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RESEARCH ARTICLE

Molecular Epidemiology of *Babesia bovis* in Bovine of Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

Babesiosis is endemic in Pakistan and is one of the most economically important bovine diseases that cause huge economic losses and high mortality in young animals. An epidemiological study was conducted to unveil the prevalence and associated risk factors of *Babesia bovis* (*B. bovis*) in distinct climatic regions. A total of 900 (cattle=479, buffaloes=421) blood samples were collected from three distinct temporal zones of Khyber Pakhtunkhwa (KPK) province, Pakistan. The samples were analyzed by polymerase chain reaction (PCR) amplifying spherical body protein-4 (BbSBP-4) gene. Chi-square test, univariate analysis and multivariate logistic regression were used to analyze the data. The overall, prevalence in three distinct temporal zones of KPK province was found to be 10.11%. A higher prevalence of *B. bovis* was recorded in cattle 11.90%, compared with buffaloes 8.08% (OR:1.537, CI:0.984-2.403). Sequencing and phylogenetic analysis of locally isolated *B. bovis* showed sequence homology with the reported Syrian strain using NCBI BLAST tool. Species of the animal, sex of animals, tick infestation status, previous tick history, and tick control status, management type and geo-location were the significant (OR>1) risk factors associated with the occurrence. This study is the first molecular evidence of *B. bovis* and its associated risk factors in climatically distinct regions of KPK province, Pakistan.

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INTRODUCTION

Genus *Babesia* (Apicomplexa: Piroplasmida: *Babesiidae*), is a tick borne intraerythrocytic protozoa which causes a disease known as babesiosis. These infections are reported predominantly in summer season followed by rainy and winter seasons (Elhaig *et al.*, 2016). Recently, the babesiosis has got significant attention in the human medicine as an emerging zoonotic disease (Savic *et al.*, 2014). Many *Babesia* species causes clinical babesiosis in bovine however, *B. bigemina, B. bovis* and *B. divergens* are the most dominate etiological species so for clinically as well as economically in bovine populations. Babesiosis mostly occurs in tropical, subtropical and temperate regions of the globe affecting millions of cattle populations (Ojeda *et al.*, 2010).

The distribution of babesiosis caused by these species (*B. bigemina, B. bovis* and *B. divergens*) is dependent on the availability of ixodid tick species, which are known to be transmitting these parasites between the bovine populations. In general, babesiosis progresses with varying intensity that can frequently be associated to the age and immunological status of the host, coexisting infections with other pathogens, and/or genetic factors. Common manifestations of acute babesial infections in different hosts can include pyrexia, anemia, hemoglobinuria, icterus, malaise, lethargy and anorexia, while the chronic status is generally asymptomatic (Schnittger *et al.*, 2012). The water buffaloes have been reported to bear subclinical infections of *B. bovis* and *B. bigemina*; however, *B. orientalis*, which is transmitted by *Rhipicephalus haemaphysaloides* in China,

is highly pathogenic to these animals and produces great economic losses (Uilenberg, 2006; He *et al.*, 2012).

The epidemiological studies in past from Pakistan have reported prevalence of babesiosis on the bases of microscopic examination of Giemsa-stained thin blood smear. The prevalence has been reported 2.85% in two research institutes i.e. NARC and BLPRI (Khan *et al.*, 2004), 2.8% in district Peshawar (Afridi and Ahmad, 2005), 2.5% in district Kasur (Zahid *et al.*, 2005), 9.67% in district Sahiwal (Rashid *et al.*, 2010), 7.2% in district Sahiwal (Niazi *et al.*, 2010) and 6.57% in district Sargodha (Atif *et al.*, 2012).

Very few studies have been conducted on molecular prevalence of bovine babesiosis. In Punjab province, some studies have reported prevalence in various areas of the province. In district Kasur 33.33% prevalence has been reported (Durrani and Kamal, 2008), 18.75% prevalence in southern Punjab (Zulfiqar *et al.*, 2012), 11% in Livestock Experimental Station, Qadirabad (Chaudhry *et al.*, 2010). To date only one study has shown molecular prevalence of *B. bovis* in KPK Province of Pakistan. The study has reported prevalence 6.30% in calves and 10.60% in cows (Shams *et al.*, 2013). The phylogenetic study on *B. bovis* has not yet been conducted anywhere in Pakistan.

The laboratory diagnosis of clinical infection by piroplasms in cattle is usually based on detection of the parasite in Giemsa-stained blood smears. Carrier animals are important contributors to the transmission of the infection by tick bites. Hence, detection of piroplasms in carrier animals is very important to control the infection. However, detection of piroplasms by microscopy is not easy and the commonly co-existing pathogenic and non-pathogenic species cannot be distinguished. Serological tests can be used to detect circulating antibodies, but cross reactivity between the species can occur (Papadopoulos *et al*., 1996). Several PCR based diagnostic procedures for the identification of these parasites have been developed (Birkenheuer *et al.*, 2003).

The data on molecular based epidemiology of *B. bovis* is still deficient in most parts of Pakistan. Therefore, this study focused the molecular epidemiology of *B. bovis* in different temporal regions of KPK province, Pakistan.

MATERIALS AND METHODS

Sampling and primary screening: This study was conducted in year 2015 (April to September). A total of 900 blood samples (n=300 per zone) were collected using multistage sampling from three temporal zones (02 districts/zone) of KPK province, Pakistan (Fig. 1). Temporal zones were selected based on agro-ecological conditions. The blood samples were collected from apparently healthy animals for microscopic examination of *Babesia*-like inclusion bodies in the Giemsa-stained thin blood smears (Fig. 2) adopting the protocol as used by Moretti *et al*. (2010). Additionally, approximately 3ml of blood was collected into vacutainers containing EDTA for DNA extraction. A piloted questionnaire was filled for each sample from the owner to study the assumed risk factors.

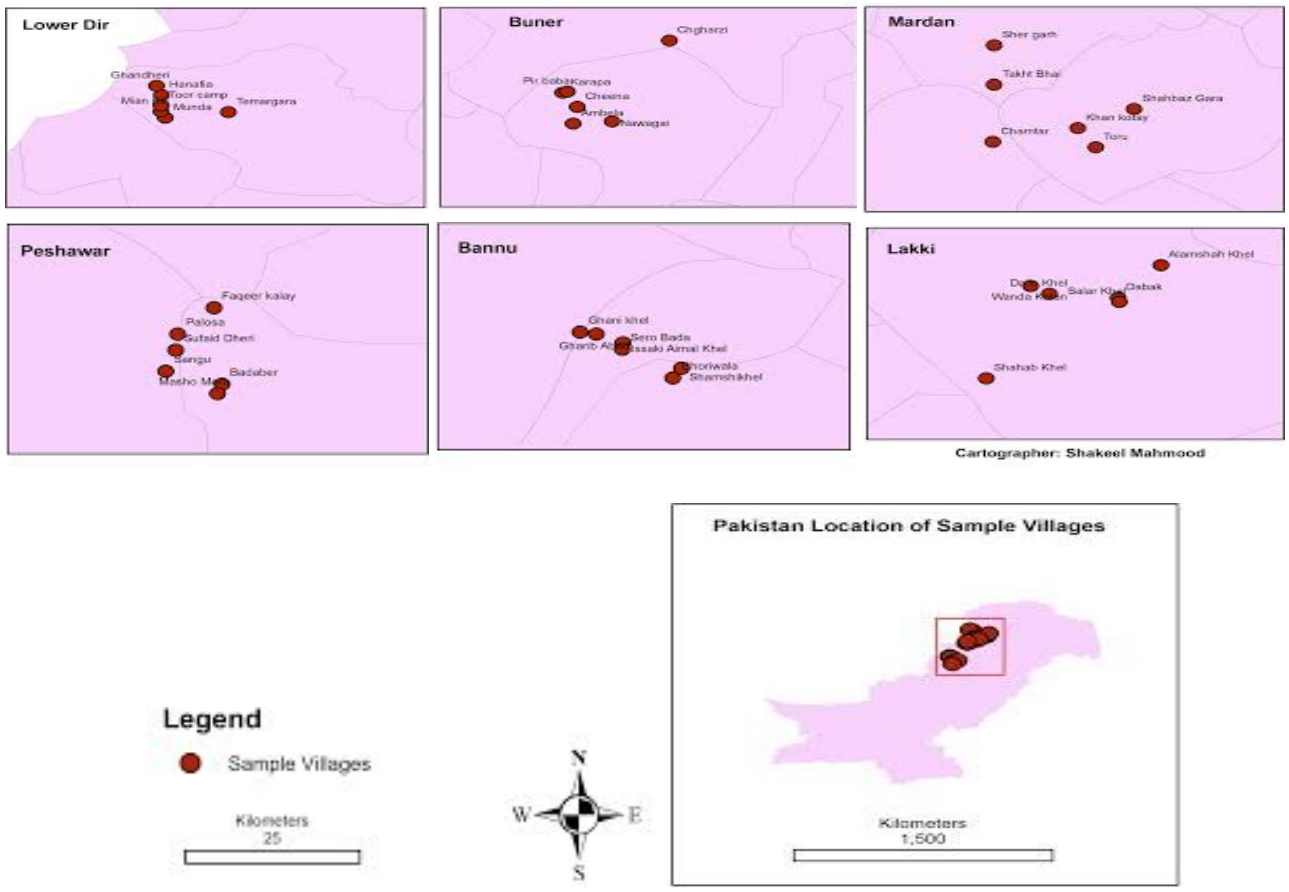

Fig. 1: Map of sampling area around six districts of KPK province.

Molecular diagnosis of *B. bovis*:

DNA extraction, quantification and purity analysis: Genomic DNA was extracted from 900 blood samples. The DNA extraction was carried out using DNA extraction kit (GeneAll®, Exgene™, 105-101) following the manufacturer's directions. The extracted DNA was checked for concentration (ng/µl) and purity by means of spectrophotometry using the wavelengths 260/280nm ratio, revealing the samples had suitable DNA for PCR amplification.

PCR for the detection of *B. bovis*: The samples DNAs isolated were subjected to PCR, which amplified BbSBP-4 gene using primers reported by Terkawi *et al.* (2011b) (forward primer; 5'-AGTTGTTGGAGGAGGCTAAT-3', and reverse primer: 5'-TCCTTCTCGGCGTCCTTTTC-3'). PCR mixture was prepared in a final volume of 20µl consisting of 10µl of TOPreal™ qPCR 2x PreMIX, 2µl of DNA sample and 1µmol of each primer. Reaction was cycled 35 times after initial denaturation at 95°C for 5 minutes with denaturation at 95°C, annealing at 60°C and extension step at 72°C, each step was given 30 seconds, a final elongation at 72°C for 10 min was performed. A positive control and a negative control were included in each PCR run. The PCR products were observed for positive bands against a 100bp molecular ladder on ethidium bromide stained1.5% agarose gel (Fig. 3). The bands observed at 907bp level were considered for further confirmation through sequencing.

Sequencing: The bands on 1.5% agarose gel were cut using cutter and were subjected to gel extraction using gel extraction kit (Catalog no. K210012), following the manufacturer's directions. DNA concentration was checked by electrophoresis on 1% agarose gel. The samples were sent for sequencing to Macrogen-USA.

Statistical analysis: The data regarding prevalence and hypothesized risk factors were subjected to chi-square test, univariate analysis and multivariate logistic regression for analyses using Statistical Package for Social Sciences (SPSS) version 20. Odds ratios were determined to prove degree of association of risk factors. P-value less than 0.05 was considered significant.

RESULTS

In this study blood samples were collected from subsistent farms of three temporal zones of KPK, Pakistan (Fig. 1). The samples were processed both classically and molecularly based (microscopy and PCR). The samples showing intracellular inclusion bodies were declared positive while in PCR the amplification of 907bp fragment of BbSBP-4 gene revealed on a 1.5% agarose gel was declared positive test. The PCR based results (Fig. 3) of 900 apparently healthy animals (cattle=479; buffaloes=421) revealed 57 (11.90%) and 34 (08.08%) positive for babesiosis in cattle and buffaloes respectively.

The expected amplicons with sizes of 907bp were observed in 91 out of 900 samples. Twenty (20) out of 900 samples from apparently healthy animals, negative on microscopy were positive for *B. bovis* on PCR. The sensitivity of PCR was identified using concentrations of 0.1ng to 0.1pg of control positive DNA. The detection limit of the PCR was 0.125pg and the primers specific for BbSBP-4 gene of this *B. bovis* did not amplify the *Anaplasma marginale* (positive), which is commonly found together infecting cattle. The PCR products for this gene were subjected to sequencing. It was carried out for the SBP-4 gene of 3 isolates collected from different hosts and geographic locations. These sequences were analyzed using BLAST and CLUSTAL W alignment. BLAST queries of the resulted sequenced nucleotides indicated the sequence identity with BbSBP-4 gene of *B. bovis*. For comparative purposes, the nucleotide sequences of *B. bovis* from NCBI database were aligned. The amplicons showed 96-99% homology with the nucleotide sequences for this gene deposited in GenBank. The phylogenetic analysis was performed using Mega 7 program by maximum likelihood algorithm with bootstrapping at 1000 replications. The phylogenetic tree was constructed based on the 3 sequenced products (B2-B Bov-F, B11-B Bov-F, B5-B Bov-F) from KPK province, Pakistan (Fig. 4).

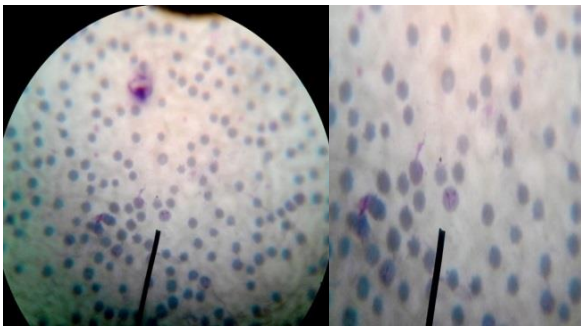

Fig. 2: Giemsa-stained thin blood smears results for intra-erythrocytic bodies resembling *Babesia*.

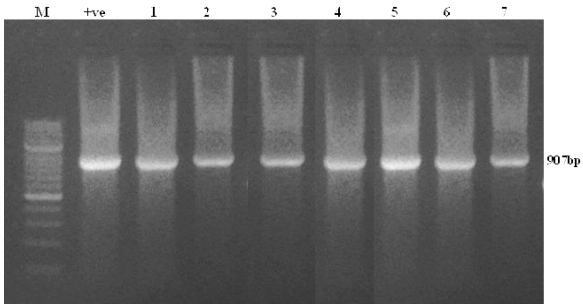

Fig. 3: PCR results of amplified 907bp DNA fragment of *B. bovis* SBP-4 gene against a known 100bp molecular weight marker. The lane M indicates molecular weight marker, lane +ve indicates control positive (*B. bovis*- USA) and lane 1-6 indicates positive samples of *B. bovis*.

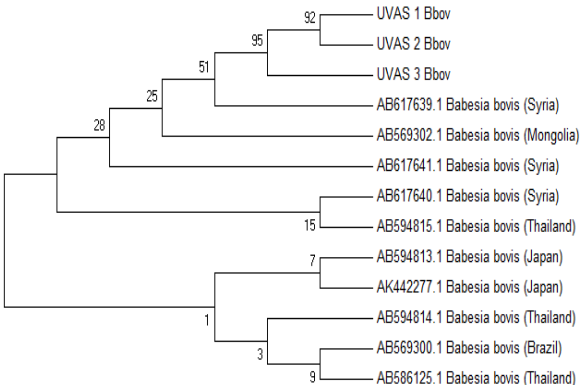

Fig. 4: Phylogenetic tree for *B. bovis* SBP-4 gene sequence.

Table 1: Univariate analysis of the potential risk factors associated with the occurrence of babesiosis in bovine

Variable	Variable level	No. Samples Examined (n=900)		OR	M-H odds ratio (OR) and 95% CI	P-value
		Positive (%)	Negative			
Location (Zone)	Northern	38 (12.67)	262	0.437	0.187-1.019	0.055
	Central	31 (10.33)	269			
	Southern	22 (07.33)	278			
Specie of Animal	Cattle	57 (11.90)	422	1.537	0.984-2.403	0.058
	Buffalo	34 (08.08)	387			
Ticks infestation status	Yes	52 (11.30)	408	1.310	0.846-2.030	0.225
	No	39 (08.86)	401			
Previous Tick History	Yes	50 (13.26)	327	0.798	1.162-2.780	0.008
	No	41 (07.84)	482			
Ticks control status	Yes	51 (08.76)	531	0.668	0.430-1.035	0.07
	No	40 (12.58)	278			
Interval Between Acaricide use	<30 days	04 (03.92)	098	2.679	0.912-7.870	0.012
	>30 days	47 (09.73)	436			
Breed of cattle	None	40 (12.70)	275	0.740	0.591-0.910	0.006
	Non descript	0 (00.00)	003			
	Sahiwal	07 (07.45)	087			
	Achai	07 (06.73)	097			
	Crossbred	16 (12.50)	112			
	Fresian	15 (18.07)	068			
	Jersey	12 (17.91)	055			
Breed of buffalo	Nili Ravi	13 (08.23)	145	1.037	0.683-1.571	0.863
	Kundi	10 (09.90)	091			
	Non-descript	11 (06.79)	151			
Gender of Animal	Male	23 (14.38)	137	1.345	0.986-2.345	0.136
	Female	68 (09.19)	672			
Management Type	Intensive	83 (10.36)	718	0.608	0.275-1.343	0.495
	Semi-Intensive	08 (08.08)	091			
	Sucklers	13 (13.68)	082			
Age of Animal	Young stalk	20 (09.09)	200	2.622	1.316-5.222	0.006
	Productive/old	58 (09.91)	527			

Table 2: Multivariate analysis for risk factor associated with babesiosis in bovine of KPK province, Pakistan

Potential Risk factors	Regression coefficient	Standard error	OR	Upper–lower limit at 95% C.I.	P-value
Zone	0.066	0.473	1.068	0.422-2.701	0.890
District	0.140	0.228	1.151	0.736-1.800	0.538
Specie	1.175	0.463	3.237	1.307-8.018	0.011
Breed	-0.193	0.101	0.824	0.677-1.004	0.055
Age	-0.109	0.171	0.897	0.641-1.254	0.524
Sex	0.539	0.270	1.714	1.010-2.910	0.046
Infestation	0.139	0.235	1.149	0.724-1.822	0.556
Previous tick history	0.376	0.242	1.457	0.907-2.339	0.120
Tick control	0.199	0.286	1.220	0.697-2.138	0.486
Interval of acaricide Usage	-0.586	0.239	0.557	0.349-0.889	0.014
Management	0.448	0.400	1.565	0.714-3.429	0.263

The cumulative prevalence of the three temporal zones recorded using PCR was 10.11%. The highest prevalence was recorded from northern zone 12.67%, followed by central and southern zones with prevalence of 10.33% and 7.33%, respectively.

The assumed risk factors like geo-location, specie of animal, tick infestation status, previous tick history, ticks control status, acaricide usage, interval between acaricide usage, gender of animal, management type, age of animal and breed of animal were statistically analyzed (Table 1).

This study showed an overall 10.11% prevalence of babesiosis in KPK. A non-significant (P>0.05; OR=0.437; CI=0.187-1.019) association was recorded between geo location and prevalence of babesiosis in bovine. However, the highest prevalence was recorded from northern zone followed by central and southern zone respectively. Species of animals also showed a non-significant association (P>0.05) with prevalence of babesiosis. The odds ratio 1.537 (CI=0.984-2.403) however suggested animal's species as a potential risk factor for the disease dynamics. Tick infestation levels also showed a non-significant (P>0.05) association with prevalence of babesiosis. The odds ratio 1.310 (CI=0.846-2.030) however, declared the infestation status as the potential risk factor for the occurrence of babesiosis. The previous tick history showed a significant (P<0.05) association with prevalence of babesiosis. However, the odds ratio 0.798 (CI=1.162-2.780) suggested that the previous tick history was not a potential risk factor for the occurrence of babesiosis in bovine. The tick control status also showed a non-significant association (P>0.05) with prevalence of babesiosis. The odds ratio 0.668 (CI=0.430-1.035) also suggested that tick control status is not a potential risk factor for the occurrence of babesiosis in bovine population of KPK province, Pakistan. The results suggested that there was a significant (P<0.05) association in interval between acaricide usage and prevalence of babesiosis. The odds ratio 2.679 (CI=0.912-7.870) also declared the acaricide usage interval as a potent risk factor for the prevalence of babesiosis in bovine population. The buffalo breeds were studied for association with disease prevalence. The results showed a significant association with disease prevalence (OR=1.037 (CI=0.683-1.57) however, the chi-square value considered it a non-significant factor (P>0.05). Similarly, cattle breeds were also studied and although the chi-square value suggested

cattle breeds as a significant factor (P<0.05) affecting the disease dynamics but the odds ratio did not agree with its inclusion as significant factor (OR=0.740 (CI=0.59-0.91). The results also showed a non-significant (P>0.05) association between species of animals and babesiosis. However, the prevalence was high in male than in female animals. The odds ratio 1.345 (CI= 0.986-2.345) declared the animal's gender as a potential risk factor for the occurrence of babesiosis. The study also shows a non-significant (P>0.05) association between the types of management with prevalence of babesiosis. The odds ratio 0.608 (CI=0.275-1.343) also declared management type as not a potential risk factor in the occurrence of babesiosis. The last factor studied was the age of animal, which showed a significant (P<0.05) association with prevalence of babesiosis. The odds ratio 2.622 (CI=1.316-5.222) suggested that age of the animal is a potential risk factor for the occurrence of babesiosis in bovine population of KPK Pakistan.

Multivariable model was developed using the backward manual step-wise elimination process removing variables with largest P-values respectively (Table 2). If a variable was found no longer significant statistically, after adjustment for the other variables was removed. Variables were removed or retained from the final model after considering their Wald Statistic with a P-value of 0.05. The incidence of confounding in data was assessed via monitoring estimated coefficient values and also by checking that values did not change more than 10% after dropping non-significant variables from the final model. Data collected on piloted questionnaire regarding 11 predicting variables were analyzed using R-statistical software and association between the dependent and independent variables was determined using univariable and multivariable analysis. Automated and manual approaches were exercised, which produced comparable models. Key risk factors identified from all the approaches included zone, district, specie, breed, age, sex, tick infestation, previous tick history, tick control, interval between acaricide usage and management, respectively.

DISCUSSION

In the present study, the samples were primarily screened for babesiosis based on slides showing intraerythrocytic inclusion bodies under light microscope. Similar, protocol was practiced by Niazi *et al.* (2008), screening the farm animals for babesiosis using light microscopy. BbSBP-4, GeneBank accession number (AB594813) gene amplification for the molecular identification of *B. bovis* on PCR. This work conforms to that of Terkawi *et al.* (2012), who also amplified the BbSBP-4 gene for the molecular identification of *B. bovis* and declares as the sensitive tool for the detection of *B. bovis* infection in bovine. These results confirm the previous findings that babesial infections are common in cattle in Asian countries (Terkawi *et al.,* 2011a, 2012) to study the phylogenetic relationship between *Babesia* species of randomly selected bovine samples, PCR primers were utilized from previously published report from Terkawi *et al.* (2011b) based on nucleotide sequences of BbSBP genes. *B. bovis*-specific PCR assay specifically identified *B. bovis* DNA sample, and no amplifications were observed for DNA samples derived from *A. marginale*. The maximum likelihood tree (boot strapping method at 1000 replications) inferred with BbSBP gene sequences of *B. bovis* isolates determined in this study. The closely related sequences of *B. bovis* SBP-4 genes retrieved from GenBank were also incorporated in the phylogeny. The phylogenetic analyses made it worth noting that, the isolates from this study were clearly distinct from other closely related taxa whose sequences were obtained from the GenBank database. Interestingly, it was observed that, the isolates of this study i.e. B2, B5 and B11, clustered positively with Syrian strain of *B. bovis* (Accession no. AB617639.1) in the GenBank. This suggests the higher homology of our isolates with the published strains.

In the present study, the overall prevalence of *B. bovis* was found 10.11% in three climatic zones of KPK province, Pakistan. Similar, findings have been reported by Zulfiqar *et al.* (2012). They found 10% prevalence of *B. bovis* in Layyah and Bahawalnagar districts of Punjab province Pakistan. The similarity in the prevalence between the two study areas can be attributed to the tick vector distribution, study period of the year and climatic similarities. In another study, the close results have been reported from district Sahiwal (Niazi *et al.*, 2008). They reported 13% prevalence of *B. bovis* infection. Similarly, Chaudhry *et al.* (2010) also reported 11% prevalence of *B. bovis* in crossbred carrier cattle kept at Livestock Experimental Station, Qadirabad, from June to August 2005. The zone wise prevalence of *B. bovis* was studied in the current research, the prevalence of *B. bovis* in northern zone was found to be 12.67% followed by central and southern zone showing prevalence of 10.33% and 7.33%, respectively these findings also positively conform to the findings of above published data.

The analysis of risk factors associated with *B. bovis* infection showed a significant association. The major risk factors revealed during the study were; specie of animal, sex of animal, breed of animal, previous history of tick infestation and interval between acaricide usage. Specie of animal and prevalence were significantly associated and cattle showed higher prevalence than buffaloes in the current study. Similar, findings have been dictated by Vahora *et al.* (2012) reporting higher prevalence in cattle than in buffaloes. This prevalence changes can be attributed to the higher vulnerability of cattle to the tick burden as compared to buffaloes. Next factor, found significantly associated with babesiosis prevalence was sex of animal. Male were found more susceptible to babesial infections as compared to the female animals. Similar, findings have been reported by Iqbal *et al.* (2011), and other scientists (Durrani and Kamal, 2008; Atif *et al.,* 2012b) reporting a higher prevalence in male than in female animals. The reason for the higher prevalence in males than females could be the negligence of farmers towards the male stock. Another factor, studied in the current project was breed of animal, found significantly associated with the disease prevalence. The exotic animals and their crosses were mostly affected as compared to the local breeds of cattle and buffaloes. These findings are in line with the results of Atif *et al.* (2012b) who also reported a higher prevalence of babesiosis in crossbred and exotic animals compared to

local breeds of animals. This could be attributed to the higher vulnerability of exotic animals and their crosses to tick infestation (Bock *et al*., 1997). Association between previous tick history and babesiosis prevalence was also studied in this project, which was statistically significant while the tick infestation status was non-significantly associated with the disease dynamics. The other scientists (Atif, 2015; Ali *et al.,* 2016), though suggested the tick infestation status as a potential factor for incidence and prevalence of hemoparasites. However the results for association of prevalence with previous tick history are in line with the finding of (Zhang *et al*., 2014), reporting the higher prevalence with direct or indirect contact to ticks. The tick control status and interval between acaricide usages also were significantly associated with prevalence of babesiosis. These findings are in agreement with results of (Kocan, 1995) who reported similar findings. In the current study, management system has no significant effect on the disease prevalence and the possible reason for this could be the lack of knowledge in the farming community about management systems as clear from the study most of the farmers are small holders and illiterate.

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Authors contribution: The blood sampling, data collection, processing and interpretation of results were made by SHF, MI, MHS, MIR and ZA. AK and SI made the data analysis. SHF, HN, AIA and KH wrote the manuscript. All the authors read the manuscript and approved the contents.

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