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Prevalence of *Borrelia burgdorferi* Sensu Lato in Pet Dogs and Associated Ticks in Pakistan

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ABSTRACT

Borrelia burgdorferi sensu lato is the etiological agent of Lyme disease. We investigated presence of *B. burgdorferi* s.l. in 600 pet dogs and 391 tick pools using PCR assay and sequencing in the Lahore City Metropolitan Area, Pakistan. Potential association of various risk factors with occurrence of Lyme borreliosis was also estimated via univariate and multivariate logistic regression. From each dog, blood and tick samples were collected and then tick species were identified. DNA extraction was followed by detection of 16S rRNA signature gene using *B. burgdorferi* s.l. specific primers through conventional PCR. We found that 4.3% dogs and 8.9% tick pools were positive for *B. burgdorferi* s.l. *Rhipicephalus sanguineus* (86.5%) was the most abundant tick species. 57.1% *I. gibbosus* and 8.4% *R. sanguineus* pools tested positive for *B. burgdorferi* s.l. Phylogenetically, our sequences clustered with *B. burgdorferi* sensu stricto, *B. bavariensis*, *B. garinii*, and *B. bissettii* sequences sourced from different hosts worldwide. Sequences showed 40.2%-99.1% sequences identity among them and 39%–98.3% with previously reported sequences on NCBI GenBank. Our sequences were submitted in NCBI GenBank under accession numbers MW547399-MW547405. Four animal-related: age (p=0.03), weight (p<0.05), sex (p=0.03), and breed (p<0.05), while three management-related risk factors: type of housing (p<0.05), tick infestation (p=0.007), and travel history (p=0.003) were significantly (p < 0.05) associated with occurrence of Lyme borreliosis. This is the first report on the presence of *B. burgdorferi* s.l. in Pakistan indicating the need for more comprehensive molecular surveys to estimate its prevalence in wider geographical areas and additional animal species as well as human population.

INTRODUCTION

Lyme borreliosis (Lb) is an emerging vector borne disease (Jánová, 2019). A spirochete, *Borrelia burgdorferi* sensu leto, is its causative agent. There are more than 52 species of *Borrelia* (Cutler *et al*., 2017) but currently 22 species are classified under *B. burgdorferi* s.l. and the most important of these 22 are *B. burgdorferi* sensu stricto, *B. afzelii*, *Bb mayonii* and *B. garinii* (Littman *et al*., 2018; Springer *et al*., 2020).

The main vectors of *Borrelia* sp. are three-host tick *Ixodes scapularis* in Mid-Atlantic, Northeastern, and upper Midwestern states (Herrin *et al*., 2017), *I. ricinus* in Europe, and *I. persulcatus* in Asia (Zintl *et al*., 2020). *Ixodes* are once considered prevalent ticks of temperate zone but their distribution and density have expanded over the years due to climate change and globalization (Bouchard *et al*., 2015). Besides, other tick species including *Rhipicephalus sanguinus, Hyalomma anatolicum, I. persulcatus, I. granulatus, I. sinesis,* and *H. longicornis, Hy. asiaticum, H. punctata, D. nuttalli, D. marginatus* and *I. persulcatus, Amblyomma americanum* have also been reported to harbor *B. burgdorferi* s.l. (Geurden *et al*., 2018; James *et al*., 2001; Livanova *et al*., 2018; Yu *et al*., 2016, 2017). Consequently, Lyme disease is becoming endemic in those areas like China, Japan, Thailand, Egypt which are previously not known to have this disease (Elhelw *et al*., 2021; Sthitmatee *et al*., 2016; Wang *et al*., 2019).

Dogs are the most common pet animals with over 470 million pet dogs in 2018 throughout the world E. Bedford, 'Global Dog and Cat Pet Population 2018', Statista: Consumer Goods and FMCG> Pets and Animal Supplies,

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2020 <https://www.statista.com/statistics/1044386/dog-and-cat-pet-population-worldwide/> [accessed 2 February 2021]. In Pakistan, pet lovers keep more than 0.2 million pet dogs as per a recent estimate. Dogs are highly susceptible to Lb and the most common clinical picture attributed to canine Lb includes history of tick infestation, anorexia, lethargy, fever, arthritis, nephritis, and neurological signs. However, most of the seropositive dogs show no clinical sign but they may act as carrier (Littman *et al*., 2018). Since pet dogs maintain a close contact with their owners, any exposure of these dogs to *B. burgdorferi* s.l or its vectors reflects corresponding risk to their owners. This is why, many previous studies used dogs and ticks as sentinels to assess the risk of Lyme disease in the corresponding human population (Liu *et al*., 2019).

Of the various tests prevalent for the diagnosis of Lyme disease in dogs such as ELISA and indirect fluorescence antibody (IFA), PCR is most sensitive and specific assay to detect different species of *B. burgdorferi* s.l. complex and it can also distinguish between active infection and past exposure (Díaz-Sánchez *et al*., 2020). Besides, its results can be subsequently used to characterize different strains, study their origin, and find out genetic modification. Above all, it is equally effective for screening of *B. burgdorferi* s.l. in dogs as well as ticks and many researchers have used it (Kocoń *et al*., 2020; Michalski *et al*., 2020).

To the best of our knowledge, previously no work has been done to diagnose and report *B. burgdorferi* s.l. in dogs and their ticks in Pakistan, despite evidence of Lb from neighboring country China as before as 1985 (Yu *et al*., 2016). Moreover, Pakistan regularly imports dogs from Europe and America where Lb is endemic. The current study was designed to investigate status of *B. burgdorferi* s.l. This study provides the first evidence of *B. burgdorferi* s.l. presence in Pakistan on the basis of PCR assay and sequencing. The nucleotide sequences of 16S rRNA gene obtained from positive DNAs were analyzed. The study also explored the distribution of tick species on dogs and explained potential association of various hypothesized risk factors with occurrence of Lb.

MATERIALS AND METHODS

Ethics statement

The study followed all essential ethical guidelines and recommendations of ARRIVE. Blood samples were collected after seeking consent from pet owners. Furthermore, Institutional Review Committee for Biomedical Research, University of Veterinary and Animal Sciences, Lahore approved the sample collection methodology (Letter number DR/894; dated 22/08/2017).

Study area

The study was carried out in Lahore district, Punjab. Lahore (31.5204° N and 74.3587° E.) is provincial capital of Punjab with a population of about 130 million (average rainfall 628.8mm and average temperature is 24.1 °C). Its metropolitan area is particularly important where expatriates bring their pet dogs with them and urban elite likes to keep pure breeds of dogs imported from Europe and America where Lyme disease is endemic. Twelve veterinary clinics, out of >100 veterinary clinics, were contacted for participation in the study.

Study design

Dogs with a history of tick infestation, anorexia, travel history, arthritis, shifting lameness, lymphadenopathy, renal issues, or neurological complications were included in the study. Blood and tick samples from 600 dogs were collected. Tick samples were collected in a sterile tube containing ethanol. Blood samples were collected from jugular vein aseptically, and stored until further processing.

Data related to hypothesized risk factors were collected in a structured questionnaire. The study lasted more than three years; from 1st May 2018 to 30th June 2021.

Genomic DNA extraction from blood and tick samples

The genomic DNA from blood and tick samples was extracted using Thermo Scientific GeneJET® Genomic DNA purification kit (catalogue number K0721) following the instructions of manufacturer. Ticks were first identified under an Olympus SZX16 stereoscopic microscope. Usually, one fully engorged tick from each dog was processed for DNA extraction. However, when multiple ticks were collected from a single dog, they were pooled and examined as single specimen. The tick specimen was grinded in liquid nitrogen using a mortar and pestle. 180uL digestion and 20uL Proteinase K solutions were added in the resultant mixture and mixed thoroughly through pipetting and vortexing. Then completely lysed tissue material was incubated for whole night in shacking incubator at 56°C. Subsequently, 20uL RNase solution, 200uL lysis solution, and 400uL 50% ethanol were added following guidelines given by the manufacturer. Finally, after addition of wash buffers and afterwards elution buffer fulfilling centrifugation and vortexing conditions, genomic DNA was obtained which was stored at -20°C until further processing. The same procedure was used for DNA extraction from blood samples.

Molecular diagnosis of Borrelia burgdorferi *sensu lato*

We used a highly specific primer set which targets 16S rRNA signature gene of all species of *B. burgdorferi*

s.l. The forward and reverse orientations of the primer are 5'ATGCACACTTGGTGTTAACTA 3' and 5' GACTTATCACCGGCAGTCTTA 3', respectively and it generated DNA fragment of 357 bases through conventional PCR (Marconi and Garon, 1992).

Thermo Scientific™ DreamTaq Green PCR Master Mix (2X) (catalog number K1081) was used for performing PCR. Each reaction mixture (25µL) was prepared by adding Master Mix 12.5µL, forward primer 1.25µL, and reverse primer 1.25µL. The volumes of DNA template and water were adjusted according to concentration of DNA in each sample. Except annealing temperature, different stages and conditions for PCR were same as suggested by manufacturer of master mix. Briefly, initial denaturation and denaturation at 95°C for 2 min and 30 seconds, while extension and final extension at 72°C each for 1 min and 12 min, respectively. Number of cycles for denaturation, annealing, and extension were set at 33. Prior to our sample analysis, optimization and validation of assay was accomplished using AMPLIRUN® BORRELIA BURGDORFERI DNA CONTROL (catalogue number MBC076).

The obtained PCR products were further evaluated using agarose gel electrophoresis (2%) with a 100 bp ladder (Invitrogen Co. Carlsbad, CA, USA). Then gel purification of amplified DNA fragments of 357bp was carried out with Wizard SV Gel and PCR Clean-Up System (Promega, Co., Madison, WI, USA). The final gel purified DNA products were processed for sequencing on 3100 DNA analyzer (Applied Biosystems) both in forward and reverse directions.

The unique 16S rRNA sequences, which were used in the phylogenetic analyses have been submitted to NCBI GenBank and assigned GenBank accession numbers MW547399-MW547405.

Phylogenetic analyses

The final nucleotide data of this study was assembled, edited, and aligned using BioEdit version 7.2.5 and only one sequence was selected from those, which were 100 alike. Using the same software, the final sequence data was phylogenetically compared with various nucleotide sequences, originating from different geographical regions and hosts, reported on GenBank. To infer evolutionary history, Maximum Likelihood (ML) method and Tamura-Nei model were used (Tamura and Nei, 1993). Phylogenetic tree centered on 16S rRNA was constructed and 1000 replications of bootstrap were used. Branches corresponding to partitions reproduced in less than 50 percent bootstrap replicates were collapsed. MEGA version X was used for this evolutionary analysis (Kumar *et al*., 2016). The same consensus sequences were used to calculate the percent identities.

Statistical analysis

The data was compiled into Microsoft Excel spreadsheets and validated using Epi Info™ software. The data was statistically analyzed using Minitab® 17.1.0 or GraphPad Prism® version 8.0 or SPSS version 20.0. Two statistical models were used to analyze association of risk factors with occurrence of Lb. In model 1, univariable exact logistic regression was used for estimating association of animal associated risk factors. In this model, *p* value was calculated using score method. Multivariate logistic regression was not possible owing to small positive sample size in each variable category. In model 2, firstly univariate mixed logistic regression test was used to determine the association of management-related risk factors with occurrence of borreliosis in dogs. All variables which had *p* values equal to or less than 0.2 were dropped and remaining were analyzed as explanatory variables using binary logistic regression. Backward elimination method was used to include variables in the model.

RESULTS

Distribution of ticks

Overall, 887 ticks were collected from 369 dogs. The most abundant tick species was *R. sanguineus* (86.5%; 95% CI = 84.06-88.57) followed *Hy. anatolicum anatolicum* (4.4%; 95% CI = 3.23-5.95), and *D. reticulatus* (1.6%; 95% CI = 0.94-2.63), whereas the least abundant species of ticks was *H. punctata* with only 9 ticks (1%; 95% CI = 0.54-1.92) identified according to keys (Keirans and Clifford, 1978; Keirans and Litwak, 1989). On average, 69 tick samples were collected from each clinic. The number of ticks infesting a dog was between 1 > 50 and 94.3% dogs were infested with a single type of ticks. The most favorable predilection site of ticks was inner side of hindleg with 430 ticks (48.5%; 95% CI = 45.2-51.77) followed by head, tail, and other body parts of the dogs as described in Table I. Tick collection also varied with the season and the most of the ticks were collected in summer season (56%; 95% CI = 52.75-59.27) followed by autumn and spring seasons while the least number of ticks (n=55) were collected in winter season. Out of 887 ticks, 783 were females, 46 nymphs while the rest were males (Table I). Most of these ticks were partially or fully engorged and 166 were unfed. Excluding presumptive repetitive sampling, partially and fully engorged female ticks and nymphs were pooled for subsequent analyses by DNA extraction and PCR.

Table I. Pertinent information about distribution of ticks, their species, life stages, seasonal recovery, and engorgement status.

Category	Percentage (number of samples/total)	95% CI
Species (n=829)		
Rhipicephalus sanguineus	86.5% (767/887)	84.06-88.57
Haemaphysalis punctata	1% (09/887)	0.54-1.92
Hyalomma anatolicum anatolicum	4.4% (39/887)	3.23-5.95
Dermacentor reticulatus	1.6% (14/887)	0.94-2.63
Ixodes gibbosus	6.5% (58/887)	5.09-8.4
Predilection site (n=887)		
Head	22.3% (198/887)	19.71-25.18
Tail	15.8% (140/887)	13.53-18.33
Inner side of hindleg	48.5% (430/887)	45.2-51.77
Other parts of body	13.4% (119/887)	11.33-15.82
Season (n=887)		
Winter	6.2% (55/887)	4.79-7.98
Spring	14.5% (129/887)	12.38-17.01
Summer	56% (497/887)	52.75-59.27
Autumn	23.4% (208/887)	20.78-26.25
Life stage of ticks (n=887)		
Male	6.5% (58/887)	5.09-8.36
Females	88.3% (783/887)	85.99-90.23
Nymph	5.2% (46/887)	3.91-6.85
Larvae	-	-
Engorgement status of (n=887)		
Partially engorged	55% (488/887)	51.73-58.26
Fully engorged	20% (177/887)	17.46-22.71
Unfed	18.7% (166/887)	16.29-21.41

Molecular detection of Borrelia burgdorferi *sensu lato*

We calculated the molecular prevalence of *B. burgdorferi* s.l. using PCR assay. 16S rRNA gene was targeted in PCR and results showed that 4.3% dogs and 8.9% tick pools were positive for *B. burgdorferi* s.l. (Table II). Among different tick species, *I. gibbosus* were 57.1% positive for *B. burgdorferi* s.l. followed by *R. sanguineus*, *Hy. A. anatolicum*, and *D. reticulatus* while we found no *H. punctata* positive for *B. burgdorferi* s.l. All of these ticks were adult females but no nymph was found positive for Lb. Breed wise positive percentage was also calculated and we found that bulldog had the highest positive percentage (13.4%; 95% CI = 7.66-22.44) while Spaniel and American Pit Bull Terrier had no cases (0%; 95% CI = 0.00-10.42 and 0%; 95% CI = 0.00-24.25) of *B. burgdorferi* s.l. Figure 1 shows positive cases of Lb in dogs.

Table II. Descriptive statistics and results of univariable exact logistic regression analyses vis-à-vis animal-related risk factors significantly associated with occurrence of lyme borreliosis in dogs.

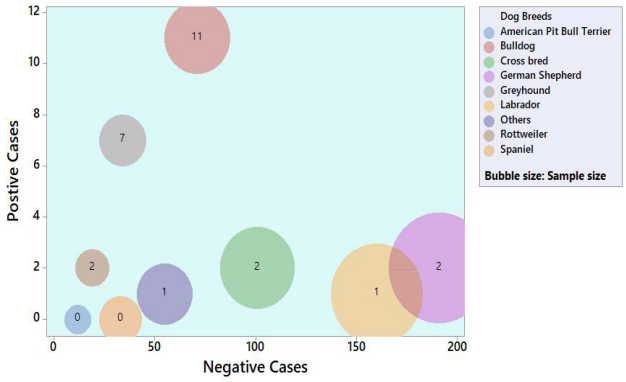
Predictor variables and their categories	Positive (%) cases	ODD ratio (CI[a] 95%)	p value[c]
0.03[b]			
<1 Y (Puppy)	211/4(1.8)	Referent	
1-7 Y (Adult)	231/16(6.9)	3.9 (1.25 to 10.74)	0.01
>7 Y (Senior)	152/6(3.9)	2.1 (0.56 to 6.76)	0.2
<0.0001			
Small (<10 Kg)	61/1(1.6)	Referent	
Medium (10-30 Kg)	416/7(1.7)	1.03 (0.17 to 11.75)	0.9
Large (>30 Kg)	123/18(14.63)	10.3 (1.70 to 109.4)	0.01
Male	293/19(6.09)	2.6 (1.08 to 6.66)	0.03
Female	281/7(2.4)	Referent	
<0.0001			
German shepherd	191/2(1.03)	1.7 (0.19 to 24.42)	0.6
Greyhound	34/7(17.1)	32.94 (5.48 to 372.6)	<0.0001
Labrador	160/1(0.6)	Referent	
Bulldog	71/11(13.4)	24.97 (4.25 to 26)	<0.0001
Rottweiler	19/2(9.5)	4.5 (0.51 to 65.65)	0.2
Spaniel	0(0.00)	-	-
American pit bull terrier	0(0.00)	-	-
Cross bred	101/2(1.9)	3.2 (0.36 to 46.16)	0.3
Others	56/1(1.79)	2.9 (0.15 to 55.55)	0.4

[a] 95% Confidence Interval; [b] Over-all significance for variables with >2 categories; [c] Significant p value (<0.05) is bold.

Association of animal-related risk factors with occurrence of Lb

Using the subset of 600 dogs, univariate exact logistic regression identified four animal related risk factors: Age, dog weight, sex, and breed, as significantly ($p < 0.05$) associated with the occurrence of Lb. The adult dogs were at significantly ($p = 0.01$) greater odds of carrying Lb while there was no significant ($p = 0.2$) difference in the odds observed between senior dogs and puppies. Similarly, the odds of having Lb was 10.3 times higher in heavy dogs (>30kg) than in light-weight dogs (<10kg), whereas the

odds of testing positive for Lb was not significant ($p = 0.9$) in medium dogs. Likewise, male dogs (6.09%) had 2.6 times greater odds of suffering from Lb than female dogs (2.4%). Lastly, the occurrence of Lb was significantly ($p < 0.05$) associated with dog breeds. Labrador had the lowest percentage (0.6%) of Lb and was used as a reference breed. The results showed that the odds of carrying Lb were 32.94, 24.9, 4.5, 3.2, 1.7 in greyhound, bulldog, rottweiler, crossbred, and German shepherd, respectively (These results are presented in Table II).



Note: Data label inside each bubble is showing the number of positive cases in that dog breed

Fig. 1. Positive cases of Lyme borreliosis in different breeds of dogs.

Association of management-related risk factors with occurrence of Lb

Results from univariate mixed logistic regression were compiled. The model identified that type of housing, tick infestation, acaricide usage, and travel history were significantly ($p < 0.05$) associated with the occurrence of Lb. Nevertheless, the predictors with p values ≥ 0.2 were further processed in the final binomial regression model with a stepwise backward elimination procedure to test their association with responses. However, the high value of variance inflation factor (VIF) for acaricide usage data showed multicollinearity and was, therefore, excluded from the final model. The remaining data adequately fitted the model (Hosmer and Lemeshow Test = 1.000) and the value of variance inflation factor (VIF = 1.00) confirmed the absence of multicollinearity. The final binomial logistic regression model was statistically significant ($\chi^2 = 35.39$; $p = 0.000$) and it explained 56.6% (R-squared) variance in Borrelia infection. The model confirmed that keeping dogs outside the house was significantly ($p = 0.000$) associated with increased likelihood of carrying Lb. Likewise, the presence of ticks and travel history within last 14 days were also significantly ($p < 0.05$) associated with increased likelihood of suffering from Lb with odds of 3.8178 and 3.6471, respectively (Table III).

Table III. Association of management related risk factors with occurrence of Lb using multivariate binominal logistic regression.

Predictor variables	Response (Frequency)	Positive cases (%)	ODD ratio (CI[a] 95%)	p value
Type of housing (n=591)	Indoor (377)	6(1.6)	Referent	0.000[b]
	Outdoor (214)	20(9.8)	0.146 (0.057, 0.374)	
Tick infestation (n=600)	Yes (369)	22(6)	3.817 (1.272-11.452)	0.007
	No (231)	4(1.7)	Referent	
Travel history within last 7 days (n=582)	Yes (270)	19(7)	3.647 (1.478-8.994)	0.003
	No (312)	7(2.2)	Referent	

[a] 95% confidence interval; [b] p values less than 0.05 are bold.

Phylogeny of isolates by maximum likelihood method

Phylogeny of our isolates from dog blood and tick was inferred using 16S rRNA sequences of *B. burgdorferi* s.l. reported on NCBI gene bank. BLAST search of our sequences generated different results and all top hits with maximum percentage identity were selected for investigating phylogeny at nucleotide level. Evolutionary tree was constructed using 20 nucleotide sequences. The percentage of replicate trees in which related sequences clustered together in 1000 bootstrap replications are displayed next to the branches (Felsenstein, 1985). Based on Neighbor-Join and BioNJ algorithms, initial trees for the investigation were attained using Maximum Composite Likelihood method followed by selection of the topology with superior log likelihood value. A sequence of *B. turicatae* (AY93460.1), which is a member of relapsing fever borrelia, was used as outer group. The final tree branched into two groups and three main clades, with bootstrap support varied from 52% to 100% (Fig. 2). Overall, divergence among our sequences varied between 0.9% and 59.8%. Sequences MW547400 and MW547404 clustered in sister clades as both shared 99.1% homology. Isolates MW547399 and MW547402 were 97.4%-98.3% similar to MW547400 and MW547404 and all of these clustered in the same clade with 52 % bootstrap consensus. However, isolate MW547399 shared 98.8% similarity with human isolates from Germany CP028872.1, MW547400 and MW547402 isolates displayed 97.1% and 98.8% resemblance with tick isolates from USA (KU598197.1), while MW547404 showed 97.7% homology with another tick isolate from USA (NR_148750.1). Contrarily, our two isolates MW547405 and MW547401 clustered in a separate branch of the tree. Both were 97.5% identical to

each other and clustered with a tick isolate from France (MW301938.1) showing 59 percent bootstrap consensus. One of our isolates (MW547403) was very divergent from all others and clustered with tick, mouse, and human isolates from Germany (CP019916.1 and CP019844.1), France (CP028861.1), and USA (CP017201.1 and CP031412.1) with 100 percent bootstrap support; this isolate MW547403 shared almost 99% homology with other isolates (Fig. 2).

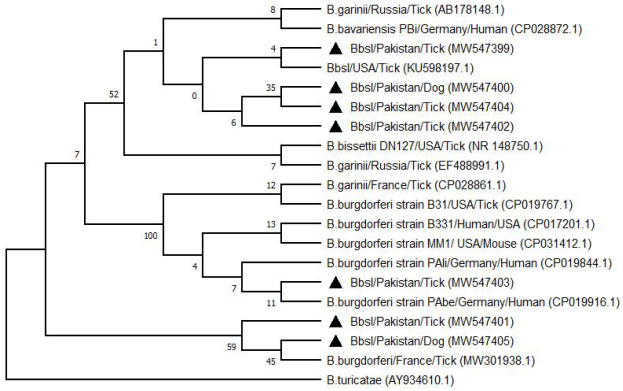


Fig. 2. Phylogenetic relationship of the *Borrelia burgdorferi* sensu lato 16S rRNA gene sequences recovered from pet dogs and their ticks. The tree was drawn using Maximum Likelihood method with 1000 bootstrap replications on MEGA X.

DISCUSSION

Investigation of emerging vector borne zoonotic diseases is a crucial component of one health program. Lyme disease has long been neglected in Pakistan. In the current study, molecular prevalence of Lb and association of its various hypothesized risk factors were estimated in pet dogs and their ticks in the Lahore City metropolitan area. The study presented first molecular evidence of *B. burgdorferi* s.l. in ticks and different dog breeds in Pakistan. 16S rRNA signature nucleotides were detected in our isolates using a special primer set designed by (Marconi and Garon, 1992). This primer set specifically amplifies 16S rRNA signature nucleotides related to all species of *Borrelia* that cause Lyme disease. The authenticity of this primer set is still established and it has been used successfully by many researchers for screening *B. burgdorferi* s.l. in different hosts (Akl *et al*., 2019; Elhelw *et al*., 2021; Liu *et al*., 2017).

This study confirmed that more than 93 percent dogs were infested with *R. sanguineus*. This finding is consistent with previous studies conducted in Pakistan (Bashir *et al*., 2009; Cabezas-Cruz *et al*., 2019; Ul-Hasan *et al*., 2012). With exception to Ul-Hasan *et al*. (2012), all the aforementioned researchers limited their studies to the genus of dog-sourced ticks, which explains why *D. reticulatus* and *H. punctata* species were not reported on dogs in Pakistan. However *Hy. a. anatolicum* which are abundantly found on other animal species including buffaloes, cows, sheep, and goats have been reported by many researchers (Alanazi *et al*., 2021; Batool *et al*., 2019; Ghafar *et al*., 2020). *Ixodes scapularis*, the most important species for *Borrelia* sp. transmission was missing in our sampling. Our study is the first to report that *Hy. a. anatolicum* also infests pet dogs in Lahore. This may be due to shared environment with other animals especially during a religious event, Eid-ul-Adha, in which pet owners buy and keep ruminants at home or in close proximity to pet dogs.

The most favorable predilection site of ticks was inner side of hindlegs (48.5%) and various factors like season, time of tick collection, and inaccessibility for grooming of this body area explain it. On the other side, seasonality of tick recovery revealed that most of the ticks were recovered during summer (56%) and autumn (23.4%). This recovery period coincides with seasonal activity of *R. sanguineus*, which is the most abundant tick species in our samples (Estrada-Peña *et al*., 2004). Interestingly, all of the recovered ticks were adults or nymphs but no larvae. Although nymph, larva, and adult of the most abundant tick species in our sampling, *R. sanguineus*, feed on dogs but no larva was recovered. The reason can be ascribed to the fact that mouth parts of larvae of *R. sanguineus* are much shorter as compared to other ticks like *Ixodes* and thus have difficulty in their attachment (Fourie *et al*., 2013). Moreover, different drop-off rhythm of larvae, nymph, and adult *R. sanguineus* can also explain it (Paz *et al*., 2008).

Overall, our study reported 4.3% and 8.9% positive percentages of *B. burgdorferi* s.l. in dogs and ticks. The most of the literature we reviewed showed that prevalence in dogs was quite high in our study as compared to previous studies which reported 0.1%-5.4% prevalence in dogs (Angelou *et al*., 2019; Galluzzo *et al*., 2020; Movilla *et al*., 2016), whereas 0.3-2.0% in dog-sourced ticks (Geurden *et al*., 2018; Parry, 2016; Zhang *et al*., 2017). Contrarily, some studies also reported higher prevalence of Lb in ticks as 7.3% by Livanova *et al*. (2018) and 18.6% by Yu *et al*. (2016) while in dogs as high as 11% by Beall *et al*. (2008). So, more elaborative studies are imperative to estimate the exact prevalence of Lyme disease in Pakistan. It is also pertinent to mention that *Ixodes* sp. are considered as the principal vector of *B. burgdorferi* s.l. (Michalski *et al*., 2020), whereas many studies, including our study, reported the presence of *B. burgdorferi* s.l. in *R. sanguineus* (Cabezas-Cruz *et al*., 2019; Elhelw *et al*., 2021), though

many of these studies implied that *R. sanguineus* acquired *B. burgdorferi* s.l. by feeding on Lb positive dogs. In addition, a few studies hypothesized and concluded that many factors like high parasite diversity (Betts *et al*., 2018), and co-infection (Cabezas-Cruz *et al*., 2019) may increase ecological innovation in ticks (Cabezas-Cruz *et al*., 2017). Considering these findings, it is indispensable to ascertain the role of *R. sanguineus* in harboring and transmitting *B. burgdorferi* s.l. in future studies.

According to our findings related to the association of various risk factors, likelihood of testing positive to Lb was significantly ($p < 0.05$) associated with age, weight, gender, and breed of dogs. Adult dogs are more prone (OR = 3.9; $p = 0.01$) to the disease probably due to their more active lifestyle. Our study also reported 2.6 times greater odds of testing Lb positive in males than in female dogs and this finding is in line with the finding of a recent study (Galluzzo *et al*., 2020). Likewise, heavy breeds of dogs also had a higher possibility (OR = 3.9; p = 0.01) of being positive to Lb. Interestingly, the same trend was observed when we compared the Lb results in different breeds of dogs individually (Table II). A possible explanation relies on the differences in the susceptibility of different dog breeds to *R. sanguinus* (Louly *et al*., 2009, 2010; Zeringóta *et al*., 2021) and *B. burgdorferi* s.l. (Gerber *et al*., 2007); however, more studies are warranted to explore it further.

Unsurprisingly, the result of our management related hypothesized risk factors confirmed that outside housing, presence of ticks, and travel history were significantly ($p < 0.05$) associated with occurrence of Lb. The reason of this association lies in a simple fact that dogs travelled in recent past or living outside had more exposure of ticks i.e., the main vectors of Lb. Guerra *et al*. (2001) also reported the same findings. We excluded the data of acaricide usage from the final regression model to avoid multicollinearity; however, its association with occurrence of Lb needs to be established in further epidemiological studies.

Phylogenetic analyses of our isolated DNAs present a grim picture of Lb in the study area. Our recovered sequences shared maximum homology with sequences of four main species of Lyme borreliosis namely, *B. burgdorferi sensu stricto*, *B. bavariensis*, *B. garinii*, and *B. bissettii*. Besides, the fact that these previous isolates were sourced from human, illustrates the corresponding zoonotic potential of our isolates. Additionally, our isolates also showed some sequence differences (39%–98.3%) with previously reported sequences on NCBI GenBank. As a previous study has already ascertained that Lb agents evolve differently in different geographies, the genetic modifications found in our isolates highlight the need of further elaborative studies to investigate complete genetic modification and resultant change in pathogenicity.

Limitations of this study include inclusion criteria-based sampling, some missing information related to risk factors, and use of *B. burgdorferi* s.l. specific, rather than species-specific, primers for amplification of the isolated DNAs. The sample selection was based on aforementioned inclusion criteria due to pioneer nature of the study and unavailability of previous data related to presence of *B. burgdorferi* s.l. not only from Lahore, our study area, but also in Pakistan. Non-random sampling limits generalization of results but it is considered necessary for more focused and valid results. Secondly, some information related to risk factors was missed due to difficulty in recalling by some owners as well as hesitation of some owners to answer questions. This limitation was unavoidable because we could not force an owner to share everything related to his pet. Nevertheless, most of the owners provided the information as risk factors data shows (Tables II and III). Lastly, *B. burgdorferi* s.l. specific primer set was used instead of species-specific primers, which could have provided detailed information about circulating species and strains of *Borrelia*; as there are more than 22 species of *Borrelia* in *B. burgdorferi* s.l. group, which causes Lyme disease so future studies are indispensable for detailed genomic analyses of circulating *Borrelia* species in Pakistan.

To conclude, this study reports presence of *B. burgdorferi* s.l. in pet dogs (4.3%) and distribution of ticks on dogs in the Lahore City Metropolitan area, Pakistan. *R. sanguineus* was ubiquitously present on dogs and also found positive for *B. burgdorferi* s.l. A potential association among hypothesized animal and management related risk factors with occurrence of *B. burgdorferi* s.l. was also studied. Furthermore, phylogenetic analyses illustrated that our sequences showed maximum identities with 16S rRNA sequences of *B. burgdorferi sensu stricto*, *B. bavariensis*, *B. garinii*, and *B. bissettii* sourced from different hosts worldwide. Clearly, these results confirm a substantial disease risk of Lb in Pakistan and this risk is likely to become more significant without better surveillance program and availability of adequate diagnostic testing. Therefore, future studies are required to characterize genospecies of *Borrelia*, and investigate unexplored geographical areas and hosts including potential human population at risk, particularly veterinarians and the pet owners.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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