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Zoonotic Implications of (*Borrelia Burgdorferi sensu lato*) in Camel (Camelus Dromedarius) Populations: Molecular Surveillance and Risk Factors Modelling in Semi-Arid Regions of Pakistan

Nadeem Raza
Para Veterinary Institute Karor Lal Eason Layyah Sub Campus, University of Veterinary and Animal Sciences, Lahore, Pakistan
nadeem.raza@uvas.edu.pk
Aneela Zameer Durrani
Department of Veterinary Medicine, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan
Hadia Mubeen
Department of Veterinary Medicine, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences Lahore, Pakistan
Muhammad Rizwan
Multan College of Veterinary Sciences, Multan University of Science and Technology, Multan Pakistan
Sabah Mansoor
Department of Allied Sciences, University of Home Economics, Lahore, Pakistan
Nargis Ambreen
Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore
Abdul Aziz
Department of Veterinary Clinical Sciences, Faculty of Veterinary and Animal Sciences, University of Poonch Rawalakot, Azad Kashmir, Pakistan
Faisal Rasool
Department of Pathobiology, Faculty of Veterinary and Animal Sciences, University of Poonch Rawalakot, Azad Kashmir, Pakistan
Muhammad Khurram
Multan College of Veterinary Sciences, Multan University of Science and Technology, Multan Pakistan
Rustam Ali Balouch
Multan College of Veterinary Sciences, Multan University of Science and Technology, Multan Pakistan

ABSTRACT
The main goal of this study was to find out if Borrelia burgdorferi sensu lato is present in camels from two areas of Punjab, Pakistan. We collected a total of 405 samples from these areas. Gathered information on things that might increase the risk of infection. We used a test to look at the 16S rRNA gene and found that 3.70% of the samples were positive for B. burgdorferi s.l. This means that out of 405 camels, 15 had the infection. When we looked at the genes of the infected camels, we found that they were similar to but not the same as genes from infected animals. We also found that some things, like the age, type, and sex of the camel and whether or not it had ticks, were linked to the infection. This study is the first to show that B. burgdorferi s.l. is present in camels in Pakistan. Our findings suggest that camels might be able to carry and spread the infection to animals and humans.

Keywords: *Camel, B. Burgdorferi S.L., Risk Factors, PCR, And Phylogenetic Analysis.*

Introduction

Livestock is a vital sector that contributes 61.9 percent of the value in the agriculture field and 14.0 percent to the national gross domestic product (GDP), 14.0 percent added during 2021-2022. The gross value of the livestock sector gradually increases from Rs 5,269 billion to Rs 5,441billion. Approximately 8

million families in rural areas are involved with the livestock sector (Ministry of Finance, Pakistan Economic Survey 2021-2022).

Dromedary (*Camelus dromedaries*) is a significant financial livestock animal in Pakistan. The genus Camelus consists of the following three species: *Camelus bactrianus, Camelus bactrianus ferus*, and *Camelus dromedaries* (Liu et al., 2015). The population of camels all around the world is 18.85 million, while in the desert areas of Pakistan, 1.2 million camels are reared. Globally, one-humped camels are 89%, while 11% are two-humped camels (Ministry of Finance, Pakistan Economic Survey 2018-19). There are about 0.328 million houses connected with camel production in Pakistan (Pasha et al., 2013).

Camels play an important role in dairy and meat production all over the world. Several vector-borne and hematophagous arthropod diseases affect camel health, meat, and milk production. In addition, numerous studies reported that camels are infected by various pathogens, including *Borrelia burgdorferi* (Sazmand et al., 2019), *Bartonella, Ana plasma phagocytophilum, Coxiella burnetii, Rickettsia* species, and *Theileria s*pecies (Loftis et al., 2006), about 17% human infectious diseases are caused by vector-borne diseases, and about 700000 deaths occur annually around the world (Athanasiou et al., 2021)**.** The world's most important reported vector-borne disease is borreliosis in camels (Mohammadpour et al., 2020)**.**

Borrelia species spread all over the world and are maintained in the environment within many vectors, avian, reptilian, and mammalian hosts (Brisson et al., 2012). B. burgdorferi spirochete affected numerous species of animals (Comstedt et al., 2006). Borreliosis is a multisystemic, vector-borne disease of animals and humans, which is caused by the *B. burgdorferi s.l* species complex. The Clinical signs and symptoms of this disease include: erythema migrans, fever, anorexia, lymphadenopathy, weight loss, myalgia, and fatigue (Rizzoli et al., 2011), but the nervous and cardiovascular systems, and also joints may be affected (Stanek et al., 2010). Ticks play an important role in the transmission of *B. burgdorferi s.l*, examples are *I. scapularis* in North America, *Ixodes ricinus* in Europe, and *I. persulcatus* in Asia (Zintl et al., 2020).

The occurrence rate of Borreliosis is gradually increasing throughout its environmental ranges worldwide, but there is little proof to support this assessment (Health Protection Surveillance Centre, 2019). The higher occurrence of Lyme disease reported in Slovenia, Austria, Sweden, Belgium, Germany, and the Czech Republic (Sykes and Makiello, 2017) is also reported in neighboring countries in Iran and China in camels (Zhai et al., 2018). Lyme disease is endemic and geographically circulated in central Asia, the USA, and Eastern Europe (WHO, 2020).

To the best of our knowledge, there is no information available regarding the presence of *B. burgdorferi s.l* in camels in Pakistan. Therefore, the objective of this study is to detect the (*B. burgdorferi s.l)* in camels along with risk factor analysis that helps in the circulation of this disease in the study area.

Materials and Methods

Ethical approval

The study was done following the rules and guidelines of the University of Veterinary and Animal Sciences in Lahore, Pakistan. We got permission from the Ethical Committee with a letter (wide letter NO. 894, dated 22-08-2017).

Study area

We did the study in two areas of Punjab, Pakistan: Bhakkar and Bahawalpur. Bhakkar has four areas, and Bahawalpur has five. We collected blood samples from camels in all of these areas. District Bhakar contains the following tehsils: Kaloorkot, Bhakar, Mankera, and Darya khan. District Bahawalpur is located in the southern Punjab. This district has five tehsils: Khairpur Tamewali, Ahmedpur Sharqia, Hasilpur, Yazman, and Bahawalpur.

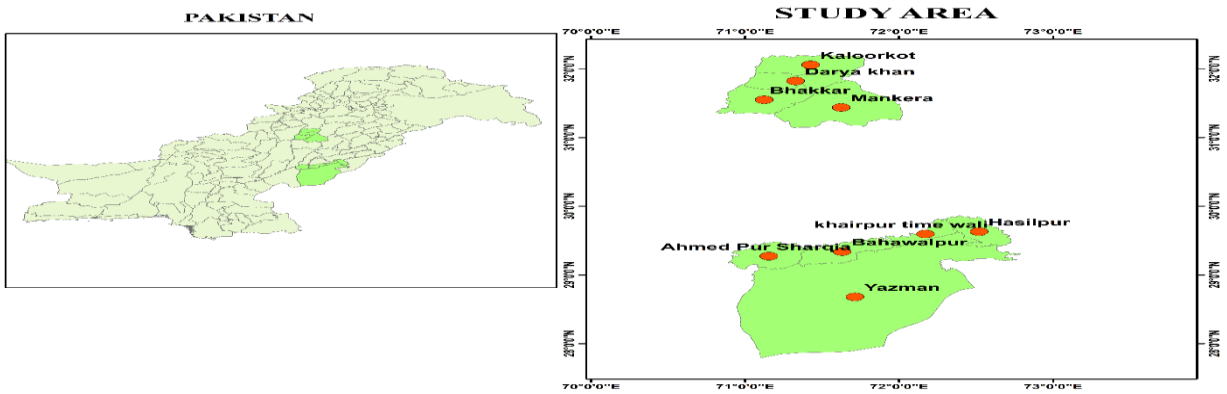


Fig.1. Blood samples collected from camels of marked area of Punjab distracts, Pakistan

Samples collection:

A four hundred and five blood samples were collected from dromedary camels during May 2020 to August 2021. 5ml of blood sample was drawn from the jugular vein of the camel in an EDTA-containing vacutainer (anticoagulant). Each vacutainer was labeled with its information and identification about its collection sites.

DNA Extraction

To test for the infection, we first extracted DNA from 405 of the blood samples using the commercial QIAamp DNeasy Blood and tissue kit (QIAGEN, Maryland, USA). We store the extracted DNA at -20 °C.

Polymerase chain reaction

A conventional PCR was used for the detection of *B. burgdorferi s.l* using primers.

Forward primer BSL 5′ AATAGGTTCTAATAATAGCCTTAATAGC 3′

Reverse primer BSL 5′ CTAGTGTTTTGCCATCTTCTTTGAAA 3′

Amplification of the 16S rRNA gene was performed as described in previous studies (Zhang et al., 2014; Wodecka, 2010; Zhai et al., 2017). PCR reactions were carried out in a thermocycler (Bio-Rad, USA) with a total reaction volume of 30 mL, comprising 15 mL of Master Mix, 1.5 mL of template DNA, 3 mL of each primer (total 6 mL), and 7.5 mL of distilled water. The PCR cycling conditions were as follows:

Initial Denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
95 ^{O}C for 3 minutes	95 ^{O}C for 3 seconds	56 ^{O}C for 30 minutes	72 ^{O}C for 1 minute	32	72 ^{O}C for 15 minutes

Then, the PCR products were separated by 2% Agarose Gel Electrophoresis. 100ml TBE buffer, 2 g powder of agarose gel were mixed in a bottle and heated in an oven for two minutes until a transparent fluid appeared. Then 17 µL ethidium bromide was added and properly mixed. Then, tray rakes and a comb were applied and kept for 10-15 minutes to solidify at room temperature. Then, 3 µl loading dye was added to each sample. The cassette is placed in the assembly, which contains the TBE buffer. DNA has a negative charge, so it must be on the blue negative electrode side because it repels the same charges to each other and moves to the red positive electrode. 3ul drops for dye with PCR product, 8ul, and loading dye sucked with pipette and loaded in the well. 3ul DNA marker was loaded in the first cell, then 120 volts of current was applied for 45 minutes to each sample. After 45 minutes, the gel was taken and observed for final results on the UV illuminator. Positive bands were selected for the sequence analysis.

Sequence Analysis

All obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information website to determine sequence similarity and identity**.**

Statistical analysis

Qualitative data were analyzed using the Chi-square test. The odds ratio (OR) was calculated to evaluate the association between potential risk factors and infection. Statistical analyses were performed using IBM SPSS Statistics (version 20.0.0).

Results

Risk Factors Analysis in the Study Area:

Risk factors were recorded in a field study, which was associated with the detection of *B. burgdorferi s.l* in camels. The results are described in a table. 1.

Table1.Factors associated with the field study with molecular detection of *Borrelia burgdorferi (sl)* in Camels

Risk factors	Category	Total	Positive (%)	Odd ratio	P-value
Gender	Male	170	2 (1.2%)	5.151	0.02
	Female	235	13 (5.5%)		
Species	Marrecha	230	12(5.22%)	3.156	0.05
	Brela	175	3(1.71%)		
Age	<1 year	57	2(3.51%)	2.082	0.0177
	(1-8) years	233	4(1.72%)	1	
	> 8 years	115	9 (7.83%)	4.861	
Herd	Herd 1	60	10(16.67%)	1.8	0.69
	Herd 2	30	3(10%)	1	
	Herd 3	15	2(13.33%)	1.385	
Infestation	Infested	405	303(74.81%)	5.125	<0.0001
	Non infested		102(25.19%)		

P< 0.05 = significant level

The ocThe occurrence of *B. burgdorferi s.l* varies among the genders of camels. It was found that female camels were 5.151 percent more infected than male camels in the study area, as presented in Table 1. It showed that there was a significant ($p < 0.02$) difference between the genders of camels. Our outcomes also showed that a significant ($p < 0.05$) difference was observed between the species of camels. Similarly, the incidence of *B. burgdorferi s.l* was highly prevalent, below one year and above eight years of age of camels as described in Table 1. Our findings also showed that manifestation of *B. burgdorferi s.l* was significantly ($p <0.0001$) associated with the rate of tick infestation in the study area, as described in Table 1.

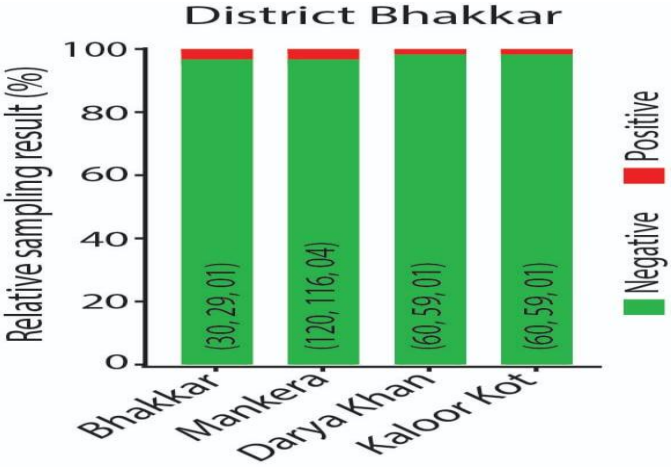
Detection of *B. burgdorferi s.l* in camels

Molecular Detection of *B. burgdorferi s.l* in Camels is described in Table 2.

TABLE 2. Molecular Detection of (*B. burgdorferi s.l*) in two Districts

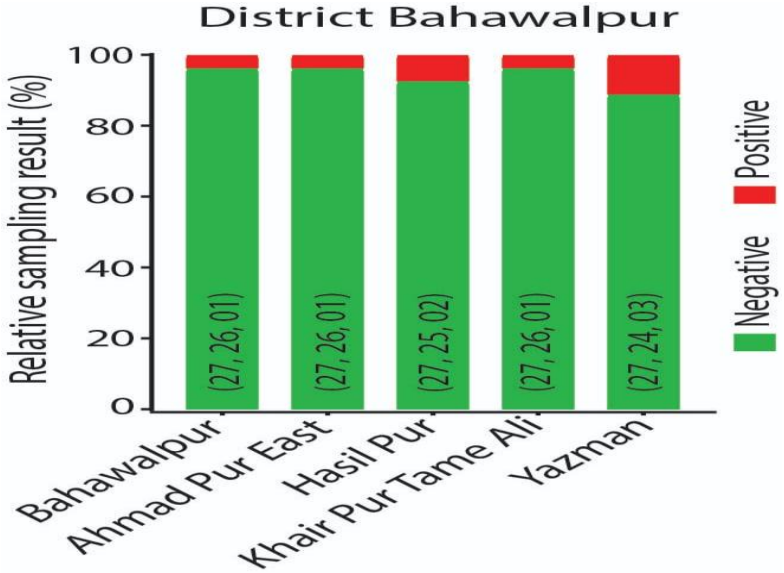
Samples Area (Districts)	Positive	Negative	Total
Bhakkar	7 (2.59%)	263 (97.41%)	270
Bahawalpur	8 (5.93%)	127 (94.07%)	135

Two hundred and seventy blood samples were collected from the district of Bhakkar. Out of 270 blood samples, 2.59% (7/270) of the samples were positive for *B. burgdorferi s.l.,* while 97.41% (263/270) of the blood samples were negative. One hundred and thirty-five blood samples were collected from the district of Bahawalpur. Out of 135 blood samples, 5.93% (8/135) were positive for *B. burgdorferi s.l.,* while 94.07% (127/135) of the samples were negative.



Graph 1: Molecular Detection of (*B. burgdorferi s.l)* from Tehsils of District Bhakkar:

In one area, Bhakkar, we collected 270 blood samples. Found that 2.59% of them were positive for Borrelia burgdorferi sensu lato. In the area of Bahawalpur, we collected 135 blood samples and found that 5.93% of them were positive. The result showed that 135 blood samples were collected from five tehsils of the district of Bahawalpur. The highest positive result was seen from tehsil Yazman 11.11% and tehsil Hasilpur 7.41% followed by tehsil Khairpur Tame Ali, Bahawalpur, and Ahmed Pur East, each showed 3.70% positive for (*B. burgdorferi s.l)* in camel.



Bottom to top from each column values showed: total number of samples, Negative samples, positive samples

Graph 2. Molecular Detection of *Borrelia burgdorferi sensu lato* from the Tehsil of District

Discussion

As we know, the Camels play an important role in meat and milk production. Production and health of camels are highly affected by tick infestation, as described by Nazifi *et al*. (2011). Ticks are a significant vector for Lyme Borreliosis in domestic and wild animals. Most parts of Pakistan, especially desert areas and Cholistan, offer favorable ecological conditions as well as a variety of hosts that can be infected and transfer diseases in animals. The main goal of this study is to detect the presence of (*B. burgdorferi s.l)* in camels, along with risk factor analysis that helps in the circulation of this pathogen in the study area.

In the molecular study, out of two hundred and seventy blood samples from Bhakkar, 2.59% blood samples were positive for (*B. burgdorferi s.l)* in camels, while out of 135 blood samples from Bahawalpur, 5.93% were positive for (*B. burgdorferi s.l)* in camels. Mourad *et al*. (2016) reported in their study that the prevalence rate of (*B. burgdorferi s.l)* 1.85 % was in camels. Zhai *et al*. (2018) reported that blood samples collected from camels, the positive rate found was 3.6% in camels in China. Similar research conducted by Said *et al*. (2016) found that Prevalence rates 1.8% were present in camels. Yang *et al*. (2015) identified

that (*B. burgdorferi s.l)* was (33.2% and 17.4%) between South and North China. Similar results were reported by Ni *et al*. (2014) that the positive rate was 4.15% in animals. Lyme disease was also reported by Letrilliart *et al*. (2005), who showed that the percentage of borreliosis differed in the same province but differed in location within the country. Difference in the percentage of *Borrelia* infection due to climate change, species, sex, age, herd size, and breed. The current study on *Borrelia* pathogens in Pakistan was conducted for the first time at the doctoral level.

During gender wise study, out of 170 blood samples collected from male camels, 1.18% samples were reported positive for (*B. burgdorferi s.l*), while out of 235 blood samples collected from female camels, 5.53% were positive for (*B. burgdorferi s.l)*. Another study in India conducted by Praharaj *et al*., 2008) reported that a higher positivity rate was shown by females as compared to male camel (15.86% and 10.95%). Male camels were fewer in number than female camels in the study area. Mostly camels are reared for milk and meat purposes rather than transportation. The transportation system in camels decreased due to other developed transportation systems.

During the field study, tick-infested camels showed that out of 405 camels, 74.81% were infested with ticks. Tick infestation, as agreed by Said *et al.* (2016), was observed in the study area during the collection of ticks. The findings of the current study are also supported by Elhelw et al (2021), who found that the infestation rate was highly significant in camels, 90%. The number of ticks was reported to be higher in Bahawalpur due to a higher camel population, a low feeding system, and a higher tick load in the study area. In a higher population, feeding management and decreasing the tick infestation rate were difficult. Significant difference (p <0.0001) was observed in tick-infested camels in the study area.

Species-wise study showed that Marrecha camels were 3.156 times more highly infected with (*B. burgdorferi s.l)* than Brela camels. A significant (p <0.05) association was found between the species of camels in the study area. During a herd-wise study, blood samples were collected from different herd sizes of camels. The herd sizes of camels were 60, 30, and 15. In the first type of herd (n=60), 16.67% (10/60) camels were found to be infected with (*B. burgdorferi s.l)*. In the second type of herd (n=30), 10% (3/30) were positive for (*B. burgdorferi s.l).* In the third type of herd (n=15), only two camel 13.33% (2/15) were positive for *B. burgdorferi s.l*. The load of ticks will be higher in big herds. The feeding system is no longer well-suited for larger herds compared to smaller herd sizes. The small size of the herd allows for the control of ticks, and a well-developed feeding system is provided to the camel.

During the age-wise study, out of 57 blood samples collected from less than one-year-old camels, 3.51% were positive for (*B. burgdorferi s.l)*. Meanwhile, when we collected samples from one to 8-year-old camels, the result was 1.72% were positive for *B. burgdorferi s.l*. from 233 blood samples. The percentage increases when we take 115 samples from camels above 8 years old; the results are 7.83% were positive for (*B. burgdorferi s.l).* A greater infection rate was detected in the age group of fifteen to thirty years in both female and male sexes, 18.69% in females and 11.48% in males, reported by Praharaj *et al*. 2008. These findings are supported by Abdalla (2007, who stated that moving camels in this age are more vulnerable to tick infestation. Diab et al (2001) stated that the rate of infestation was high during the months of March to November in Egypt. The age effect on the load of ticks was observed, where older camels carried significantly higher loads of ticks than the younger camels. Significant difference (p <0.0177) of *B. burgdorferi s.l* was observed between different age stages of camels.

When we looked at the areas, we found that some had a higher rate of infection than others. In Bhakkar, the areas of Mankera and Bhakkar had a rate of infection. In Bahawalpur, the area of Yazman had a rate of infection. Borrelia burgdorferi sensu lato was found in camels in all of the areas we studied. This suggests that the infection is widespread and that camels might be playing a role in spreading it to animals and humans.

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