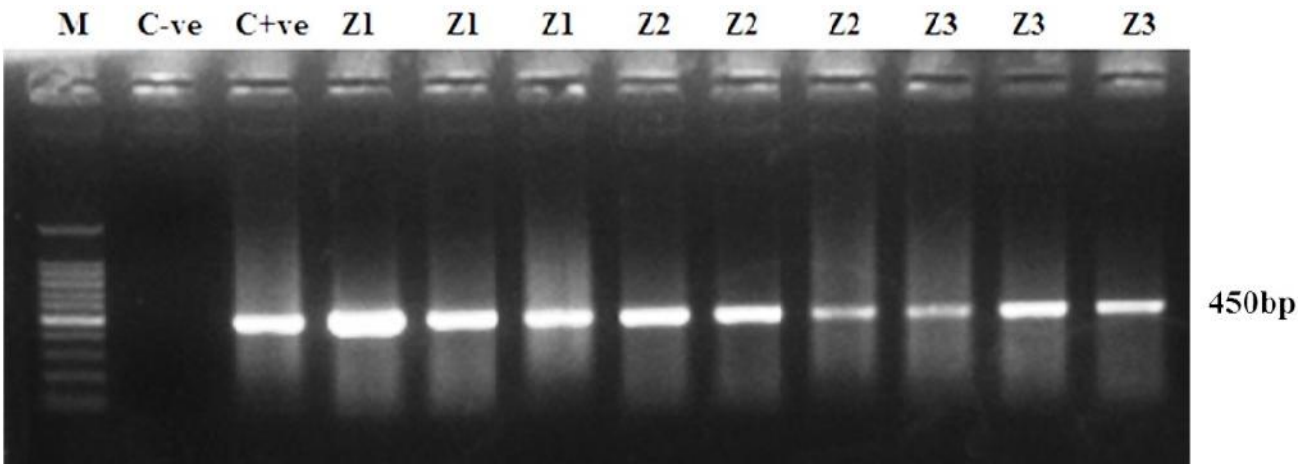

Figure 1. Shows PCR results for amplification of *T. annulata* cytochrome b gene amplification on ethidium bromide stained 2% agarose gel. Lane M indicates molecular weight marker, lane C-ve indicates negative control, Lane C+ve indicates positive control, Lane Z1 (consecutive three) indicates representative positive samples from northern zone, Z2 indicate central zones and Z3 indicates the southern zone representative positive samples.

Table.1 Showing the prevalence of *T. annulata* in cows and buffaloes in distinct zones of KPK Pakistan.

KPK Zone	Districts	Cows				Buffaloes			
		No. of samples examined	No. of sample Positive (%)	Confidence interval	*P* value	No. of samples examined	No. of sample Positive (%)	Confidence interval	*P* value
Northern	Buner	79	14.0 (17.72)	0.000-0.000	0.000	71	5.0 (07.04)	0.003-0.062	0.13
	Dir	75	23.0 (30.66)			75	7.0 (09.33)		
Central	Mardan	77	27.0 (35.05)			73	16 (21.91)		
	Peshawar	75	13.0 (17.33)			75	7.0 (09.33)		
Southern	Bannu	84	19.0 (22.61)			66	8.0 (12.12)		
	Lakki	89	16.0 (17.97)			61	13 (21.31)		
Total		479	114 (23.79)			421	56 (13.30)		

Table. 2 Showing the univariate analyses of risk factors affecting the prevalence of *T. annulata.*

Variable	Levels	No. of samples examined	No. of sample Positive (%)	Logistic regression *Odds ratio	Confidence interval	*P*- value
Zone	Northern	300	49.0 (16.33)	1.108	0.374-3.287	0.273
	Central	300	65.0 (21.66)			
	Southern	300	56.0 (18.66)			
Breed	Sahiwal	95	11.0 (11.57)	2.047	0.660-6.351	0.000
	Achai	104	10.0 (09.61)			
	Crossbred	128	51.0 (39.84)			
	Fresian	83	24.0 (28.91)			

	Jersey	67	16.0 (23.88)			
	Nili Ravi	156	18.0 (11.53)			
	Kundi	100	14.0 (14.00)			
	Non-descript	167	26.0 (15.56)			
Sex	Male	160	42.0 (26.25)	0.305	0.157-0.593	0.031
	Female	740	128 (17.29)			
Age	1-2 months	95	21.0 (22.10)	1.198	0.443-3.236	0.000
	3-12 months	220	59.0 (26.81)			
	1-6 years	473	51.0 (10.78)			
	Above 6 years	112	39.0 (34.82)			
Management	Intensive	801	152 (18.97)	1.054	0.614-1.809	0.036
	Semi-intensive	99	18.0 (18.18)			
Tick infestation	Yes	460	123 (26.73)	3.052	2.116-4.402	0.000
	No	440	47.0 (10.68)			
Previous Tick history	Yes	377	168 (44.56)	209.39	51.465-851.98	0.000
	No	523	2.00 (0.003)			
Tick Control	Yes	582	68.0 (11.68)	0.280	0.198-0.396	0.000
	No	318	102 (32.07)			
Type of Acricide	Cypermethrin	213	44.0 (20.65)	1.895	0.644-5.579	0.000
	Ivermectin	371	24.0 (06.46)			
	None	316	102 (32.27)			
Interval of Use	>30 days	101	11.0 (10.89)	1.120	0.388-3.227	0.000
	31-60 days	343	29.0 (08.45)			
	<60 days	141	27.0 (19.14)			
	None	315	102 (32.38)			

DISCUSSION

Tick borne diseases (TBDs) have been reported to affect both water buffaloes (*Bubalus bubalis*) and cattle (*Bos indicus* and *Bos taurus*) in Pakistan (Henson and Campbell, 1977; Haider and Bilqees, 1988; Durrani and Kamal, 2008). *T. annulata* causes a serious, potentially fatal disease in bovine, leading to substantial economic losses in enzootic countries in Asia and Africa, and is mainly transmitted by ticks of the genus *Hyalomma* (Vishvanath and Kole, 2008). In general, tropical theileriosis is more severe in exotic and cross-bred cattle (*Bos taurus*) than indigenous animals (e.g., *Bos indicus*) (Lefevre *et al.,* 2010). Recently Polymerase Chain Reaction (PCR) has been recommended as the best diagnostic tool for the detection of *T. annulata* in epidemiological studies due to its high sensitivity and specificity than any other techniques in practice to date (Almeria *et al.,* 2001; Bhoora *et al.,* 2009; Shahnawaz *et al.,* 2011) .

This study reported 18.88% overall prevalence of *T. annulata* in three temporal zones of KPK these findings are in line with the results of (Shahnawaz *et al.,* 2011), who found 19% prevalence of *T. annulata* in different districts of Punjab, Pakistan. Sajid *et al.* (2009) reported the higher prevalence in male than in female animals in various areas of lower Punjab, these findings positively corresponds with the current study which reported a higher prevalence in males 26.25% compared to female 17.29%. The reason could be the negligence of farmers to the male stock which are mainly used for draught and meat purpose. In the current study it was found that the prevalence of *T. annulata* is higher in cows than in buffaloes, these findings are in line with the findings of (Khattak *et al.,* 2007; Khan *et al.,* 2013) who also reported higher prevalence of *T. annulata* in cattle than in buffalo in various areas of the country. This can be attributed to the similarities of the environmental conditions among the study areas. A significant difference in prevalence of *T. annulata* was found among the study districts of this project. These results are in line with the findings of (Dumanli *et al.,* 2005; Atif *et al.,* 2012) who also reported significant association of different areas with the prevalence of *T. annulata.* Breed wise study showed highest prevalence in exotic cattle and their crosses than the indigenous breeds which positively corresponds with the findings of (Singh *et al.,* 2001; Glass *et al.,* 2005; Nazifi *et al.,* 2010). Among buffaloes non-descript showed highest prevalence than other breeds these findings are not in line with the findings of (Sajid *et al.,* 2009, Sajid *et al.,* 2014) They have studied kundi and Nili Ravi buffalo with no significant difference in the order of prevalence between the breeds and have not mentioned non-descript buffaloes. The highest prevalence

in non-descript buffaloes can be attributed to the negligence of farmers in taking proper care of these animals and this might be due to the lower cost of non-descript animals as compared to the registered breeds i.e. Nili Ravi and kundi. Secondly the reason might be the haphazard crossing of the buffaloes which mask the effect of breeds on the prevalence of disease. Significant difference in the prevalence of *T. annulata* was recorded in this study in which the 3-12 months and > 6 years aged animals were having the higher prevalence than 1-2 months aged and 1-6 years aged animals these findings are in line with the findings of (Sajid *et al.,* 2009) for the higher prevalence in 3-12 months aged. The higher prevalence in the > 6 years aged animals could be due to inefficient immunity boosting in old age. Management systems also affected the prevalence of *T. annulata* significantly in this study these findings do agree with the reports of (Salih *et al.,* 2007) who declared the management system as the potential risk factor in the epidemiology of *T. annulata.* Tick infestation also had a significant effect on the prevalence of *T. annulata*and this is in line with findings of (Inci *et al.,* 2008; Sajid *et al.,* 2009). A reduction pattern in the prevalence of theileriosis in herds was recorded with proper tick control measures and varying betterments with scheduled tick control strategies in this study. Kocan (1995) have also dictated the similar findings. In this study we found that parentral ivermectin gave better control in tick infestation compared to the pour on cypermethrin these findings are not in line with the findings of (Sajid *et al.,* 2009) who found cypermethrin as the better solution for tick control in filed. The lower efficacy of cypermethrin could be attributed to the faulty usage of this preparation due to the lack of knowledge regarding proper use of acricide. Secondly this might be due to lack of resistance to ivermectin in our study area which make it the better choice in controlling the tick population and ultimately reducung the prevalence of *T. annulata* and other piroplasms. Prevalence of *T. annulata* was low with repeated usage of acaricides in tick abundance period these findings are in line with (Hungerford, 1990) who also stated that repeated and scheduled acaricides application can protect animals from tick infestation.

From this study this can be concluded that tropical theileriosis is an important disease of livestock in KPK province of Pakistan and PCR is the most sensitive technique for ruling out the accurate diagnosis of *T. annulata.* Effective hemoparasite control can be achieved by considering the role of various factors affecting the dynamics of parasites and their vectors.

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