Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Molecular Detection and Characterization of *Coxiella burnetii* and Associated Risk Factors in Camels in Southern Punjab Pakistan

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Abstract

CAMELS are particularly vulnerable to *Coxiellosis* in countries with high camel population densities, where it remains one of the most widespread zoonotic diseases. This study, the first of its kind in Pakistan, aims to assess the incidence of *Coxiellosis*, characterize and analyze *Coxiella burnetii* isolates from camel populations, and investigate co-infection with other vector-borne haemoparasites, along with associated risk factors. A total of 300 clinically suspicious camels, mostly infested with ticks, were selected for blood sampling using a convenience sampling technique. DNA extracted from camel blood and tick samples was subsequently subjected to polymerase chain reaction (PCR). The incidence of *C. burnetii* in camel blood and tick samples in District Bhakkar was 18% and 30.94%, respectively, while in District Bahawalpur it was 21.33% and 34.51%. Phylogenetic analysis of *Coxiella burnetii* isolates from camels revealed similarities to isolates from aborted sheep fetuses in India and goats in Germany. Co-infections with *Trypanosoma*, *Anaplasma*, *Theileria*, and *Babesia* spp. were detected in District Bhakkar at rates of 20%, 10%, 2.66%, and 2%, respectively. Similarly, in District Bahawalpur, co-infection rates with *Trypanosoma*, *Anaplasma*, *Theileria*, and *Babesia* spp. were 24%, 12%, 4%, and 3.3%, respectively. Risk factor analysis indicated that age, sex, history of abortion, body condition and housing type were significant contributors to the occurrence of the disease. The outcomes of current study will help in designing effective control strategies against *C. burnetii* infection in camels of Pakistan.

Keywords: *Coxiella burnetii*, Coinfection, Phylogenetic analysis, Risk factors.

Introduction

Camels are vital to rural livelihoods in developing countries but face health threats from vector-borne protozoan and bacterial infections that reduce productivity [1] *Coxiellosis*, a zoonotic disease caused by *Coxiella burnetii*, significantly affects camel reproductive health [2]. In Pakistan, where about 8 million families rely on livestock for over 35% of their income, such diseases pose serious economic risks [3, 4].

In arid regions, camels provide milk, meat, transport, and medicinal benefits [5]. Camel milk, especially vital during droughts, has greater dairy potential than cow milk [6]. Pakistan annually produces 0.24 million tons of camel milk and 50 thousand tons of meat, with the Mareecha breed known for high milk yield [7].

As concerns over food security rise, camels are increasingly seen as "food security animals" [8]. However, close human-camel interactions and breeder unawareness increase zoonotic risks, challenging the "One Health" framework [9].

C. burnetii causes fever, infertility, abortion, and reduced milk and meat production. Even subclinical infections lead to major economic losses [10]. The bacteria spread via birth products, milk, aerosols, semen, and tick bites [11] [12]. Ticks, particularly *Hyalomma* spp., are key vectors, and camel

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(Received 27 July 2025, accepted 07 November 2025)
DOI: 10.21608/ejvs.2025.407875.2999

movement during rutting season's increases transmission risk [13].

Serological studies report high *C. burnetii* prevalence in camels: 66% in Egypt, 80% in Chad, 62% in Saudi Arabia, and up to 100% in Ethiopia [14] [15]. In Pakistan, *Coxiellosis* remains underreported due to limited diagnostics and misdiagnosis [16] [17].

While earlier Pakistani studies have focused on serology or pooled molecular data, no work has addressed the phylogeny of *C. burnetii* in camels or its co-infection with haemoparasites. This study aims to detect *C. burnetii* at the molecular level, analyze its phylogeny, explore associated risk factors, and assess co-infection with vector-borne haemoparasites in dromedary camels from two key districts of Southern Punjab.

Material and Methods

This study was conducted from July 2022 to June 2023 in the Bhakkar and Bahawalpur districts of Punjab, Pakistan.

A total of 300 clinically suspected camels, mostly tick-infested (150 from each district), were selected using convenience sampling to investigate tick-borne haemoparasites and *Coxiella burnetii* infection. Approximately 5 mL of blood was collected aseptically from the jugular vein using disposable syringes and transported to the lab under a cold chain at 4°C.

For haemoparasite detection, blood smears were prepared on clean slides using the method of Durrani and Goyal [18]. Giemsa's stain (1:10 dilution) was applied for 30 minutes after fixation in methyl alcohol. Smears were examined under a 100x oil immersion lens to identify haemoparasites based on morphology [19].

For molecular detection of *C. burnetii*, DNA was extracted using the GeneJET DNA Purification Kit (Thermo Fisher Scientific, Cat. #K0721). Blood (200 µL) was mixed with 400 µL lysis buffer and 20 µL Proteinase K, incubated at 56°C, washed, and eluted. DNA was quantified using a Nanodrop 2000 spectrophotometer and stored at -20°C [20].

For tick molecular detection, 421 fully engorged female ticks were collected from the camels using forceps and preserved in 70% ethanol. Tick identification to the genus level was done using a stereomicroscope (Olympus, Japan) and morphological keys [21]. Figure (1). All procedures conducted in the Laboratory of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore.

Molecular Investigation and Data Analysis

Ticks processed for DNA extraction using the GeneJET DNA Purification Kit (Thermo Fisher Scientific, Cat. #K0721). Ticks were rinsed with 70% ethanol and sterile water to remove contaminants [22]. Mortars and pestles decontaminated with bleach and sterilized.

Ticks were flash-frozen, ground using chilled equipment, and suspended in 500 µL PBS. A 200 µL aliquot was mixed with 400 µL lysis buffer and 20 µL Proteinase K, vortexed, and incubated at 56°C overnight. DNA was eluted with 200 µL of elution buffer [23]. DNA from both blood and tick samples was quantified using a NanoDrop spectrophotometer (230 nm). Samples with >100 µg DNA were used for PCR.

PCR Analysis

Performed using Thermo Scientific DreamTaq Green PCR Master Mix (2X, Cat. #K1081). The 25 µL reaction mixture contained primers, master mix, DNA, and water. The IS1111 gene of *C. burnetii* was amplified with the following cycle conditions: denaturation at 95°C for 3 minutes, followed by 32 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes.

PCR products were visualized using 1.5% agarose gel electrophoresis and examined under UV light. Positive bands were excised, purified, and sequenced by Applied Biosystems. Sequences were submitted to GenBank, aligned using BioEdit, and analyzed phylogenetically in MEGA 6.0 using Maximum Likelihood with 1000 bootstrap replicates.

Statistical Analysis

Conducted using SPSS 23.0, and associations between risk factors and *C. burnetii* infection assessed using the Pearson chi-square test.

Results

Molecular testing of samples confirmed *Coxiella burnetii* through *the IS1111* transposase gene amplification with an amplicon size of 294 bp. which is represented in Figure 2.

Coxiellosis detected in 59 out of 300 blood samples, showed 19.66% overall occurrence of *Conclusion* in both districts (Bhakkar and Bahawalpur). *C. burnetii* was present in 27 out of 150 blood samples which showed an 18% occurrence of *Coxiellosis* in district Bhakkar. 32 out of 150 blood samples were found positive for *C. burnetii* showing a 21.33% occurrence of *Coxiellosis* in district Bahawalpur. The findings revealed a notable positive percentage of *Coxiellosis* in both Districts Bhakkar and Bahawalpur. Bahawalpur exhibits a slightly higher occurrence compared to Bhakkar (Table 1).

PCR results for tehsil-wise occurrence of *Coxiellosis* in each district revealed 13.33% (4/30) in tehsil Bhakkar, 23.33% (14/60) in tehsil Mankera,

13.33% (4/30) in tehsil Darya Khan and 16.67% (5/30) in tehsil Kaloorkot. Similarly, 6.67% (1/15) in tehsil Bahawalpur, 12% (3/25) in tehsil Ahmadpur east, 26.67% (8/30) in tehsil Hasilpur, 13.33% (4/30) in tehsil Khairpur and 32% (16/50) occurrence were recorded in tehsil Yazman as shown in Table 2.

The graphical representation of the positive percentage of *Coxiella burnetii in* tehsils of District Bhakkar and Bahawalpur is given in Figure 3 which showed the highest positive percentage of *C. burnetii* in tehsil Mankera of District Bhakkar and tehsil Yazman of district Bahawalpur.

χ^2 (8, N = 300) = 11.25, p = 0.188, A chi-square test of independence was conducted to examine the association between tehsil and *Coxiellosis* positivity. The test was not statistically significant, χ^2 (8, N = 300) = 11.25, p = 0.188, suggesting no significant variation in positivity rates among different tehsils.

Figure 4 depicts a phylogenetic tree constructed from identified isolates and sequences already reported in the NCBI-GenBank database. The ID information includes the sample source, country of origin, year of isolation, and accession number. The alignment of our sequences based on their characterization from camel revealed that the sequences were 98% similar to sheep aborted fetus isolates from India and 82% to goat isolates from Germany (Accession number AB8448993.1 and MG385668.1).

For Molecular Detection of *Coxiella burnetii* in Ticks of Camel the tick samples were taken from the same animals that had previously been blood sampled. On genus-based identification, Camels were infested with *Hyalomma* spp and *Rhipicephalus* spp. The overall occurrence of *Hyalomma* and *Rhipicephalus* ticks was 66.66% (140/ 210) and 33.33% (70/210) respectively in district Bhakkar. In District Bahawalpur, the overall occurrence of *Hyalomma* and *Rhipicephalus* ticks were found at 69.67% (147/211) and 30.33% (64/211) respectively as represented in Figure 5 which indicated the highest distribution of *Hyalomma* ticks in the district Bahawalpur and *Rhipicephalus* in district Bhakkar.

The frequency of *Coxiella burnetii* in two tick species, Hyalomma spp., and Rhipicephalus spp., in Districts Bhakkar and Bahawalpur is shown in Table 3. Of the 210 tick samples examined in Bhakkar, 24.28% (51) *Hyalomma* ticks tested positive for *Coxiella burnetii,* while 6.6% (14) *Rhipicephalus* ticks tested positive. On the other hand, out of 211 tick samples obtained in Bahawalpur, 7.5% (16) of *Rhipicephalus* spp. and 27.06% (57) of *Hyalomma* spp. were positive for *Coxiella burnetii*. Tick samples indicated 30.95% occurrence of *Coxiella burnetii* in district Bhakkar and 34.59 % in District Bahawalpur.

Hyalomma spp.χ^2 (1, N = 287) = 0.083, p = 0.773, *Rhipicephalus* spp.: χ^2 (1, N = 134) = 0.236, p = 0.627 Chi-square tests revealed no significant association between district and tick species detection rates.
Infection with one type of disease agent can decrease or increase the likelihood of infection with another. Coinfection with different haemoparasites in the same camel were common in this study. Blood samples from both districts were subjected to microscopic examination to find out the coinfection of *Coxiella burnetii* with other haemoparasites. Results indicated 20% (30/150) occurrence of *Trypanosoma* in Bhakkar and 24 % (36/150) in district Bahawalpur. The overall occurrence of *Trypanosoma* out of 300 samples from both districts was 22%. The study revealed a 10% (15/150) occurrence of *Anaplasma* in Bhakkar and 12 % (18/150) in district Bahawalpur. Out of 300 samples from both districts, the overall occurrence of *Anaplasma* was 11%. Microscopic examination of blood samples revealed a 2.66% (4/150) occurrence of *Theileria* spp. in Bhakkar and 4 % (6/150) in district Bahawalpur. The occurrence of *Theileria* spp. out of 300 samples from both districts was found to be 3.33%. *Babesia* was another tick-borne haemoparasite that was found in camel blood samples. The study revealed a 2% (3/150) occurrence of *Babesia* spp. in district Bhakkar and 3.33 % (5/150) in district Bahawalpur as shown in Table 4. The occurrence of *Babesia* out of 300 samples from both districts was found at 2.66%.

Data on animal age, gender, species, tick presence, abortion history, body condition, feeding system, and housing were collected to assess potential risk factors for *Coxiella burnetii* infection in camels. The occurrence of infection is varied by breed with the Brela breed, being more affected than the Mareecha breed. *C. burnetii* showed no significant variation across breeds ($p>0.05$). The infection rate was 17.27% (19/110) in Mareecha camels and 21.05% (40/190) in Brela camels, with an overall rate of 19.66% (59/300).

Gender was a significant risk factor ($p<0.05$), with more female camels (26.6%, 48/180) infected than males (9.1%, 11/120). Infection rates also increased with age, peaking in camels older than 8 years ($p=0.001$). Older camels had a higher infection rate than those less than 1 year.

Ticks were evaluated as potential vectors, but their association with infection was not significant ($p=0.1$). Camels with tick infestations had a slightly higher infection rate, but tick history did not show a significant effect (20.41% vs. 16.66%).

Abortion history was significantly associated with *C. burnetii* ($p<0.05$). Infected camels with a history of abortion had a higher prevalence (25.55%, 46/180) compared to those without (10.83%,

13/120), indicating that abortion history is a key risk factor.

Feeding management (grazing, stall feeding, or both) did not show a significant correlation with infection (p=0.103). However, body condition was a significant factor (p=0.004), with emaciated camels having a higher infection rate (33.3%, 30/90) compared to healthy (14.2%, 20/140) or fatty camels (12.85%, 9/70).

Housing type was also a significant risk factor (p=0.045). Camels housed in confined spaces had a higher infection rate (26.66%, 32/120) compared to those in open housing (15%, 27/180). This suggests that confined housing increases the risk of *C. burnetii* infection. The association of various risk factors with disease occurrence has been shown in Table 5.

Discussion

The occurrence of *C. burnetii* in camels has been reported to vary by geographical region. In the current study, the overall PCR based prevalence of *C. burnetii* was reported 19.66% in camel blood samples. Similar to the previous findings which represented the molecular prevalence of *C. burnetii* 10.76% in Iran and 16.05% in Pakistan, respectively [24] [9]. However, conflicting with present study, a higher seroprevalence (62%) was reported in Saudi Arabia [2] and (66%) in Egypt [14]. The higher prevalence in those regions may reflect greater camel susceptibility and vector exposure. Our detection rate could be influenced by targeted sampling and camel susceptibility, alongside tick vector preferences. The findings also revealed 30.94% occurrence of *Coxiella burnetii* in ticks in district Bhakkar and 34.51% in District Bahawalpur, supporting earlier research [25]. The co-infection of *Coxiella burnetii* with other tick-borne parasites in camel has also been reported. These findings are in agreement with the previously conducted studies [26] [27] [28] [29] in which the co-infection of *Coxiella burnetii* with other tick-borne pathogens was found in tick species.

However, this is the first study conducted in Pakistan to investigate the co-infection of coxiellosis with other hemoparasitic diseases in the camel population. This presence of co-infection was explained by the presence of heavy tick infestation in the camel population of Southern Punjab, which poses zoonotic risks. The current study also confirmed the link between Coxiellosis and various animal-related risk factors. Tick infestation was a major contributor, consistent with the previous findings which indicated ticks as reservoirs and vectors of *C. burnetii* [30]. Higher infection rates were observed in animals currently infested with ticks compared to those with a past history of infestation, as previously observed in a study in which tick infestation had been identified as a potential risk factor associated with disease occurrence [9]. The current report also indicated the relationship of breed of animal with the occurrence of coxiellosis in camels. According to the current study findings, the Brela camel breed had a higher infection rate (21.0%) than Mareecha camels (17.3%), which alighns with earlier research in which higher seroprevalence rate (38.9%) was observed in Barela than in Marecha (28.6%) [9]. The animal's gender was found statistically significant ($p<0.05$) in disease occurrence. In this study, higher disease prevalence was observed in female camels (26.6%) as compared to male camels (9.1%) consistent with prior research [9]. Females, particularly older females are more vulnerable, possibly due to *C. burnetii's* preference for the Placenta, udder, and other reproductive tissues.

The disease occurrence was higher in older camels than in younger camels under one year of age. The results revealed a higher prevalence (38.57%) in aged camels (> 8 years), which was in agreement with the prior studies [31][32]. This may perhaps be conceivable because exposure time to infection increases with age. A history of abortion was significantly associated with *C. burnetii* infection in the current study, alighning with the previously conducted research [33] while conflicting with the findings of earlier research which showed no association between abortion history and disease occurrence [9]. This might be due to various factors such as management practices and immune status of animals.

Body condition was critical, with emaciated camels showed significantly higher infection rates than healthy or well-conditioned ones, contradicts with the previous findings in which no significant association between body condition and disease was reported [9]. Poor nutrition and weakened immunity may contribute to this vulnerability. Finally, husbandry practices influenced disease occurrence. Camels in confined housing had a higher infection rate than those in open environments, supporting studies linking intensive husbandry to higher disease prevalence due to close contact and shared resources. The assessment of risk factors showed that body condition, abortion history, and housing type were significant determinants influencing disease dynamics. The findings of this study will help in designing effective control strategies against *C. burnetii* infection in camels of Pakistan.

Conclusion

The findings of the current study confirm the presence and circulation of *Coxiella burnetii* among the camel population in Southern Punjab, highlighting its role as a significant source of Q fever outbreaks in both rural and urban human populations. Camels infected with *Coxiellosis* were frequently co-infected with other vector-borne hemoparasites, compounding the severity of the disease and contributing to substantial production losses. Given

the complexity of *C. burnetii* transmission and persistence in the environment, complete eradication remains challenging. Therefore, emphasis should be placed on improving management practices and biosecurity measures to mitigate the spread. Since ticks serve as the primary vectors for disease transmission, future research should focus on evaluating the effectiveness of various tick control strategies and their role in reducing the incidence of *Coxiellosis* in camels.

Acknowledgments

The authors acknowledge the expert guidance and technical support provided by supervisory committee and Dr Zahid Iqbal which significantly contributed to the conception, design and execution of this study.

Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

The present study was conducted in acquiescence with the official ethics committee's recommendations Office Of Research Innovation And Commercialization University of Veterinary and Animal Sciences, Lahore, Punjab, Pakistan (No.DR/ 332/ 08/2021).

TABLE 1. Molecular Detection of Q fever in District Bhakkar and Bahawalpur

Sample Area (Districts)	Positive	Negative	Total
Bhakkar	27(18%)	123(82%)	150
Bahawalpur	32(21.33%)	118(78.66%)	150

$\chi^2 = 0.592$, p-value = 0.441: The p-value > 0.05 indicates that the difference in Q fever positivity between Bhakkar and Bahawalpur is not statistically significant.

TABLE 2. Tehsil-wise distribution of *Conclusion* in District Bhakkar and Bahawalpur

District	Tehsil	Positive	Negative	Total
Bhakkar	Bhakkar	4 (13.33%)	26 (86.67%)	30
	Mankera	14 (23.33%)	46 (76.67%)	60
	Darya khan	4 (13.33%)	26 (86.67%)	30
	Kaloorkot	5 (16.67%)	25 (83.33%)	30
Bahawalpur	Bahawalpur	1(6.67%)	14(93.33%)	15
	Ahmedpur East	3(12%)	22(88%)	25
	Hasilpur	8(26.67%)	22(73.33%)	30
	Khairpur Tame wali	4 (13.33%)	26(86.67%)	30
	Yazman	16(32%)	34(68%)	50

TABLE 3. Positive percentage of *Coxiella burnetii* in ticks

District	Total Sample	*Hyalomma spp*			*Rhipicephalus spp.*		
		Positive	Negative	Positive percentage (%)	Positive	Negative	Positive percentage (%)
Bhakkar	210	51	89	24.28	14	56	6.66
Bahawalpur	211	57	90	27.01	16	48	7.5

TABLE 4. Co-infection of *Coxiella burnetii* with other tick-borne haemoparasites

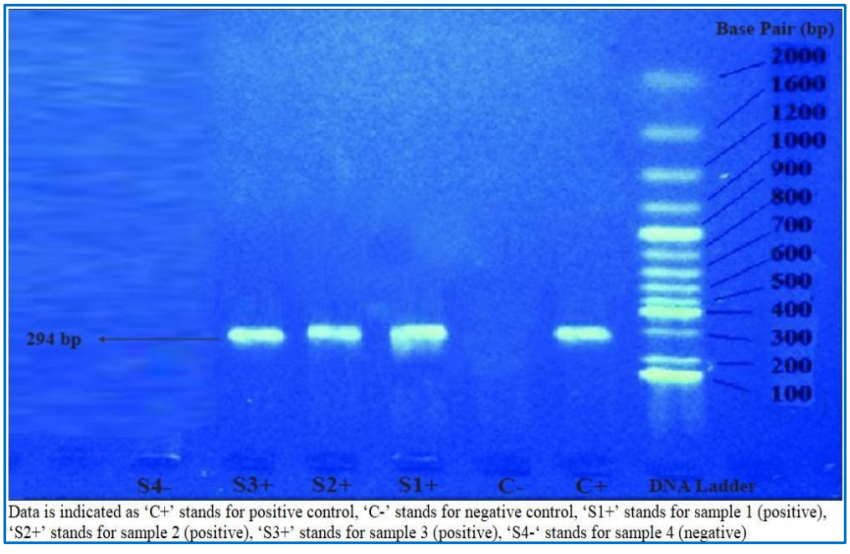
Sample area (District)	Total Sample N=300	Status	*Trypanosoma* spp.	*Anaplasma* spp.	*Theileria* spp.	*Babesia* spp.
Bhakkar	n=150	Positive	30	15	4	3
		Negative	120	135	146	147
		Occurrence	20%	10%	2.66%	2%
Bahawalpur	n=150	Positive	36	18	6	5
		Negative	114	132	144	145
		Occurrence	24%	12%	4%	3.33%

TABLE 5. Association of Risk Factors with the occurrence of *Coxiellosis* in camel.

Study Variable	Category	No of samples	Observed Positive samples	Expected Positive samples	Observed Negative samples	Expected Negative samples	P value
Specie/Breed	Mareecha	110	19	17.27	91	82.7	
	Brela	190	40	21.05	150	78.95	0.73
Sex	Male	120	11	9.16	109	90.8	
	Female	180	48	26.66	132	73.33	0.001
Age	< 1 years	120	6	5	114	95	
	1-8 years	110	26	23.63	84	76.36	0.001
	>8 years	70	27	38.57	43	61.42	
Ticks' infestation	Yes	240	49	20.41	191	79.58	
	No, but previous history of tick infestation	60	10	16.66	50	83.33	0.1
Abortion history	Aborted	180	46	25.55	134	74.44	
	Non-Aborted	120	13	10.83	107	89.16	0.004
Feeding	Grazing	160	41	25.62	119	74.37	
	Grazing +Stall Feeding	110	14	12.72	96	87.27	0.103
	Stall Feeding	30	4	13.33	26	86.66	
Body Condition	Emaciated	90	30	33.33	60	66.66	
	Healthy	140	20	14.28	120	85.71	0.004
	Fatty	70	9	12.85	61	87.14	
Housing	Confined	120	32	26.66	88	73.33	
	Open	180	27	15	153	85	0.045

P-value (p<0.05) indicates significant result**s.**

Fig. 1. Morphological identification (Dorsal and Ventral view) of Ticks under stereomicroscope



Data is indicated as 'C+' stands for positive control, 'C-' stands for negative control, 'S1+' stands for sample 1 (positive), 'S2+' stands for sample 2 (positive), 'S3+' stands for sample 3 (positive), 'S4-' stands for sample 4 (negative)

Fig. 2. Amplification of *IS1111* transposase gene with an amplicon size of 294 bp

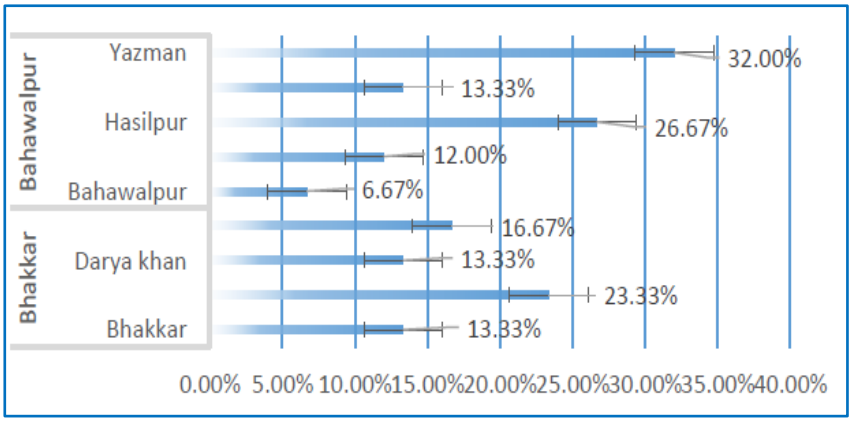


Fig. 3. Tehsil-wise distribution (Positive percentage) of *Coxiella burnetii* in District Bhakkar and Bahawalpur

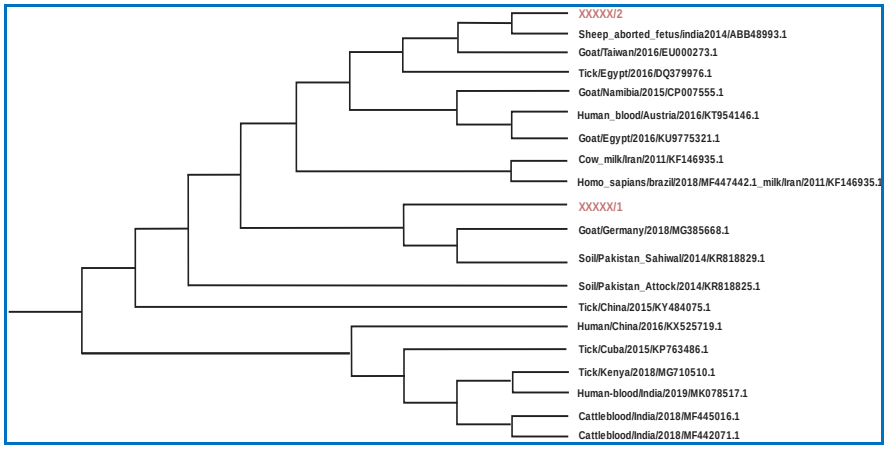


Fig. 4. Phylogenetic relationship of Camel isolates based on *IS1111* gene of *Coxiella burnetii*

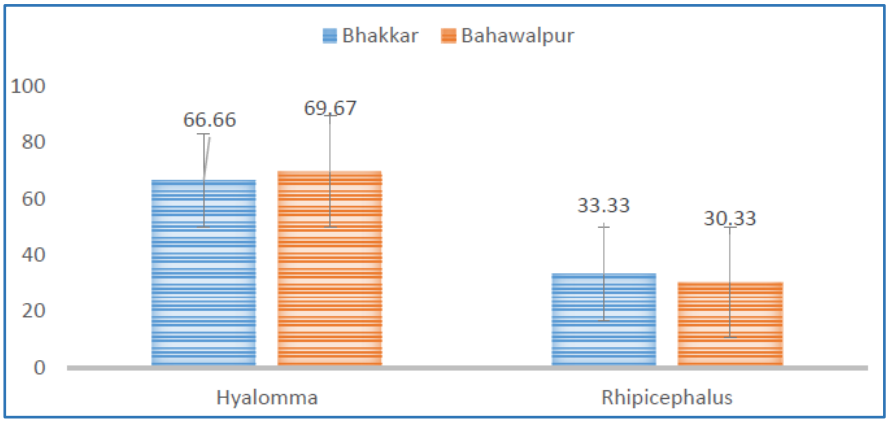


Fig. 5. Distributions of Ticks in District Bhakkar and Bahawalpur

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