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Molecular occurrence of canine babesiosis in rural dog population in Pakistan

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Abstract. Canine babesiosis is an important tick-borne protozoal disease of dogs that poses major health problem worldwide. Farm dogs in rural areas are the companion animals, that not only watch the livestock herds but also guard the house of the owners. Each farmer keeps his companion dog to get all the services. In our study, a total of 450 blood samples of farm dogs from three different ecological zones (Southern, Central and Northern regions of the province; Punjab) of Pakistan, were collected to examine through microscopy and PCR. Examination of thin blood smears revealed an overall prevalence of 12.8% (58/450) of canine babesisal parasites. However, PCR analysis revealed 46.8% (211/450) and 7.3% (33/450) samples positive for *B. gibsoni* and *B. vogeli*, respectively. The amplicons of 671 bp and 590 bp were amplified for the detection of *B. gibsoni* and *B. vogeli*, respectively through PCR. The results of multivariate analysis showed that the occurrence of canine babesiosis is higher in the Central Punjab and younger age of the dogs, while breed and sex of the host were not significantly associated with the occurrence of the disease. Mixed infection of *B. gibsoni* and *B. vogeli* was observed only in 3 dogs each in district Kasur and Rawalpindi. Our study is the first report to observe the occurrence of canine babesiosis in rural dogs in Pakistan through PCR.

INTRODUCTION

The babesial parasites belong to the phylum Apicomplexa, which infect wild and domestic animals including human (Köster *et al.*, 2015). Till date, twelve species of *Babesia* have been reported from dogs (Irwin 2009) with widespread dispersal of *B. vogeli* and *B. gibsoni* in Asia (Inokuma *et al.*, 2004; Miyama *et al.*, 2005; Lee *et al.*, 2009; Mokhtar *et al.*, 2013; Terao *et al.*, 2015). Ticks of the genus *Ixodes* are responsible for the transmission of babesiosis in dogs and Canine babesiosis is the cause of fever, anemia, jaundice, dullness and thrombocytopenia (Boozer and Macintire, 2003, Oyamada *et al.*, 2005).

Farm dogs are the companion animals of a livestock farmer which not only protect the herd, but also guard his house. Pakistan is an agriculture-based country and most of the people living in rural areas, earn their livelihood by keeping livestock animals. Each farmer keeps a dog to protect his herd from losing the animal so it has a pivotal role for the sustained earning of the farmer (Marker *et al.*, 2005). Farm dogs are more likely to contract diseases as compared to household dogs because the latter are kept in a great care by the pet-lovers. Rural dogs are more prone to get parasitic diseases like helminthiasis than household dogs (Dubná *et al.*, 2007) as the former are kept in the field with other animals.

Previous studies from pet dogs of Pakistan based on microscopic examination revealed the prevalence of canine babesiosis ranges from 2.62% to 13.23% (Ahmad *et al.*, 2007, Bashir *et al.*, 2009). However, no information regarding occurrence of the disease and involvement of *Babesia* spp. is available for rural dogs of Pakistan. Pakistan is located in sub-tropics climatically and South-East of Asia geographically, extending from the Arabian Sea in the South and the Himalayas in the North (Sheikh *et al.*, 2015). Due to the favourable climatic conditions for the transmission of parasitic diseases especially the tick-borne diseases, the occurrence of the parasites are more likely to be present in animals (Durrani & Kamal, 2008; Bashir *et al.*, 2009; Rehman *et al.*, 2011; Zulfiqar *et al.*, 2012; Khan *et al.*, 2013; Hussain *et al.*, 2014).

The aim of this study was to determine the molecular occurrence of *Babesia* spp. in rural dogs and their association with age, sex and breed in three different ecological areas of Punjab, Pakistan.

MATERIAL AND METHODS

Collection of samples

Blood samples were collected randomly from farm dogs of South Punjab (Avg. temperature in summer and winter 33.3°C to 17.1°C with average annual rain fall 219.2 mm), Central Punjab (Avg. temperature in summer and winter 31.6°C to 15.4°C with average annual rain fall 741.9 mm) and North Punjab (Avg. temperature in summer and winter 30.2°C to 15.0°C with average annual rain fall 947.1 mm), as shown in Figure 1. Total of 450 samples (n=150 from each area) were collected in EDTA coated vacutainer between May and October 2016. Thin blood smears were prepared and the blood samples were stored at -20°C for DNA extraction. The temperature and average rainfall of the study areas were obtained from Pakistan Metrological Department (PMD, 2016). This study was approved by the Institutional Ethical Committee of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

Microscopic Examination

Microscopic examination of thin blood smears were done by Giemsa staining as per standard protocol (Coles, 1980) and observed under oil immersion lens at 1000X.

DNA extraction and PCR amplification

Total genomic DNA was isolated from 200 µl of EDTA treated blood by using the DNA extraction kit (GeneAll®, Exgene™, 105-101) according to the protocol provided by the manufacturer and DNA was eluted in 200 µl buffer. For *B. gibsoni*, 18S rRNA gene was amplified by using previously published primers (Inokuma *et al.*, 2004) and *B. vogeli* was confirmed by using reported primer pair (Duarte *et al.*, 2008). PCR reactions were carried out in 20 µl of reaction mixture containing 2 µl of 10 pmol of each primer, 2 µl of DNA, 10 µl of 2X AmpMaster™ Taq (GeneAll®, Exgene™, 541-001) and 4 µl of UltraPure™ DEPC water (Invitrogen 750023). A conventional PCR was carried out for the amplification of *B. gibsoni* and *B. vogeli* in thermal cycler (G- STORM GS482). The reaction conditions for *B. gibsoni* were; initial denaturation at 95°C for 5 min followed by 40 cycles having initial denaturation at 95°C for 30 secs for 40 cycles, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The final extension was carried out at 72°C for 5 min. For *B. vogeli*, initial denaturation at 95°C for 5 min followed by 40 cycles having denaturation at 95°C for 30 sec, annealing at 55°C for 30 and extension at 72°C for 30 sec, final extension at 72°C for 5 min, were the reaction conditions. The positive and negative controls were run for each reaction.

The amplified DNA was electrophoresed in 1.5% agarose gel (120 V, 200 mA, 45 min) stained with Ethidium bromide (Invitrogen 15585-011) and observed under GelDoc 100 imaging system.

Statistical Analysis

Data of predesigned questionnaire were entered in Excel data sheet. Data were validated by crosschecking all the computerized records with the original hard copy of complete data. Boundaries for the

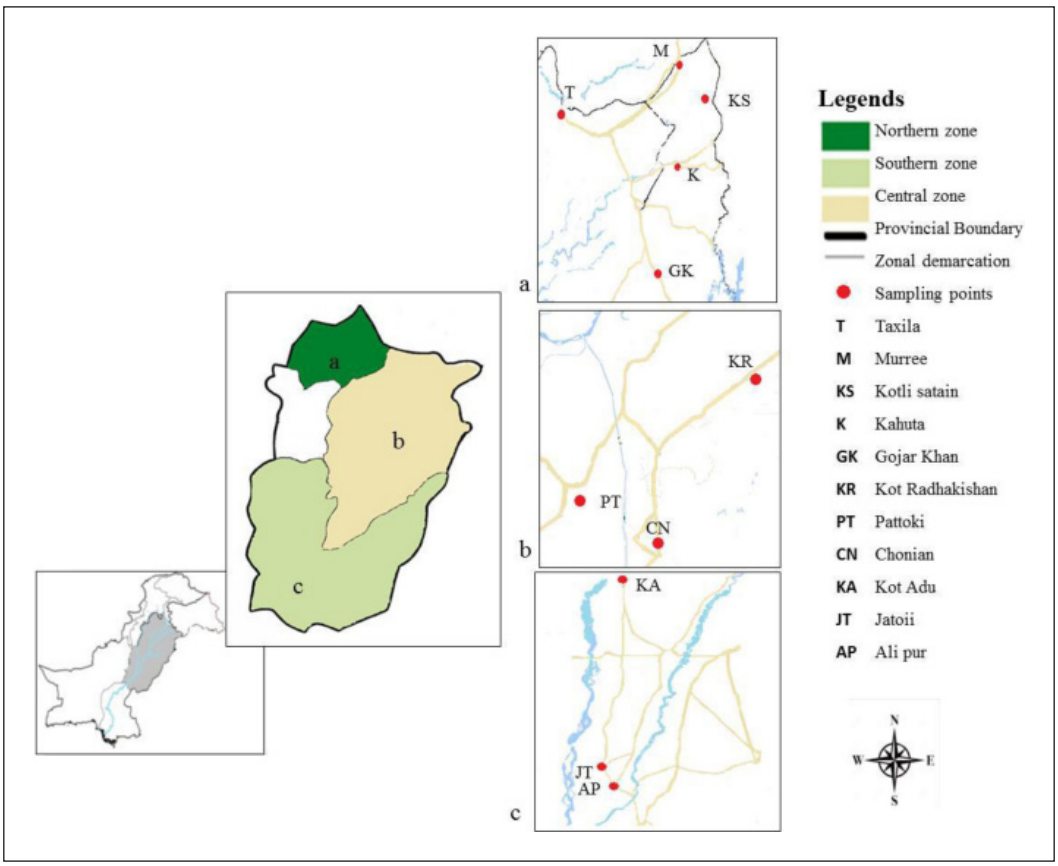

Figure 1. The map representing the sampling site from three different agro ecological zone of Punjab Pakistan.

categorization were chosen on the basis of predefined categories for the variables in questionnaire (Turner *et al.*, 2010). Data normality was assessed using Kolmogorov-Smimov and Shapiro-Wilk test. The predefined levels were based on the knowledge about the variables by reviewing of the available literature. Categorical variables having more than two categories were included as dummy variables. All biologically relevant and plausible variables from questionnaire were screened using SPSS version 22.00. Using the standard screening approach suggested by Hosmer *et al.* (2013) variables with a *P*-value of <0.25 were passed on from the univariable analysis for use in development of the multivariable model. This subset of variables was then checked for collinearity using Spearman rank correlation.

Multivariable regression was conducted using a manual stepwise elimination process (Dohoo *et al.*, 1997) removing the one with the largest *P*-value respectively. If a variable was no longer statistically significant after adjustment for other variables, it was removed (*P*-value = 0.05). Variables were retained or removed from the model after considering the log likelihood ratio test for categorical variables with 3 or more levels with a *P*-value of 0.05. The presence of confounding in the data was assessed by monitoring the estimated coefficient values and checking that they did not change by more than 10% when statistically non-significant variables were dropped from the model (Dohoo *et al.*, 1997).

RESULTS

Occurrence of Canine Babesiosis

This study revealed an overall 12.88% (58/450) occurrence of canine babesiosis through microscopic examinations of intraerythrocytic inclusion bodies as shown in Table 1. Specific primer pairs for *B. gibsoni* amplified 671 bp fragment as shown in Figure 2, and for *B. vogeli* amplified 590 bp fragment through PCR as shown in Figure 3. The PCR results revealed 54.22% (244/450)

Supplementary Table 1. Sensitivity and specificity of microscopy detecting *B. gibsoni* positive samples

Statistic	Formula	Value	95% CI
Sensitivity	$\frac{a}{a+b}$	11.85%	7.82% to 16.99%
Specificity	$\frac{d}{c+d}$	86.19%	81.16% to 90.30%
Positive Likelihood Ratio	$\frac{Sensitivity}{1-Specificity}$	0.86	0.53 to 1.39
Negative Likelihood Ratio	$\frac{1-Sensitivity}{Specificity}$	1.02	0.95 to 1.10
Disease Prevalence	$\frac{a+b}{a+b+c+d}$	46.89% (*)	42.20% to 51.62%
Positive Predictive Value	$\frac{a}{a+c}$	43.10% (*)	31.79% to 55.18%
Negative Predictive Value	$\frac{d}{b+d}$	52.55% (*)	50.78% to 54.31%
Accuracy	$\frac{a+d}{a+b+c+d}$	51.33% (*)	46.61% to 56.04%

Sensitivity and specificity of microscopy detecting *B. gibsoni* positive samples

Statistic	Formula	Value	95% CI
Sensitivity	$\frac{a}{a+b}$	12.12%	3.40% to 28.20%
Specificity	$\frac{d}{c+d}$	87.05%	83.44% to 90.12%
Positive Likelihood Ratio	$\frac{Sensitivity}{1-Specificity}$	0.94	0.36 to 2.42
Negative Likelihood Ratio	$\frac{1-Sensitivity}{Specificity}$	1.01	0.88 to 1.15
Disease Prevalence	$\frac{a+b}{a+b+c+d}$	7.33% (*)	5.10% to 10.14%
Positive Predictive Value	$\frac{a}{a+c}$	6.90% (*)	2.78% to 16.10%
Negative Predictive Value	$\frac{d}{b+d}$	92.60% (*)	91.65% to 93.46%

occurrence of canine babesiosis. Through PCR analysis, the occurrence of *B. gibsoni* was 46.88% (211/450) and *B. vogeli* was 7.33% (33/450). The mixed infection was observed only in six dogs, in the district Kasur and Rawalpindi, three dogs in each district as shown in Table 3.

Risk Factor Analysis of Canine Babesiosis

The analysis of risk factors and for their association with occurrence of canine babesiosis was carried out using univariate and multivariate logistic regression as shown in Table 1 and Table 2. The univariate analysis (Table 1) of risk factors based on microscopy showed that the association of the disease with age, sex and breed was significant statistically (OR>1). The analysis found breed as the only significant risk factor for the molecular occurrence of *B. gibsoni* (OR>1) however, the geographical locations showed a promoting effect on disease dynamics based on chi-square test ($P<0.05$). In case of *B. vogeli*, age was found

Table 1. Univariable analysis to identify risk factors based on microscopic and PCR results for *B. gibsoni* and *B. vogeli*

Univariable analysis for Microscopic results					
Variable	Description	Positive/Negative	Odd ratios	C.I (95%)	*P-Value
Age	6 month	14/112	1.479	0.70-3.09	0.300
	7-12 month	22/137	1.043	0.54-1.98	0.898
	>12 month	22/143	–	Ref	–
Sex	Male	37/217	1.421	0.80-2.51	0.143
	Female	21/175	–	Ref	–
Breed	Nondescript	52/324	1.819	0.75-4.40	0.122
	Others	6/68	–	Ref	–
Location	Central Punjab	23/127	0.516	0.24-1.07	0.075
	North Punjab	21/129	0.590	0.28-1.22	0.159
	South Punjab	14/136	–	Ref	–
Univariable analysis for *B. gibsoni* based on PCR					
Age	6 month	60/66	0.993	0.60-1.62	0.978
	7-12 month	79/80	0.814	0.52-1.27	0.366
	>12 month	72/93	–	Ref	–
Sex	Male	118/136	0.961	0.66-1.39	0.455
	Female	93/103	–	Ref	–
Breed	Nondescript	179/197	1.193	0.72-1.97	0.288
	Others	32/42	–	Ref	–
Location	Central Punjab	87/63	0.495	0.30-0.79	0.004
	North Punjab	63/87	0.958	0.59-1.53	0.858
	South Punjab	61/89	–	Ref	–
Univariable analysis for *B. vogeli* based on PCR					
Age	6 month	7/119	2.683	1.04-6.86	0.040
	7-12 month	8/151	2.552	1.06-6.13	0.036
	>12 month	18/147	–	Ref	–
Sex	Male	17/237	0.807	0.39-1.64	0.339
	Female	16/180	–	Ref	–
Breed	Nondescript	27/349	0.877	0.34-2.20	0.467
	Others	6/68	–	Ref	–
Location	Central Punjab	16/134	0.363	0.14-0.90	0.030
	North Punjab	9/141	0.699	0.25-1.90	0.483
	South Punjab	8/142	–	Ref	–

P-value is based on Likelihood test ratio.

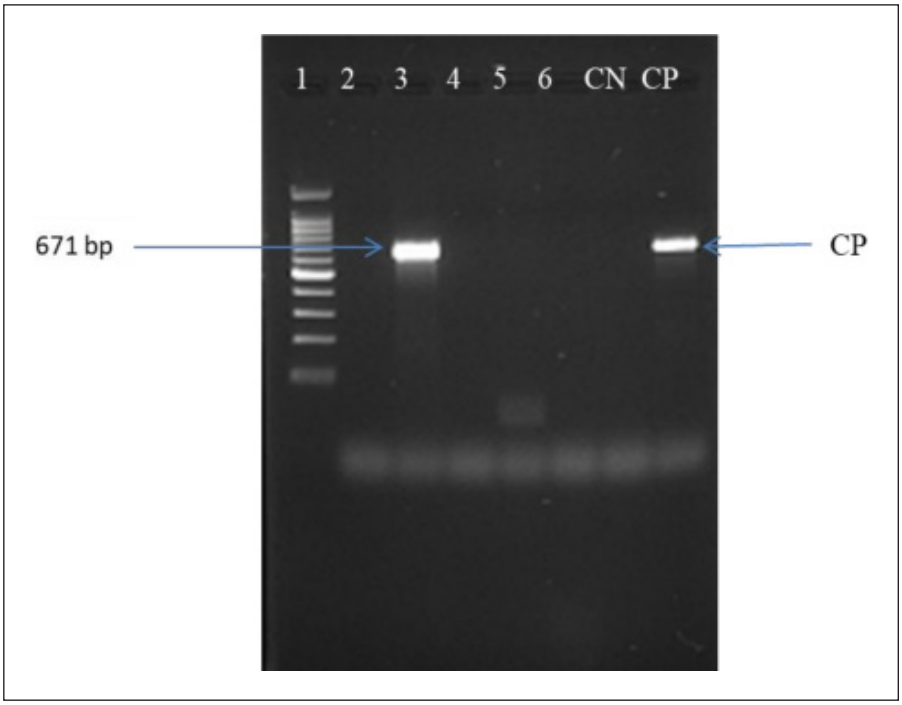

Figure 2. PCR amplification of 18S r RNA gene of *B. gibsoni*, Lane 1 represents 100bp DNA ladder, Lane 3 represents the positive sample, 1, 3-6 *B. gibsoni* negative, CN represents *B. gibsoni* control negative and lane CP represents control positive of *B. gibsoni*.

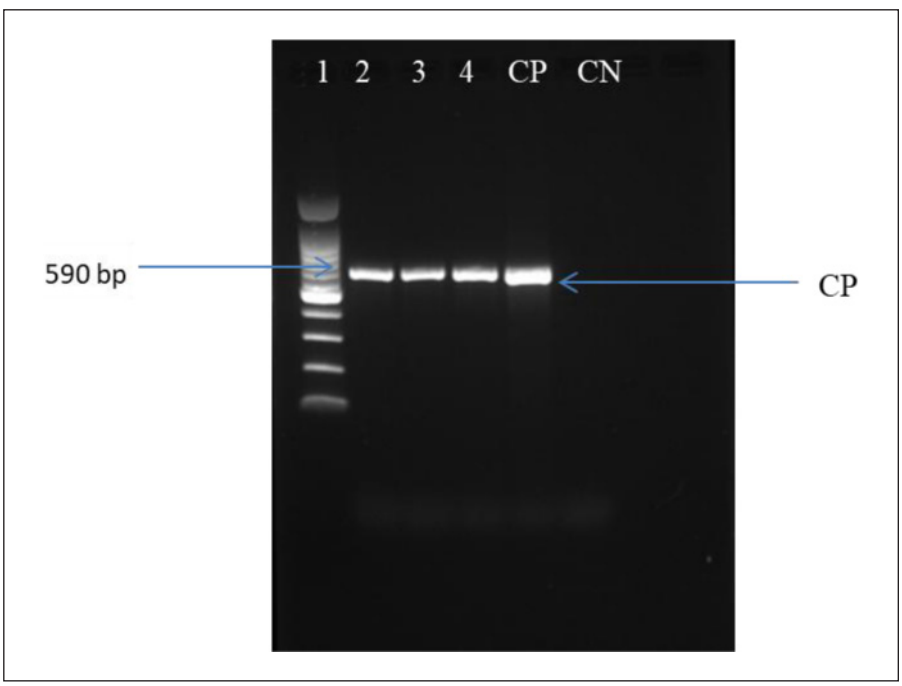

Figure 3. PCR amplification of 590 bp of 18S rRNA, 5.8S rRNA, 28S rRNA, ITS1 & ITS2 region of *B. vogeli* Lane 1 represents 100 bp DNA ladder, 2-4 *B. vogeli* samples positive sample, CP represents *B. vogeli* control positive and lane CN represents control negative of *B. vogeli*.

Table 2. Multivariable analysis to identify the potential risk factors based on microscopy and PCR for *B. vogeli* and *B. gibsoni*

Variable	Description	Coefficient	S.E	OR (C.I)	P-Value
Multivariable analysis for Microscopic results					
Sex	Male	-0.363	-0.363	0.69 (0.39-1.27)	0.21
	Female	–	–	Ref	
Breed	Nondescript	-0.651	0.454	0.52 (0.21-1.27)	0.152
	Others	–	–	Ref	–
Location	Central Punjab	-0.614	0.363	0.54 (0.26-1.10)	0.091
	North Punjab	-0.478	0.369	0.62 (0.30-1.27)	0.196
	South Punjab	–	–	Ref	–
Multivariable analysis for *B. gibsoni* based on PCR					
Location	Central Punjab	-0.701	0.235	0.49 (0.31-0.78)	0.003
	North Punjab	-0.055	0.235	0.94 (0.59-1.49)	0.815
	South Punjab	–	–	Ref	–
Multivariable analysis for *B. vogeli* based on PCR					
Age	6 month	-0.987	0.479	0.37 (0.14-0.95)	0.040
	7-12 month	-0.937	0.448	0.39 (0.16-0.94)	0.036
	>12 month	–	–	Ref	–
Location	Central Punjab	1.014	0.468	2.75 (1.10-6.89)	0.030
	North Punjab	0.359	0.511	1.43 (0.52-3.90)	0.483
	South Punjab	–	–	Ref	–

P-Value based on likelihood test ratio.

Table 3. The occurrence of canine babesiosis in dogs

Pathogen	Age (month)	Kasur (n)	Rawalpindi (n)	Muzaffargarh (n)
B. gibsoni	6	6.88% (31)	5.55% (25)	2% (9)
	7-12	6.88% (31)	5.77% (26)	4.44% (20)
	>12	5.33% (24)	3.33% (15)	7.11% (32)
B. vogeli	6	1.1% (5)	0.4% (2)	0.2% (1)
	7-12	0.2% (1)	0.8% (4)	0.6% (3)
	>12	2.2% (10)	0.6% (3)	0.8%% (4)
Mixed infection	6	0% (0)	0.2% (1)	0% (0)
	7-12	0% (0)	0.2% (1)	0% (0)
	>12	0.6% (3)	0.2% (1)	0% (0)

as a significant factor based on univariate analysis (OR>1), however, geographical location was found a promoting factor for occurrence of *B. vogeli* infection on chi-square bases ($P<0.05$).

The multivariate analysis (Table 2) revealed geographical location as a key risk factor for the occurrence of *B. vogeli*, however none of other factors was found as a key risk factor in all the categories i.e. microscopy and molecular occurrence of *B. gibsoni* and *B. vogeli*. The *P*-values of certain factors revealed a promoting trend in some factors like; geographical location for *B. gibsoni* while, age in case of *B. vogeli* ($P<0.05$).

DISCUSSION

The present study is the first report on molecular occurrence of *B. gibsoni* and

B. vogeli from rural dogs in Pakistan. The tick-borne parasites i.e. *B. gibsoni* and *B. vogeli* are known to cause canine babesiosis posing serious threat to the health around the world in dogs (Beck *et al.*, 2009; Irwin, 2009). In Pakistan, canine babesiosis is frequently diagnosed through microscopic observation of inclusion bodies in thin blood smears (Ahmad *et al.*, 2007; Bashir *et al.*, 2009). However, this does not allow accurate detection of species. PCR based diagnosis can detect *B. gibsoni* and *B. vogeli* as it is the reliable tool for the detection of *Babesia* species in dogs (Jefferies *et al.*, 2003).

Our study showed an overall 12.8% occurrence of canine babesiosis on the bases of microscopic observation of intra-erythrocytic inclusion bodies as compared to previous study in which Bashir *et al.* (2009) observed 2.62% prevalence of canine babesiosis in Punjab, Pakistan (Bashir *et al.*, 2009). The higher occurrence of canine babesiosis in our study could be due to; (i) selection of dogs (farm dogs vs pet dogs) (ii) abundance of ticks on farm dogs as compared to pet dogs. In fact, farm dogs integrate more regularly with other dogs when they go with grazing animals which increase their chances of getting infection. However, pet dogs are kept under good care and management by the owners which results lower tick-infestation hence, lower prevalence of canine babesiosis in previous study (Bashir *et al.*, 2009).

The overall molecular occurrence of *B. gibsoni* (46.8%) and *B. vogeli* (7.3%) were reported in our study, while Rani *et al.* (2011) observed occurrence of 0.2% *B. gibsoni* and of 5.5% *B. vogeli* in abandoned dogs in India. They collected blood samples of 525 stray dogs from 4 different cities, each located at different ecological zone (Sikkim in subtropical highland; Ladakh in montane; Delhi in monsoon- influenced humid subtropical region and Mumbai in tropical region) (Rani *et al.*, 2011).

The prevalence of *B. gibsoni* in our study was significantly higher as compared to some reports from Japan 8.8% (Inokuma *et al.*, 2004), China 9.23% (Cao *et al.*, 2015), Japan 10.9% (Ikadai *et al.*, 2004), India 15.42% (Singh *et al.*, 2014), Croatia 21% (Becket *et al.*, 2009), and for *B. vogeli;* Nigeria 1% (Adamu *et al.*, 2014), South Africa 4.3% (Matjila *et al.*, 2004), India 5.5% (Rani *et al.*, 2011). Similarly, some studies from Japan and India revealed higher prevalence (83% and 86%) of *B. gibsoni* in dogs than our study (Goo *et al.*, 2008; Mandal *et al.*, 2014).

This study revealed a significantly higher disease incidence pattern in younger age group as compared to older ones. Several other authors found similar findings (Abdullahi *et al.*, 1990; Samradhni *et al.*, 2005; Singh *et al.*, 2014), that could be due to transplacental transmission of *B. gibsoni* from mother to offspring (Fukumoto *et al.*, 2005). Brikenheuer *et al.* (1999) also diagnosed *B. gibsoni* in puppies as young as 10 days of age, a time interval that is shorter than the prepatent period following tick transmission. This over-representation may be due to an increased susceptibility to infection or less immunity. In some protozoan infections, most of the neonates of chronically infected mothers showed a high degree of immunity to the homologous parasites compared to those born of normal mothers (Palmer 1978).

Babesia infection was observed more in young animals of less than three years of age. Although, the older dogs of more than three years of age were also prone to babesial infection. Sero-positivity for babesiosis first increases and then declines with age, reaching a maximum in case of 3.1-to 5 years old dogs (Hornok *et al.*, 2006). It has also been reported that age does not have any influence on the animals' susceptibility to the disease (Martinod *et al.*, 1986). Non-specific or innate factors (genetic or age) possessed by the hosts can act as natural protective elements (Johnston *et al.*, 1978; Levy *et al.*, 1982).

Sex of the dogs were found non-significantly associated with disease incidence. These results are in line with reported findings from previous studies (Amuta *et al.*, 2010; Singh *et al.*, 2014). In contrary to the findings of this study, Bashir *et al.* (2009) found that male dogs were more prone to disease than the females, which could be attributed to bite wounds or blood transmission during fighting contact in male

dogs as possible routes of transmission for *B. gibsoni*.

We have observed mixed infection of *B. gibsoni* and *B. vogeli* in six dogs from two districts (Kasur from Central Punjab and Rawalpindi from North Punjab). Different researcher found coinfection of *B. gibsoni* and *B. vogeli* with other pathogens, for example *B. vogeli* was found with *B. rossi* (Adamu *et al.*, 2014), with *Ehrlichia canis*, *Hepatozoon canis*, *Anaplasma platys* and *Mycoplasma haemocanis* (Kamani *et al.*, 2013; Spolidorio *et al.*, 2011; Rani *et al.*, 2011). While, coinfection of *B. gibsoni* was found with *B. rossi* (Nalubamba *et al.*, 2015) and with *H. canis* (Xu *et al.*, 2015). To the best of our knowledge, we have not found co-infection of *B. vogeli* and *B. gibsoni* in dogs in literature. Either the authors reported *B. gibsoni* or *B. vogeli* only because there was occurrence of the parasites in different locations. *B. gibsoni* and *B. vogeli* might not be present together at one geographical location.

CONCLUSION

This study is the first report on molecular occurrence of canine babesiosis in rural dogs from Pakistan. The occurrence of the disease was high in all three locations studied regardless of sex, breed and age of the dogs. Further studies are required to characterize the tick vector(s) involved in the transmission of canine babesiosis in Pakistan.

Conflict of Interest

The authors declare no conflict of interest.

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