Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.284

RESEARCH ARTICLE

Molecular epidemiology and phylogeny of *Theileria annulata* based on cytochrome b gene in the bovine population of Pakistan

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ARTICLE HISTORY (24-609)

Received:	October 6, 2024
Revised:	November 30, 2024
Accepted:	December 1, 2024
Published online:	December 3, 2024

Key words:
Buffalo
Cattle
Phylogenetic evaluation
Risk factor analysis
Cytochrome b gene
Theileria

ABSTRACT

Theileria (T.) annulata stands out among *Theileria* species due to its severe impact on bovine health and productivity, highlighting the need for focused epidemiological research in Pakistan. To investigate the prevalence of *T. annulata* in district Pattoki, 188 blood samples were collected from cattle (n=131) and buffaloes (n=57) through convenient sampling method. All the samples were screened by microscopy for the presence of *Theileria*-like inclusion bodies and PCR for molecular confirmation of *T. annulata*, targeting the cytochrome b (*cyt b*) gene to estimate the prevalence of this pathogen. Moreover, the PCR products were sequenced and analyzed for possible variations in the cytochrome b gene using different bioinformatics tools. Microscopic examination revealed that out of 188 blood samples, 31 samples (16.49%) tested positive for *Theileria* while 50 samples were confirmed positive as *T. annulata* by PCR with an overall prevalence of 26.60%. The BLAST alignment revealed variations at multiple positions in the cytochrome b gene of *T. annulata* isolates. The sequence analysis showed 99.55% similarity between Cyt b/Pak 1 and Cyt b/Pak 4 while 100% similarity between Cyt b/Pak 2 and Cyt b/Pak 3. Cyt b/Pak 5 showed comparatively less similarity with other study sequences. A varying similarity of study isolates was found with the isolates of other neighboring countries including Japan (LC431535), China (KP731977), India (MG787979), Turkey (MK693128), Iran (MT812969), Egypt (PP920504), and Tunisia (KF732030). Among different risk factors, previous tick history, previous anti-theileria treatment, tick control and education status of the owner were found as significant ($p<0.05$) risk factors associated with the prevalence of theileriosis in bovines. It was concluded that *T. annulata* is a prevalent pathogen of bovines in the study area and is significantly related to various assumed risk factors. Moreover, variations in the sequences of the cytochrome b gene of *T. annulata* are evident, necessitating further study to explore any potential correlation with buparvaquone resistance in theileriosis.

To Cite This Article: Ghani U, Ijaz M, Farooqi SH, Ahmed A, Javed MU, Jabir AA, Rasheed H, Batool M and Talha M, 2024. Molecular epidemiology and phylogeny of *Theileria annulata* based on cytochrome b gene in the bovine population of Pakistan. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2024.284

INTRODUCTION

In developing countries, infectious protozoal diseases pose a serious threat to animal health and production (Khan *et al.*, 2013). Among infectious diseases, haemoparasitism is considered a major problem (Demessie and Derso, 2015). Tick-borne diseases cause huge economic losses, being a serious threat to the dairy industry, worldwide (Javed *et al.*, 2014). Theileriosis is a haemoprotozoal disease of domestic and wild ruminants caused by *Theileria* species, primarily transmitted through trans-stadial transmission by tick vectors. However, mechanical and iatrogenic routes of transmission may also occur (Inci *et al.*, 2016). The most pathogenic species of *Theileria* in bovines are *Theileria (T.) parva* and *T. annulata*. *Theileria* spp. are distinct from other apicomplexans due to their ability to transform host leukocytes into immortalized, hyperproliferating and invasive cells, which contribute in the rapid progression of the disease and can lead to

the death of infected animals (Villares *et al.*, 2022). *T. annulata* is a highly prevalent vector-borne parasite of livestock in many parts of the globe including Pakistan (Durrani *et al.*, 2010). Bovine tropical theileriosis, caused by *T. annulata*, mostly occurs in the summer season because of the high tick burden in this season (Beniwal *et al.*, 1989). High ambient temperature provides a conducive environment for tick growth and multiplication (Saeed *et al.*, 2016).

Numerous studies have identified several risk factors associated with *T. annulata* infection in bovines. Among these, agro-climatic zone and geographical location are considered significant risk factors as the tropical and subtropical regions provide a hot humid environment for the growth of ticks (Asif *et al.*, 2022). Similarly breed, age, gender, farming practices and implementation of tick-control strategies at farm are also important risk factors contributing to the prevalence of *T. annulata* infection (Ullah *et al.*, 2022). Clinical signs associated with tropical theileriosis are anorexia, high rise in body temperature, lymph node enlargement, weight loss, decreased milk production, anemia, and jaundice. In acute cases, *T. annulata* can easily be detected through microscopic and molecular techniques (Chauhan *et al.*, 2015).

In Pakistan, several studies have been conducted on different tick-borne pathogens including *Theileria* spp. by Javed *et al.* (2014), Farooqi *et al.* (2017), Ali *et al.* (2020), Ghaffar *et al.* (2020), and Parveen *et al.* (2021). However, these studies mostly relied on conventional diagnostic approach and focused on the diagnosis and identification of parasites. The current study was primarily planned to investigate the molecular epidemiology and phylogenetic analysis of *T. annulata* based on *cytochrome b* gene which may also help to find a correlation with buparvaquone resistance against theileriosis.

MATERIALS AND METHODS

Study plan: The current study was conducted in tehsil Pattoki of district Kasur-Punjab, Pakistan (Fig. 1). This study was ethically approved by the Advanced Studies Research Board (ASRB) of the University of Veterinary and Animal Sciences Lahore. For this study, the animals were selected based on the presence of ticks, fever and swollen superficial lymph nodes. A total of 188 blood samples including 131 samples from cattle and 57 samples from buffaloes were collected from the jugular vein of animals through convenient sampling method. The blood smears were prepared from all samples and *Theileria*-like intra-erythrocytic inclusion bodies were detected through microscopy by following the protocol devised by Moretti *et al.* (2010). DNA was also extracted from these samples for molecular analysis.

Data capturing for risk factor analysis: A questionnaire was also filled out during sampling to collect information regarding possible risk factors that can be associated with bovine theileriosis. The risk factors, for which the information was collected, included species, sex, age, tick infestation, previous tick history, previous exposure to disease, previous anti-theileria treatment, tick control, and the education status of the owner.

DNA extraction of blood samples: DNA was extracted from all blood samples with the help of genomic DNA extraction Kit (GenAll®, Exgene™ Catalogue No.105-101) by following the manufacturer's instructions. DNA was quantified and checked for purity by Nanodrop technique at 260/280nm (Ghauri *et al.* 2021).

Amplification of cytochrome b gene through PCR: DNA was subjected to PCR for the amplification of cytochrome b gene (1092bp) of *T. annulata* by using the previously published primers (F CAGGGCTTTAACCTACAAATTAAC and R-CCCCTCCACTAAGCGTCTTTCGACAC (Mhadhbi *et al.*, 2015). The PCR reaction was cycled 35 times after initial denaturation for 5 minutes at 95°C followed by denaturation for 1 minute at 95°C, annealing for 45 seconds at 61°C, an extension step for 45 seconds at 72°C, and a final elongation step for 10 min at 72°C. The amplified products were separated on 1.5% agarose gel and visualized by gel documentation system as per the guidelines of Ghauri *et al.* (2021). The samples showing a clear band at 1092 bp were considered as positive samples for *T. annulata*.

Purification and sequencing of PCR products: The specific bands were sliced on the UV illuminator using a sterile blade for product purification. GenAll® Expin™ Gel extraction kit (Catalogue no. 112-102) was used following the manufacturer's guidelines for DNA purification from the gel (Ghauri *et al.*, 2021). The purified PCR products were sent to 1st BASE, JTC MedTech Hub, Singapore for sequencing.

Sequence and phylogenetic evaluation of cytochrome b gene of *T. annulata*: Following sequencing, the obtained nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis on NCBI to confirm the *T. annulata* spp. Furthermore, the related sequences were downloaded for sequences analysis. After sequence alignment with BioEdit software, the obtained sequences were compared with each other as well as with the previously deposited cytochrome b gene sequences of *T. annulata* from different geographical regions. Variations in the cytochrome b gene of the study isolates and the reference sequences were identified. This detailed analysis provided insights into the genetic diversity of the study isolates in relation to other reported isolates of *T. annulata*.

Moreover, the phylogenetic analysis of study isolates was performed using MEGA11 software of bioinformatics to study the evolutionary relationship of study sequences with NCBI-retrieved sequences. For this purpose, a phylogenetic tree was constructed using the Maximum Likelihood method with bootstrap value of 1000 replicates (Fadel *et al.* 2023). Moreover, isolate of *Babesia bovis* (Accession No. OQ818627) was added as an out-group in the tree.

Statistical Analysis: The prevalence of *T. annulata* was calculated by following the methodology described by Ghaffar *et al.* (2020). The association between the prevalence of *T. annulata* and various risk factors was statistically analyzed by logistic regression analysis at 5% probability using SPSS statistics version 22.0.

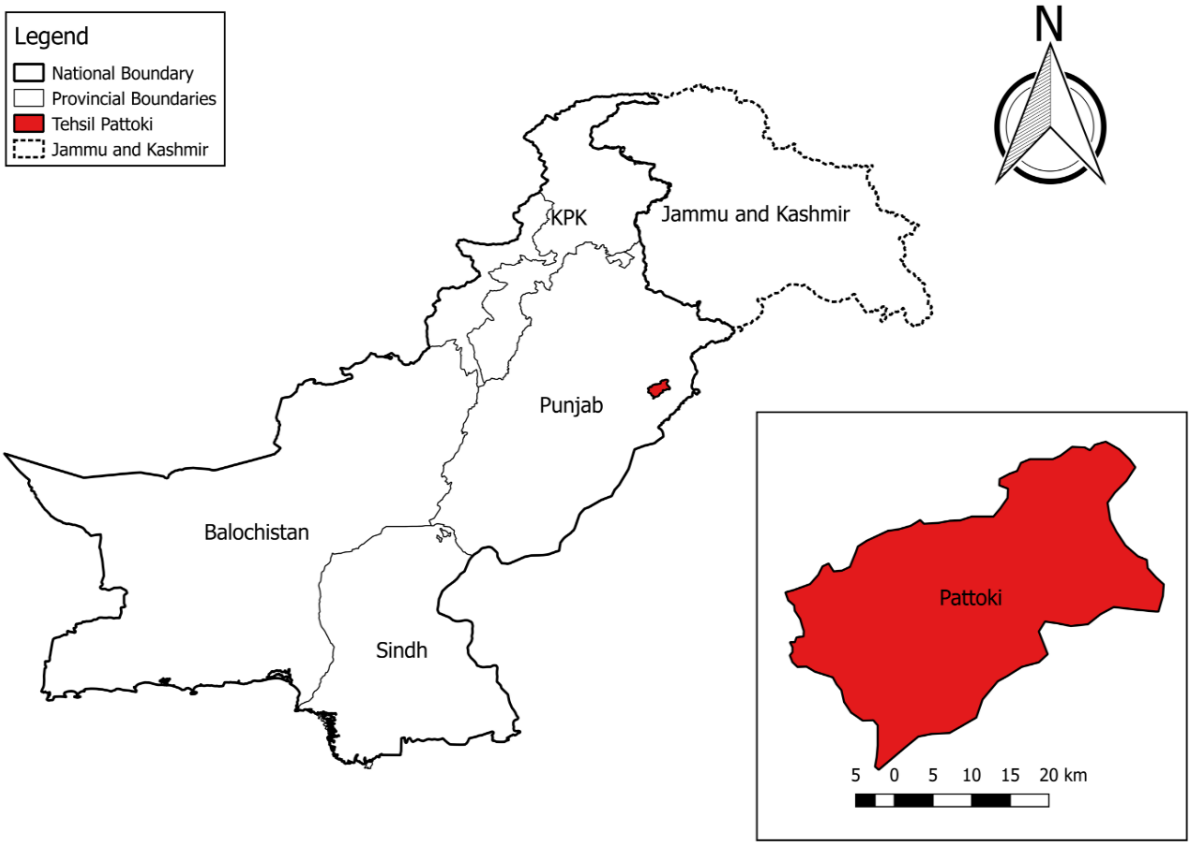

Fig. 1: Map showing the sampling area.

RESULTS

Prevalence of *T. annulata* in bovines: The microscopic examination of blood smears revealed 31 samples as *Theileria* positive with overall prevalence rate of 16.49%. However, the molecular detection by PCR confirmed 50 samples to be *T. annulata* positive with overall prevalence rate of 26.60%. *T. annulata* was found to be more prevalent in cattle (29.77%) in comparison to buffalo (19.30%) (Table 1).

Table 1: Prevalence of *T. annulata* in cattle and buffalo.

Species	No. of Samples	Prevalence N (%)	
		Theileria by microscopy	*T. annulata* by PCR
Cattle	131	23 (17.56)	39 (29.77)
Buffalo	57	08(14.04)	11 (19.30)
Total	188	31(16.49)	50 (26.60)

Risk factors analysis: The analysis of possible risk factors using the Chi-square test revealed that previous anti-theileria treatment, tick control, and education status of owner showed a significant association (P<0.05) with the prevalence of *T. annulata* in bovines while the species, age, sex, and previous exposure to disease were found to be insignificantly associated (Table 2).

Regression analysis of significant risk factors associated with *T. annulata*: Significant risk factors for *T. annulata* infection were further analyzed by logistic regression. The analysis revealed that previous anti-theileria treatment contributed 2.77 times more towards *T. annulata* infection in comparison to animals without treatment. In contrast to that, tick control and the education status of the owner have significant effects on the reduction of *T. annulata* infection in bovines with an odds ratio of 0.014 and 0.230, respectively (Table 2).

Sequence analysis: Among the sequences obtained, five representative sequences were selected for further analysis including three from cattle (Cyt b/Pak 3, Cyt b/Pak 4, and Cyt b/Pak 5) and two from buffalo (Cyt b/Pak 1 and Cyt b/Pak 2). Using BLAST, the sequence similarity between the sequenced nucleotides and the already reported sequences of cytochrome b gene of *T. annulata* on NCBI was accessed. Sequence of Cyt b/Pak 1 showed 98.14% similarity with Indian isolate of *T. annulata* (OP056626) while sequences Cyt b/Pak 2 and Cyt b/Pak 3 showed 99.35% similarity with cytochrome b gene of *T. annulata* (PP920503) isolate from Egypt with a difference of 7bp. Sequence of Cyt b/Pak 3 also showed 99.26% similarity with cytochrome b gene of *T. annulata* (MK693132) isolated from Turkey with a difference of 8bp. Sequence of Cyt b/Pak 4 showed 98.05% similarity with OP113848 with a difference of 21bp and sequence of Cyt b/Pak 5 showed 97.67% similarity with MG787979 with a difference of 25bp, both retrieved sequences reported from India.

Among the study sequences, Cyt b/Pak 1 showed 97.54% similarity with sequences Cyt b/Pak 2 and Cyt b/Pak 3 while a similarity of 99.55% and 97.55% was noted with the sequences of Cyt b/Pak 4 and Cyt b/Pak 5, respectively. The sequence Cyt b/Pak 2 showed 100% similarity with Cyt b/Pak3 while 97.54% and 98.27% similarity with Cyt b/Pak 4 and Cyt b/Pak 5, respectively. The inter-sequence similarity analysis of Cyt b/Pak 3 showed 97.54% similarity with Cyt b/Pak4 and 98.27% with Cyt b/Pak5. Moreover, 97.55% similarity was observed between sequences Cyt b/Pak4 and Cyt b/Pak5.

Table 2: Summary of various risk factors associated with *T. annulata* infection in bovines.

Parameter	Variable	No. of samples examined	No. of positive samples (% Prevalence)	Odds ratio (95% CI)	*p*-value
Species	Cattle	131	39 (29.77%)	1.773 (0.832-3.779)	0.135
	Buffalo	57	11 (19.30%)		
Age	< 2 years	33	13 (39.40%)	2.037 (0.941-4.567)	0.067
	>2 years	155	37 (23.87%)		
Sex	Male	35	8 (22.86%)	0.783 (0.330-1.86)	0.579
	Female	153	42 (27.45%)		
Previous disease exposure	Yes	72	20 (27.78%)	1.103 (0.569-2.138)	0.773
	No	116	30 (25.86%)		
Previous anti-theileria treatment	Yes	44	19 (43.18%)	2.770 (1.353-5.673)	0.004
	No	144	31 (21.53%)		
Tick control	Yes	131	6 (04.58%)	0.014 (0.005-0.040)	<0.001
	No	57	44 (77.19%)		
Owner education status	Educated	116	18 (15.52%)	0.230 (0.116-0.455)	<0.001
	Illiterate	72	32 (44.44%)		

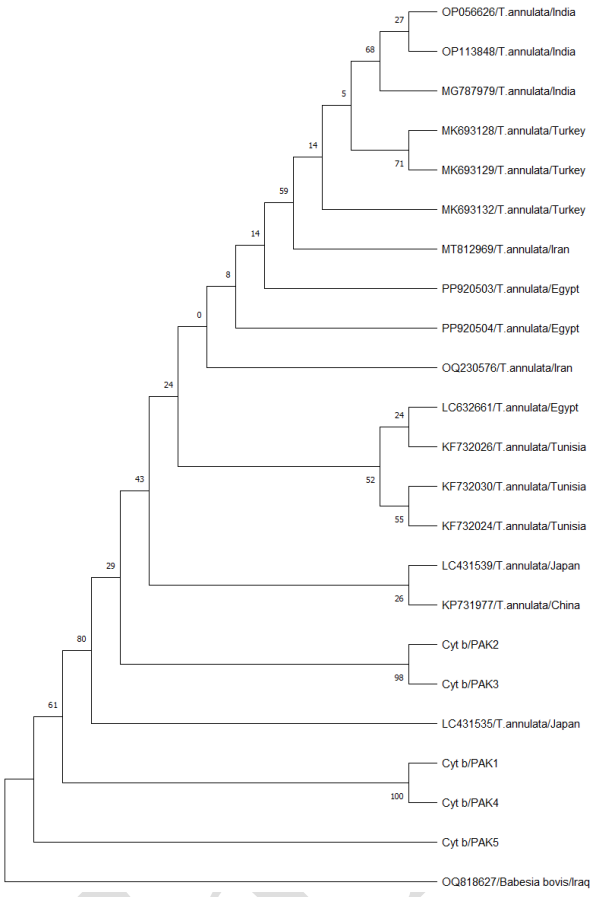

Fig. 2: Phylogenetic tree of local isolates of *T. annulata* with NCBI reported sequences.

Phylogenetic analysis: The comparison of the current study isolates revealed that the sequences of local isolates showed more similarity with each other than the isolates of other countries. Moreover, Cyt b/PAK2 and Cyt b/PAK3 showed a more genetic association with each other and exhibited somewhat homology with the isolate of Japan having accession number LC431535. Cyt b/PAK1 and Cyt b/PAK4 showed highest homology with each other exhibiting a close evolutionary relationship. While Cyt b/PAK5 showed less similarity with the other local and reference isolates. In the phylogenetic tree, Cyt b/PAK2 and Cyt b/PAK3 as well as Cyt b/PAK1 and Cyt b/PAK4 were making two separate sister taxa showing close relationship. Furthermore, the NCBI retrieved sequences of *T. annulata* isolated from India (OP056626, OP113839, MG787979), Turkey (MK693128, MK693129, MK693132), Egypt (PP920503, PP920504) and Iran (MT812969) were appeared as descendants of our study sequences (Fig.2).

DISCUSSION

Theileriosis is a vector-borne disease of tropical and sub-tropical areas of the world including Pakistan with huge economic loses in the form of morbidity and mortality. Current study was planned to investigate the prevalence of *T. annulata* which is most common specie associated with theileriosis. In this study, the prevalence of *T. annulata* is observed higher in cattle (29.77%) as compared to buffalo (19.30%). Our results coincided with the results of Waskel and Gaur (2015) in which *T. annulata* showed a higher prevalence in cattle (51.92%) compared to buffaloes (47.91%). Khan *et al.* (2013) also reported higher prevalence of *T. annulata* in cattle than buffaloes. Likewise, Memon *et al.* (2016) also observed higher prevalence of *T. annulata* (85%) in cattle as compared to buffaloes (76.00%), which also supports the findings of the current study. The higher prevalence of *T. annulata* in cattle compared to buffalo may be attributed to the greater susceptibility of cattle, particularly exotic and crossbred breeds, to tick-borne infections. Additionally, differences in tick preference, managemental practices and the immunity of buffalo likely contribute to *T. annulata* infection.

Among different age groups, young animals (<2 years) showed higher prevalence of theileriosis (39.40%) than adult (>2 years) animals (23.87%). In contrast, the study of Abaker *et al.* (2017) showed a higher prevalence rate among adults than young cattle. These findings are also contrary to the findings of Utech and Wharton (1982) who reported higher prevalence in old age animals compared to the young age animals. The higher prevalence of *T. annulata* in older animals can be attributed to prolonged exposure to tick vectors over time, increasing the likelihood of infection. Additionally, the higher prevalence in younger animals might be due to poor immunity, making them more susceptible to infection.

In this study, the prevalence of theileriosis in female animals (27.45%) was non-significantly higher than male animals (22.86%). These findings are not in line with the study of Farooqi *et al.* (2017) which reported a higher prevalence in males (26.25%) compared to females (17.30%). The higher prevalence of *T. annulata* in females might be associated with the continuous high stress of production and reproduction.

The association between the incidence of theileriosis and previous exposure to the disease of the animal was recorded as non-significant (p>0.05). However, the odds ratio suggested that previous exposure to the disease is a significant risk factor for the prevalence of theileriosis (OR>1). This might be due to the reason that when animals are exposed previously to theileriosis, they may remain carriers if not treated with an adequate amount of anti-theileria drug. Moreover, the study showed a positive association (p<0.05) between the disease occurrence and previous anti-theileria treatment. The odds ratio of 2.770 also suggests that previous anti-theileria treatment was a significant risk factor for the prevalence of theileriosis. This might be due to the reason that resistance may occur due to repeated anti-theileria treatment.

The study showed a significant association (p<0.05) between the incidence of theileriosis and tick control status which coincided with the findings of the International Laboratory for Research on Animal Diseases (ILRI) reporting that tick control is an important factor that influences the spread of bovine theileriosis (Perry, 2015). Similarly, the owner's education status is a significant risk factor for the incidence of theileriosis. Educated farmers know about farm management and tick controlling techniques so there are fewer chances of disease occurrence (Salih *et al*. 2007). The veterinarian educates the farmer about farm management, prevention of animals from diseases, and how to control vectors like ticks (Namgyal *et al*. 2021).

The sequence analysis and phylogenetic evaluation of cytochrome b gene of *T. annulata* revealed variations in the nucleotide sequences of our study isolates compared to sequences retrieved from neighboring countries. The genetic studies provide clear evidence that the Qo domain of cytochrome b is the target site of buparvaquone in *T. annulata* and mutations in this region can develop resistance against buparvaquone (Hacılarlıoglu *et al.*, 2023) which might be associated with the decreased binding affinity of buparvaquone due to the amino acid substitution in the mitochondrial cytochrome b protein. In the present study, variations among nucleotide sequence of study isolates of cytochrome b gene of *T. annulata* might be associated with high number of substituted mutations in amino acid sequence of cytochrome b protein. Our findings are consistent with the research of Sharifiyazdi *et al.* (2012) that found single point mutations in the cytochrome b gene of *T. annulata* isolates.

Molecular characterization of cytochrome b gene revealed considerable variation among field isolates of Pakistan from the isolates of other countries including Tunisia, Egypt, Turkey, Egypt and India. These variations may be due to different ecological zones having different environmental conditions. The higher similarity of local isolates of cytochrome b gene with the Japan isolates might be due to similar environmental conditions which may exerted similar selective pressures on the parasite, leading to convergent evolution and genetic similarities (Suzuki *et al.*, 2008).

Conclusions: This study concludes that *T. annulata* is a prevalent tick-borne pathogen in bovines which is significantly associated with various risk factors. Moreover, the cytochrome b gene of *T. annulata* revealed variations relative to already reported sequences in NCBI. These variations in cytochrome b gene needs to be further studied along with clinical trials to interpret its role in decreased interaction with the buparvaquone binding leading to resistance in *T. annulata.*

Conflict of interest: The authors declared no conflict of interest in publishing data.

Authors Contribution*:* The initial draft of the manuscript was prepared by UG. SHF did sampling and laboratory analysis. MI did conceptualization; AA did write-up. HR and MUJ performed data analysis. AAJ, MB, and MT did editing and reviewing of manuscript

Acknowledgments: The authors are highly thankful to the Medicine Research Laboratory (MRL), Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore for the provision of laboratory goods and chemicals for the smooth conduction of research work.

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