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Molecular Identification of *Coxiella burnetii*, and Incidence and Risk Factors of Coxiellosis in Bovines of Punjab, Pakistan

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ABSTRACT

Coxiella burnetii causes query (Q) fever in bovines. Its role has never been elucidated in bovines from Pakistan. The current study was designed to determine the incidence of coxiellosis in bovines, evaluate the association of various risk factors in the occurrence of *Coxiella burnetii*, determine phylogeny and genetic variability of various isolates identified during the study, and report hematology of the affected bovines. The incidence of coxiellosis was estimated as 32.12% and 12.5% in cows and buffaloes, respectively. The association of selected clinical biomarkers was also ascertained and all of these were found significantly (p<0.05) associated with the incidence of coxiellosis. The results of animal-related risk factors indicated that with increasing age and parity number, poor BCS, or a history of disease, the incidence of coxiellosis became significantly (p<0.05) higher. Various farm-related risk factors were also found significantly (p<0.05) associated with Coxiellosis. Moreover, phylogeny of isolated bacteria showed genetic variability (47%-99%). Lastly, the hematology profile of *Coxiella burnetii* positive buffaloes showed significant (p<0.05) lymphocytopenia (45.95 ± 13.4), neutrophilia (32.80 ± 11.75), leukocytosis (11.25 ± 3.62), eosinopenia (4.95 ± 2.14), thrombocytopenia (232.50 ± 211.74), and decreased hemoglobin (7.005 ± 0.90) and PCV levels (21.29 ± 2.28). Whereas in affected cows only low hemoglobin level (7.25 ± 1.18), decreased PCV (22.29 ± 3.34), eosinopenia (9.6 ± 5.49), and thrombocytopenia (365.25 ± 227.32) were statistically significant (p<0.05). As far as we know, this study provides the first molecular evidence of coxiellosis in bovines from Pakistan.

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Authors' Contribution
MZI conducted the research. AZD conceptualized and supervised the study. JAK and NA were members of supervisory committee. MU assisted in data analysis and write up. AJ helped in designing map. SU and AA helped in in laboratory work. MU, NR and AH collected and processed samples.

Key words
***Coxiella burnetii*, Molecular evidence, Bovines, Risk factors, Phylogeny, Hematology.**

INTRODUCTION

Coxiellosis in ruminants is caused by *Coxiella burnetii*, an intracellular, Gram-negative, coccobacilli bacterium, that is highly zoonotic in nature and is categorized as type B biological warfare agent (Shabbir *et al*., 2016). *Coxiellosis* distributed across the globe except in New Zealand and Antarctica and recent epidemiological studies have shown raising public health apprehension (Cruz *et al*., 2018; Cardinale *et al*., 2014).

Pakistan has almost 47.80 million cattle and 40.0 million buffalo and both species are the major contributor in livestock economy (11.2 % share in GDP) of the country (GoP, 2019). In Pakistan, the first case of Coxiellosis was reported in camel in 1955. *C. burnetii* causes infertility, premature birth, dead or weak calves, sporadic abortions with necrotizing placentitis and sub clinical mastitis in ruminants and resultant gigantic economic impact (Guatteo *et al*., 2010). Clinically infected cows may also develop infertility, metritis and mastitis. Cows have been known to shed *C. burnetii* in milk for up to 32 months, which indicates its public health significance (Grist, 1959).

Due to the lack of diagnostic support, the cases of Q fever remain undiagnosed and tend to be clustered with other diseases like fever and abortions in Pakistan (Shabbir *et al*., 2016). According to literature, the prevalence of coxiellosis ranges from 4.6% to 40% in all livestock species and 10.2% to 26.8% in humans (Ullah *et al*., 2019). To the best of our knowledge, only two studies regarding *C. burnetii* in cattle and buffalo are available since 1955 to 2019 and in those studies this antigen was detected serologically (ELISA and CFT). Currently, not a single molecular study on coxiellosis in cattle and buffalo

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is available from Pakistan.

Keeping in view the previously mentioned paucities and dearth in coxiellosis studies in Pakistan, the current study was designed to investigate molecular evidence of *C. burnetii* in cows and buffaloes. In addition, different associated risk factors were also taken into account. The study will lead to establish diagnosis of coxiellosis in Pakistan and provide a needful insight to policy makers because coxiellosis is not only an issue of livestock sector but also a major public health concern.

MATERIALS AND METHODS

Study area and target population

Cattle (n=160) and buffaloes (n=160) were selected from the 09 Union councils (Pattoki I, Pattoki II, Pattoki III, Wan Adhan, Shahikham, Ghumanke, Awan Chak, Baharwal, and Rodhay) located in close proximity to Veterinary Teaching Hospital, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, District Kasur Pakistan. Most cases of subclinical mastitis, endometritis and abortion were reported from these UCs (Fig. 1).

Inclusion criteria for selecting animals were tick infestation or its history, subclinical mastitis, endometritis, and abortion or recent history of these problems. The animals that fulfilled any of the two criteria were selected.

Collection of samples

A total of 320 blood samples (n=160 cows, n=160 buffaloes) were collected aseptically in plain vacutainers and after proper labeling transported to Medicine Laboratory, University of Veterinary and Animal Sciences, Lahore, where these samples were immediately centrifuged for separation of buffy coat and stored at -20°C until further processing. Sampling for the second phase were conducted from *C. burnetii* positive (n=40) and negative (n=40) cows and buffaloes after confirmation of coxiellosis during the first phase of study. These samples were collected in EDTA mixed vacutainers and immediately processed for hematology. During sample collection, information about all the potential risk factors was also collected in a questionnaire.

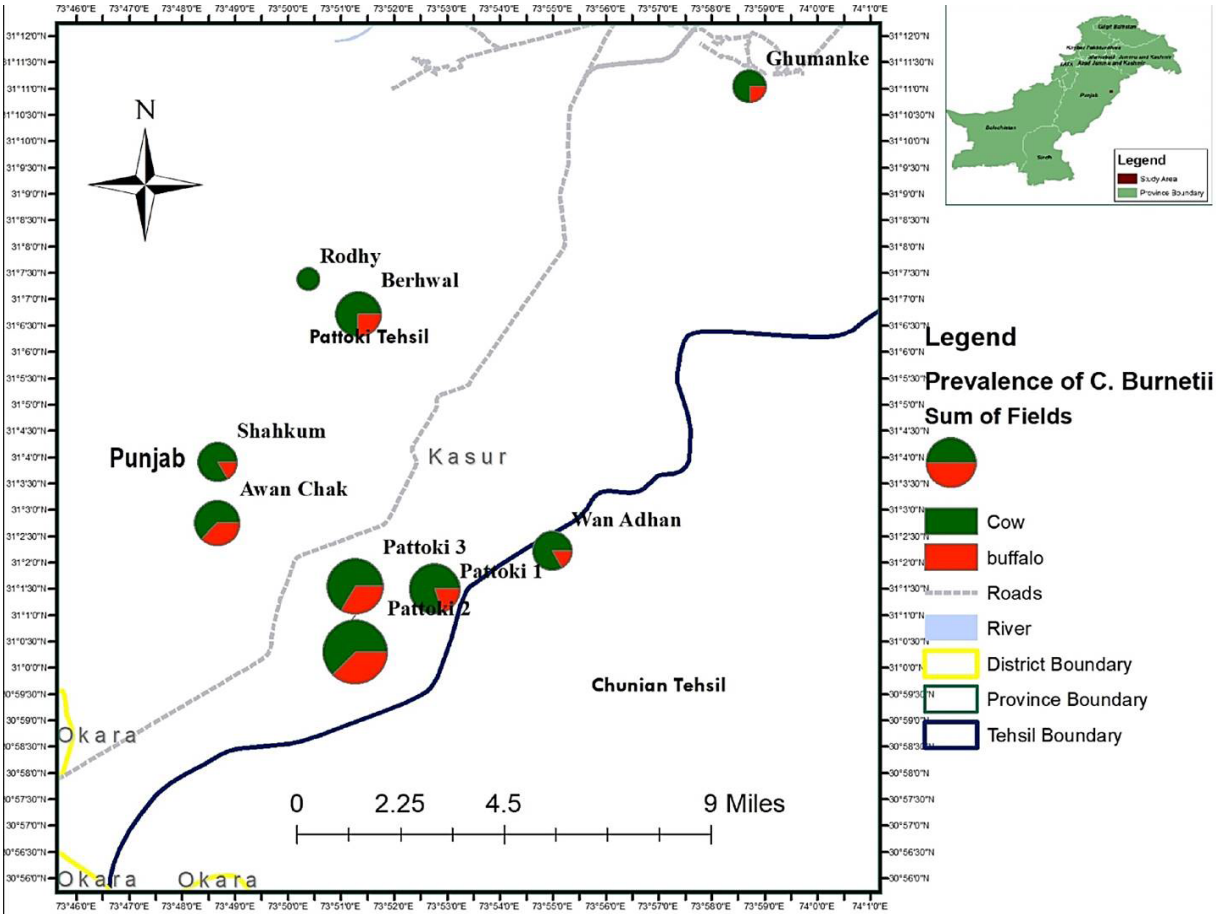

Fig. 1. Map showing the prevalence of coxiellosis in study area.

Molecular identification and phylogenetic analysis of C. burnetii

The genomic DNA from the samples was extracted using Thermo Scientific GeneJET Genomic DNA purification kit (catalogue # K0721) according to manufacturer's guidelines. In 200 µL serum, 400 µL lysis and 20 µL of Proteinase K solution were added and mixed by vortexing. The incubation was done at 56°C for 10 min and then 500 µL wash buffer 1 and 2 were added according to guidelines given. At the end, 200 µL elution buffer was added to elute the genomic DNA. The concentration of the genomic DNA was measured by using spectrophotometer (Nanodrop, USA).

The amplification of the DNA fragment was done by using highly specific *C. burnetii* primers targeting IS1111 (Transposase gene) by conventional PCR. The primers IS1111 F (5'-CGCAGCACGTCAAACCG-3') and IS1111 R (5'-TATCTTTAACAGCGCTTGAACGTC-3') were designed to generate 294 bp amplification fragment.

PCR was performed using Thermo Scientific™ DreamTaq Green PCR Master Mix (2X) (catalog number K1081). A total volume of 25 µl reaction mixture was composed of forward primer 1.25 µl, reverse primer 1.25 µl, Master mix 12.5 µl, DNA 2 µl, and water 8 µl. The thermal cycle comprised initial denaturation at 95C for 3 min followed by 32 cycles, each of denaturation at 95C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. It was followed by final extension at 72°C for 7 min. Prior to our sample analysis, the assay was optimized and validated using Amplirun® *Coxiella burnetii* DNA Control (catalogue number MBC018).

The resultant PCR product was further evaluated with agarose gel electrophoresis (1.5%) with apposite controls and a 50bp ladder (Invitrogen Co. Carlsbad, CA, USA). The PCR products of IS1111 transpose gene were submitted for DNA sequencing to Applied Biosystems, Foster City, CA, USA. The resulting sequences will be submitted to NCBI gene bank after the publication of this paper. These sequences were edited and aligned using BioEdit software version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/page2.html). If many sequences were found similar in alignment then only one of these sequences was processed for phylogeny. The phylogenetic tree was constructed by comparing with the already reported *C. burnetti* strains from Pakistan and other countries on GenBank NCBI. The analysis was done using Maximum Likelihood statistical method and Tamura-Nei Model and with the bootstrap analysis for 1000 replicates onto MEGA version 6.0 software (Tamura *et al*., 2013).

Statistical analysis

Pearson Chi-square test was applied to determine the association with potential risk factors. The odds ratio was also calculated by Fisher's exact test in order to find out the probability of risk within the study area and other risk factors. Hematology results were tested using two sample t-test. Level of significance was accepted at 95% confidence interval in every method. Statistical analysis was performed at IBM SPSS version 23.0 software.

RESULTS

The study reported an overall 22.5% prevalence of coxiellosis in bovines from the study area. The prevalence was higher in cows (32.12%) than in buffaloes (12.5%). Samples were collected from nine UCs and there were significant (p>0.05) differences in the prevalence of coxiellosis in all UCs (Table I). Table I shows the prevalence of coxiellosis in different UCs.

Table I.- Area wise positive percentage of coxiellosis in bovines.

Area	n	Positive cases (%)
UC1	36	12 (33.3)
UC2	36	10 (27.8)
UC3	36	6 (16.7)
UC4	36	8 (22.2)
UC5	36	16 (44.4)
UC6	36	8 (22.2)
UC7	36	6 (16.7)
UC8	34	4 (11.8)
UC9	34	2 (5.9)

Association of clinical biomarkers with occurrence of coxiellosis

Association of five potential clinical biomarkers of coxiellosis in bovines was studied and results showed that all of these *i.e.,* subclinical mastitis, abortion, post-abortion infertility, subclinical mastitis and abortion, subclinical mastitis and post-abortion infertility, were significantly (p>0.05) associated with coxiellosis (Table II).

Association of these clinical biomarkers was also computed with Fisher's exact test and odds of having coxiellosis were calculated. Risk of occurrence of coxiellosis was 6.3 times more in animals with subclinical mastitis than those without it. Animals having abortion were 5.9 times more likely to be infected with coxiellosis than those without it. Odd ratio of animals with post-abortion infertility showed 13 times more likelihood of having coxiellosis than animals, which did not have this problem. The risk of coxiellosis was 11 times more in animals suffering from subclinical mastitis and abortion in comparison with those that did not suffer from these issues.

The most significant (p>0.05) finding was in bovines which had subclinical mastitis as well as post-abortion infertility. In this case, risk of being *C. burnetii* positive was 20.8 times more when compared with animals without these problems (Table II).

Table II.- Association of clinical biomarkers with occurrence of coxiellosis.

Clinical biomarkers	Status	Positive % (positive cases/ total cases)	Odd ratio	p value
Subclinical mastitis	Yes	25% (70/280)	6.3	0.003
	No	5% (2/40)	1	
Abortion	Yes	26.9% (68/252)	5.9	<0.0001
	No	5.8% (4/68)	1	
Post-abortion infertility	Yes	43.7% (62/142)	13	<0.0001
	No	5.6% (10/178	1	
Subclinical mastitis and abortion	Yes	34.7% (66/190)	11	<0.0001
	No	4.6% (06/130)	1	
Subclinical mastitis and post-abortion infertility	Yes	55.6% (60/108)	20.8	<0.0001
	No	5.7% (12/212)	1	

Association of animal related risk factors with coxiellosis

Association of age, species, parity number, disease history, and body condition score (BCS) as animal related risk factors with occurrence of coxiellosis in bovines was verified (Table III). The occurrence of coxiellosis significantly (p<0.05) increased with age and became 252 times more in animals above ten years of age. Similarly, prevalence of coxiellosis varies significantly (p<0.05) with species and cows were 3.4 times more at risk than buffaloes. Interestingly, parity number also found to be significantly (p<0.05) associated with coxiellosis but only after the second parity and became highest at 5th parity with 19 times more probability of having coxiellosis. Animals with disease history were also 2.9 times more at risk of having coxiellosis than healthy ones. Similarly, BCS 1 was significantly (p<0.05) associated with coxiellosis however BCS 2 and 3 showed non-significant (p>0.05) association. Chances of coxiellosis were 6.4 times more in BCS 1 animals than those with BCS 3. And, finally, tick infestation or a previous history of it were also significantly (p<0.05) associated with coxiellosis and, interestingly, animals with previous history of tick infestation were 2.3 times more prone to coxiellosis than those which had tick infestation at the time of sampling (Table III).

Association of coxiellosis with risk factors related to management practices at farms

Data of different risk factors related to management practices at farms were also collected in a predesigned questionnaire. These data were evaluated for computing statistical association with the occurrence of coxiellosis. From different hypothesized risk factors, type of farm *i.e.,* smallholder, commercial, or progressive, presence of goat and dog, and carcass disposal *i.e.,* burning or burial, were found non-significantly (p>0.05) associated with occurrence of coxiellosis in bovines. The rest of risk factors showed mixed results (Table IV).

Table III.- Association of animal related risk factors with coxiellosis in bovines.

Risk factor	Components	Positive % (positive cases/ total cases)	Odd ratio	p value
Age	1-5	4.8% (8/134)	1	
	5-10	21.1% (32/152)	4.2	<0.001
	>10	94.1% (32/34)	252	<0.001
Species	Cattle	32.5% (52/160)	3.4	<0.001
	Buffalo	12.5% (20/160)	1	
Parity	1st	5.2% (4/76)		
	2nd	6.3% (5/80)	1.2	0.9
	3rd	19.2% (10/52)	4.3	0.02
	4th	40% (16/40)	12	<0.001
	5th	51.4% (37/72)	19	<0.001
Disease history	Yes	34.4% (42/122)	2.9	<0.001
	No	15.1% (30/198)	1	
Body condition Score	1 (1.5-2.5) or Emaciated	34.3% (44/128)	6.4	<0.001
	2 (2.5-3.5) Healthy condition	15% (12/80)	1.9	0.1
	3 (3.5 to 4.5) Fatty condition	14.3% (16/112)	1	
Ticks Infestation	Yes	10% (28/280)		0.1
	No, but previous history of tick infestation	20% (8/40)	2.3	

Type of feeding was found significantly (p<0.05) associated with coxiellosis and farms which practiced grazing were 28.7 times more at risk of having *C. burnetii* positive animals than farms exercising stall-feeding. Similarly, drinking water source was also significantly (p<0.05) associated with our disease of concern and probability of risk was 13.75 times more in farms which offered canal water than those which offered only underground water to their inhabitant animals. The most notable result was significant (p<0.05) association of

Table IV.- Association of coxiellosis with risk factors related to management practices at farms.

Risk factors	Components	Positive % (positive cases/ total cases)	Odd ratio	p value
Type of farm	Small holder	24.3 % (34/140)	3.8	0.3
	Commercial	14.2% (1/7)	2	0.9
	Progressive	7.7% (1/13)	1	
Feeding	Grazing	85.7% (6/7)	28.7	0.001
	Grazing+Stall	25% (18/72)	1.6	0.3
	Stall feeding	17.3% (14/81)	1	
Drinking water source	Canal	73.3% (11/15)	13.75	<0.001
	Both	31% (17/55)	2.2	0.06
	Underground	16.7% (15/90)	1	
Sheep	Presence	41.8% (28/67)	7.6	<0.001
	Absence	8.6% (8/93)	1	
Goat	Presence	21.6% (19/88)	1	0.8
	Absence	23.6% (17/72)	1.12	
Dog	Presence	26% (19/73)	1	0.34
	Absence	19.5% (17/87)	1.5	
Disinfection at farm	Presence	3.5% (1/28)	1	0.006
	Absence	26.5% (35/132)	9.7	
Carcass disposal	Burning	19.5% (23/118)	1	0.13
	Burial	31% (13/42)	1.8	
Manure management	Yes	17.6% (22/125)	1	0.01
	No	40% (14/35)	3.1	
Separate parturition area	Yes	9% (6/67)	1	<0.001
	No	32.3% (30/93)	4.8	

coxiellosis with the presence of sheep at farm. Animals living in proximity to sheep within the same farms were 7.6 times more at risk than those that lived in solitary farms. Disinfection, manure and parturition management were significantly (p<0.05) associated with coxiellosis and animals residing in farms which lack these practices were 9.7, 3.1, and 4.8 times more prone to coxiellosis, respectively (Table IV).

Phylogenetic analysis

A phylogenetic tree of our isolates and along with those in GenBank database is depicted in Figure 2. When our sequences were aligned based on their characterization from buffaloes and cattle, it was found that sequences isolated from the same species were 100 percent similar. Hence, only one sequence from each species was included to determine phylogeny of *C. burnetii* isolated from bovines in our study area. The strain *C. burnetti* Pak/buffalo/blood isolated from blood of buffalo is labelled with red triangle while the strain *C. burnetti* Pak/cattle/blood isolated from blood of cattle is labelled with blue triangle. Our isolate *C. burnetti* Pak/cattle/blood falls in the same clade with the strains isolated from cattle blood (accession No. MF445016.1) and human blood (accession No. MK078517.1) samples in India. While the isolate *C. burnetti* Pak/buffalo/blood falls in different clade with the strains isolated from the environmental samples from different regions of Pakistan and with the isolates from goat and bovine milk samples of Indian origin.

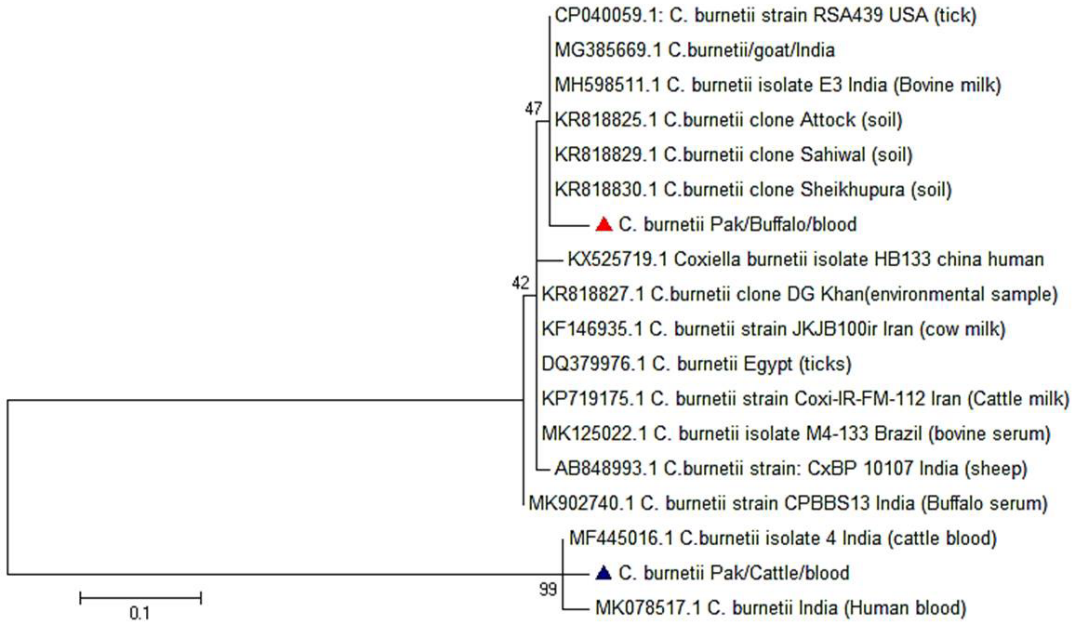

Fig. 2. Phylogenetic tree of isolated sequences of IS1111 gene of *Coxiella burnetii* and other reported sequences.

Table V.- Hematological parameters (Mean ± SD) in *C. burnetii* positive and negative cows and buffaloes.

Parameters	Buffalo			Cows		
	Positive cases	Negative cases	p value	Positive cases	Negative cases	p value
RBCs ($\times 10^6$/µl)	7.545 ± 1.27	7.705 ± 1.31	0.69	7.35 ± 1.52	6.99 ± 1.63	0.47
HGB (g/dl)	7.005 ± 0.90	9.80 ± 2.59	<0.001	7.25 ± 1.18	11.36 ± 2.23	<0.001
PCV (%)	21.29 ± 2.28	28.56 ± 7.62	<0.001	22.29 ± 3.34	34.84 ± 6.33	<0.001
Lymphocytes (%)	45.95 ± 13.4	54.20 ± 8.00	0.03	42.5 ± 8.06	49.85 ± 14.32	0.06
Neutrophils ($\times 10^3$/µl)	32.80 ± 11.75_	23.10 ± 6.02	0.003	33.9 ± 12.26	28.55 ± 10.97	0.15
TLC ($\times 10^3$/µl)	11.25 ± 3.62	8.15 ± 2.62	0.004	11.37 ± 4.49	9.65 ± 10.97	0.17
Basophils (%)	1.5 ± 1_	1.0 ± 1.11	0.24	1.45 ± 0.68	1.75 ± 1.01	0.28
Monocytes (%)	6.45 ± 2.76	6.0 ± 3.09	0.63	7.15 ± 3.01	6.4 ± 3.51	0.47
Eosinophil (%)	4.95 ± 2.14_	11.90 ± 7.15	<0.001	5.9 ± 4.48	9.6 ± 5.49	0.03
Platelets ($\times 10^3$/µl)	232.50 ± 211.74	361.2 ± 171.4	0.04	236.6 ± 171.39	365.25 ± 227.32	0.05

Hematology of C. burnetii *positive and negative bovines*

The results of various hematological parameters of *C. burnetii* positive and negative bovines were also recorded (Table V). In cows, significant (p<0.05) variations were found in only four parameters showing low hemoglobin level (7.25 ±1.18), decreased PCV (22.29 ±3.34), eosinopenia (9.6 ± 5.49), and thrombocytopenia (365.25 ± 227.32), whereas all other parameters *i.e.,* red blood cells (RBCs), lymphocytes, neutrophils, total leukocytes count (TLC), eosinophils, and platelets count were found non-significantly (p<0.05) different. In buffaloes, the trend was quite different. Only three parameters: RBCs, Basophils, monocytes, and platelet counts, were found non-significantly (p<0.05) different in *C. burnetii* positive and negative buffaloes; while the rest of the parameters were changed significantly ((p<0.05) presenting lymphocytopenia (45.95 ±13.4), neutrophilia (32.80 ±11.75), leukocytosis (11.25 ± 3.62), eosinopenia (4.95 ± 2.14), thrombocytopenia (232.50 ± 211.74), low hemoglobin level (7.005 ± 0.90), and decreased PCV (21.29 ± 2.28).

DISCUSSION

This is the first study in Pakistan that provided a comprehensive account of molecular prevalence of coxiellosis and its associated risk factors in bovines. Owing to the lack of studies in humans and animals and thereby unavailability of genetic information related to the strains of *C. burnetii* circulating in Pakistan, we used the primer set which targeted IS1111 gene that is present abundantly in the entire genome of *C. burnetii.* Thus, targeting IS1111 gene enhanced the sensitivity of our PCR as compared to single copy target gene PCR (Torez *et al*., 2014). We hypothesized that in Pakistan, coxiellosis has been causing huge damage to bovines since long. Because it is not reported yet so, its cases tend to mix with other diseases and thereby managed improperly.

According to this study, the overall prevalence of coxiellosis in bovines was 22.5%, slightly higher than the previous studies in which prevalence ranged from 7% to 19.3% (Keshavamurthy *et al*., 2019; Klemmer *et al*., 2019). This trivial surge in prevalence is probably due to different sampling strategies: selective sampling technique was adopted in this study while other studies used random sampling method. Apart from this, prevalence at species level was nearly similar to erstwhile studies conducted by De Biase *et al*. (2018) and Gache *et al*. (2017) who reported 25% and 26% prevalence of coxiellosis in cows, respectively. However, this approximate similarity is due to the similar sampling strategies. As far as prevalence of coxiellosis in buffaloes is concerned, there are very few studies available and these studies documented the prevalence range from 4.8 to 17.5% and results of our study lies within this range (Klemmer *et al*., 2018). The low prevalence rate of coxiellosis in buffaloes might be due to less susceptibility of buffaloes to infection as compared to cow (Dua, 2003; Rashid *et al*., 2019).

We expected that animals having the issues of subclinical mastitis, abortion, post-abortion infertility, subclinical mastitis and abortion, subclinical mastitis and post-abortion infertility are likely to be *C. burnetii* positive. This is confirmed by the results of this study and all of these clinical biomarkers were found significantly (p<0.05) associated with coxiellosis. De Biase *et al*. (2018) and Gache *et al*. (2017) also reiterated that coxiellosis is significantly associated with mastitis and reproductive problems; whereas Agerholm (2013) and López-Gatius *et*

al. (2012) reported the contrary findings.

Association of the different animal-related risk factors with occurrence of coxiellosis was also verified in our study. The occurrence of the coxiellosis was higher in older animals and those with high parity number. This might be due to the fact that with increasing age exposure time to infection increases. Our results coincide with those of Ullah *et al*. (2019) who also confirmed the higher prevalence of coxiellosis in small ruminants with high parity number. Besides, animals having previous history of any disease or those with low BCS also become vulnerable to *C. burnetii* due to their compromised immune system compared with healthy animals having intact immune system. These findings are consistent with those of Dhaka *et al*. (2019) and Ullah *et al*. (2019). We hypothesized that tick infestation is the most crucial factor in the occurrence of coxiellosis in bovines due to the role of ticks in the lifecycle of *C. burnetii* (Duron *et al*., 2015). Our findings confirmed that *C. burnetii* was found significantly (p<0.05) higher in animals which had tick infestation at the time of sampling rather than those with previous history of tick infestation. This finding is compatible with those of Knobel *et al*. (2013) and Galay *et al*. (2020).

The management practices at farm were also taken into account to establish their association with occurrence of coxiellosis. The disease incidence was significantly (p<0.05) higher in smallholder farms, and those which lack manure management, disinfection, outside carcass disposal and separate calving area. To the best of our knowledge, no previous study reported the association of these factors with the occurrence of coxiellosis. Likewise, the incidence of the disease was found significantly (p<0.05) higher in grazing animals than in stall-fed animals. The previous studies also reiterated that grazing is associated with acquiring and transmission of infection among livestock (Rizzo *et al*., 2016). We also sought to inquire the role of other animals; namely, dog, sheep, and goat, in the occurrence of coxiellosis in bovines. Of these animals, the presence of sheep in the farm were found significantly (p<0.05) associated with the occurrence of this disease in bovines. Our results are partially compatible with those of Rashid *et al*. (2019) who reported that mixing of small ruminants with bovines increased the incidence of coxiellosis in the latter species. However, our study did not confirm that goat companionship led to the significant (p<0.05) disease incidence in bovines. Regarding the role of dogs, our results differ from those of Porter *et al*. (2016) who reported that dog was a source of transmission of coxiellosis in bovines.

The distinct clustering of *C. burnetii* sequences from this study indicates potential genetic variability in the isolates from different species in the same geographical area. The findings of *C.burnetti* Pak/cattle/blood are particularly striking which clades with Indian isolates contrary to *C.burnetti* Pak/buffalo/blood of the same area that shows genetic similarity with environmental isolates of the same geographical origin. A previous study confirmed that the genetic variability is associated with the pathogenicity of different strains in domesticated ruminants (Martinov, 2007). So, the genetic variability reported in the current study not only confirms the circulation of different strains on *C. burnetii* in cows and buffaloes but also emphasizes the requirement of further studies to explore these strains and their pathogenesis.

Following the confirmation of coxiellosis in bovines, hematology profile of bovines was also investigated. This hematology profile describes that *C. burnetii* affects various parameters differently in cows and buffaloes. This variation among cows and buffaloes may be partly due to different strains and partly due to some other factors. However, due to non-availability of any study related to exact pathogenesis of *C. burnetii* and its correlation with hematology in bovines, it is premature and erroneous to describe any reason of this change. To the best of our knowledge, this is the first study that reported the hematology profile of *C. burnetii* positive cows and buffaloes. In small ruminants, one study is available which described that hematology remains unchanged in *C. burnetii positive* animals but these results are dissimilar with our results (Čebulj-Kadunc *et al*., 2014).

Further studies are required to characterize the strains of *C. burnetii* by strain-specific genes, validate hematology of *C. burnetii* positive bovines, and evaluate the potential impacts of coxiellosis on animals and humans in Pakistan.

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Ethics approval and consent to participate

The study was not based on any clinical trial. Institutional Review Committee for Biomedical Research, University of Veterinary and Animal Sciences, Lahore approved the sample collection methodology. Moreover, permission of animal owners was sought before sample collection.

Statement of conflict of interest

The authors declare no conflict of interest regarding publication of this manuscript.

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