Pakistan J. Zool., pp 1-10, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230516170541

Molecular Detection of *Anaplasma marginale* and *Theileria annulata* in Buffaloes by Using Duplex PCR from Sheikhupura, Jhang, and Rawalpindi Districts of Punjab, Pakistan

Saba Mehnaz[1]*, Farhan Ahmad Atif[2], Rao Zahid Abbas[1], Muhammad Kasib Khan[1] and Muhammad Saqib[3]

[1]*Department of Parasitology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan.*
[2]*Medicine Section, Department of Clinical Sciences, College of Veterinary and Animal Sciences, Jhang (Sub-Campus), University of Veterinary and Animal Sciences, Lahore, Pakistan.*
[3]*Department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan.*

Article Information
Received 16 May 2023
Revised 25 May 2023
Accepted 08 June 2023
Available online 26 June 2023
(early access)

Authors' Contribution
SM convinced the research and wrote the initial draft. RZA and FAA supervised the research project. MKK and MS contributed in proof reading the manuscript. All authors approved and contributed equally to the final version of the manuscript for its publication.

Key words
***Anaplasma marginale, Theileria annulata*, Ticks, Duplex PCR, Phylogenetic analysis**

ABSTRACT

Tick and tick-borne pathogens are among the major threats to animal health and production causing huge economic losses in the form of morbidity and mortality throughout the world. Due to the scarcity of epidemiological data and appropriate diagnostic methods, the study was carried out to detect *Anaplasma marginale* and *Theileria annulata* from buffaloes of Sheikhupura, Jhang, and Rawalpindi districts by using duplex PCR. A total of 1,152 blood samples of buffaloes were screened for epidemiological investigations. DNA extracted from blood samples was used for the amplification of the *msp1β* gene and *cytochrome b* gene by using single and duplex PCR. The prevalence of these pathogens and associated risk factors were observed through the multiple logistic regression method. The overall prevalence of a single infection of *A. marginale* was found to be 14.26% and for *T. annulata* it was 15.28%. The mixed infection, through duplex PCR in the buffalo population, was observed as 12.92%. Different associated risk factors were evaluated to assess their association with the prevalence of these pathogens. Generally, the prevalence was significantly higher in female buffaloes (P=0.0001) during summer season (P=0.0001). Sequencing and phylogenetic analysis of *A. marginale* showed that Sequence Pak-4* was closely similar to sequences CP006847.1 and appeared in the same clade. Sequence Pak-4* was ascended to Pak-3* isolate and descendant to MK792344.1 Pakistani isolate. On the other hand, phylogenetic analysis of *T. annulata* showed that the Pak-2* sequence appeared to be descendants of Indian isolate MH78945.1. In the future, this study will be helpful for large-scale analysis of multiple species by using duplex and multiplex PCR and also for improving the control of associated tick-borne diseases in endemic regions through vaccine development.

INTRODUCTION

Tick and tick-borne diseases (Anaplasmosis and Theileriosis) are the primary constraints in animal health and productivity throughout the globe. It has been estimated that the production losses caused by tick and tick-borne diseases cost billions of dollars per year. Tick-borne pathogens (TBPs) have been reported throughout the world including Pakistan (Zhou *et al*., 2019; Zeb *et al*., 2020; Ali *et al*., 2020; Zaman *et al*., 2020; Ceylan *et al*., 2021; Basit *et al*., 2022).

Anaplasmosis is an important tick-borne disease, which affects wild and domestic bovines, ovines, caprines, equines, canines, felines, and humans as well. The causative agent of this disease is *Anaplasma* which is an obligatory intra-erytrocyctic proto-bacteria (Rehman *et al*., 2019; Mahmoud *et al*., 2022; Kozhabaev *et al*., 2023). The clinical signs include fever, loss in weight gain, decreased milk production, jaundice, hyperexcitability,

* Corresponding author: raouaf@hotmail.com, sabamehnaz2012@gmail.com
0030-9923/2023/0001-0001 $ 9.00/0

abortion, or mortality (Aubry and Geale, 2011). *Theileria annulata* leads to tropical theileriosis in livestock. It is characterized by high fever, anorexia, anemia, weakness, jaundice, enlarged lymph nodes, and mortality (Jabbar *et al*., 2015; Aslam *et al*., 2023). Theileriosis is found in Southern Europe, the Mediterranean coast through North Africa, the Middle East, and Asia (Spickler *et al*., 2010). Ali *et al*. (2013) examined 50% of ticks to be positive for theileriosis and 20% cattle population with *T. annulata* infection in Pakistan.

Diagnostic techniques mostly employed have been conventional techniques, such as stained blood smear microscopy, serology, or simple PCR (Ziam *et al*., 2020). However, there were problems with sensitivity, specificity, and reliability with these diagnostic techniques. Conventionally, microscopic detection was considered as gold standard but it has limited utility for the detection of subclinical or chronic cases due to low sensitivity. Indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and immunoblot assay have problems with cross-reactivity and have less ability to differentiate past and present infections. Furthermore, species characterization is not possible with serological techniques.

However, molecular detections like conventional PCR and real-time PCR assays showed greater sensitivity and specificity for pathogen detection. Duplex PCR studies were used to detect single and concurrent infections, particularly, tick-borne infections (Thomas *et al*., 2022). The duplex PCR has the obvious benefit of inexpensive and rapid concurrent detection of tick-transmitted pathogens in a single assay. The co-existence of TBDs in dairy animals is very common in Pakistan (Karim *et al*., 2017). Nonetheless, the present study aimed to assess the suitability of duplex PCR for the simultaneous detection of *A. marginale* and *T. annulata* in buffaloes from two agro-ecological zones of Punjab, Pakistan, as well as to evaluate the genetic diversity of the circulating isolates.

MATERIALS AND METHODS

Blood sampling and data collection

A total of 1152 blood samples were collected from the buffaloes of Sheikhupura and Rawalpindi districts of Punjab, Pakistan. 384 blood samples were collected from each study district. The sampling was done from asymptomatic buffaloes by using a predesigned questionnaire having close-ended questions to collect information from owners related to different associated risk factors. These factors were including age (< 1 year to > 5 years), breed of animal (Nili-Ravi, Kundi and non-descript), sex (male and female), tick infestation (yes and no), history of TBDs (yes and no), body condition score (1 to 5), herd size (1 to >40), farm type (dairy, beef and mix), feeding pattern (grazing, stall feeding and mix), type of acaricide use (avermectin, organophosphates and pyrethroids), and season (summer, autumn, winter and spring). Sampling was done from the Juglar vein of the animals which were transported overnight to the respective laboratory.

DNA extraction

A total of 0.2ml of blood was collected from each animal. DNA was extracted from collected blood samples by using the whole blood genomic DNA extraction kit; Catalog No. K0782 (Thermo Fisher Scientific). To check the purity and concentration of DNA, Nano-drop was used (Catalog no. ND-8000-GL; Thermo Scientific, USA). The DNA was then stored at -20°C for further use. Families of multigene copies were identified to select targets for the amplification of selected pathogens.

PCR amplification of msp1β *and* cytochrome b *gene*

Single as well as duplex PCR were performed for detection of *A. marginale* and *T. annulata*. For the detection of *A. marginale*, the *msp1β* gene was selected with primer (Forward-5'-GCTCTAGCAGGTTATGCGTC-3' Reverse-5'CTGCTTGGGAGAATGCACCT-3') to amplify a fragment of 265bp length (Bilgic *et al*., 2013). However, for the detection of *T. annulata cytochrome b* gene was selected with a primer set (Forward-5'-ACTTTGGCCGTAATGTTAAAC–3'Reverse-5'-CTCTGGACCAACTGTTTGG–3') to amplify 312bp (Bilgic *et al*., 2013). PCR primer set of both genes was used for separate species detection as well as for duplex PCR.

The PCR primer set *msp1β* and *cytochrome b* were validated separately to determine their specificity. Single PCR detection was performed in a final volume of 50µl containing 10mM tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl2, 0.001% gelatin, 250 µM of dNTPs, 1U of Taq DNA polymerase enzyme, 10µM of each primer and 2µl of template DNA. The whole reaction mixture was incubated in a thermal cycler (T-100, BIORAD). Initial denaturation was performed at 94°C for 3 min, followed by 30 thermal cycles, each of denaturation at 95°C for 50 sec, annealing of primers at 50°C for 50 sec and then extension at 65°C for 50 sec. Final extension was done at 65°C for 10 min.

The duplex PCR reaction was carried out in total volume of 50µl containing 13mM tris-HCl (pH 8.3), 65mM KCl, 2mM $MgCl_2$, 0.0013% gelatin, 300µM of each dNTPs, 1U of Taq DNA polymerase enzyme, 0.5µM of *msp1β* gene primer, 0.6µM of each *cytochrome b* gene primer and 2µl of template DNA. The whole

reaction mixture was incubated in a thermal cycler (T-100, BIORAD). In the first step, initial denaturation was performed at 94°C for 5 min. Denaturation was done at 95°C for 50 sec. The annealing of primers was done at 56°C for 50 sec and the extension was done at 65°C for 50 sec, with the final extension at 65°C for 5 min. The PCR reaction was carried out in 30 cycles.

The PCR products were visualized on 1.8% agarose gel after mixing with 2µl of loading dye (6X-DNA loading dye, Thermo Fisher Scientific, USA).

Sequencing and phylogenetic analysis

The PCR products were sequenced by Advanced Bioscience International Laboratory Malaysia. The obtained sequences were searched in the BLASTn at NCBI to find the similarity indices and to download related sequences (Altschul *et al*., 1990). The downloaded sequences and query sequences were opened in MEGA10 software and multiple sequence alignment was done. For construction of the phylogenetic tree, the aligned sequences file was opened in BioEdit 7.2 software, and extra sequences and gaps were deleted. For the selection of the best-fit model, the jModel test was used. Finally, the MEGA10 software was used for the construction of a phylogenetic tree through maximum parsimony.

RESULTS

The overall prevalence in buffaloes for a single infection of *A. marginale* was 14.26% and for *T. annulata* it was 15.28% (Fig. 1). However, the prevalence was 12.92% positive for the mixed infection of *A. marginale* and *T. annulata* as shown in Table II. The primers used in the current study for the detection of *A. marginale* and *T. annulata* were specie-specific (Bilgic *et al*., 2013). We detected DNA products of 312 and 265bp length for *T. annulata* and *A. marginale*. Area-wise prevalence of single infection of *A. marginale* and *T. annulata* was highest in district Sheikhupura at 20.83 and 23.96%, respectively. A summary of the results for a single infection is shown in Table I. However, area-wise prevalence of mix infection was 16.92% (65/384) in Sheikhupura district followed by Jhang 11.97% (46/384) and Rawalpindi 9.89% (38/384) district. The highest diversity of mix-infection was observed in the district of Sheikhupura. While the lowest prevalence of mix as well as single infection was observed in district Rawalpindi. Among different associated risk factors, the prevalence of infection was higher in the < 1 year aged group of the buffalo population (23.98%) with a highly significant association (P=0.0001). However, the lowest prevalence was observed in > 5-year animals (4.51%). Age had a significant effect on the prevalence of mix infection. Single infection of *A. marginale* was significantly higher (P=0.0001) in the >5-year-aged group of animals. While single infection of *T. annulata* was higher in the > 1-year-aged group of buffalo population. As far as the breed was concerned, the prevalence was higher in Kundi (18.66%) while the lowest prevalence was observed in the Nili-Ravi breed of buffalo (7.66%) with a significant association (P=0.0001). The prevalence of mix infection was high in female animals (17.38%). Animals infested with ticks (P=0.0021) and history of tick-borne diseases (P=0.0002) were statistically more significant compared to no tick infestation and history of tick-borne diseases. Our results were highly significant (P=0.0026) with 2 body condition-scored animals. In different herd sizes, 6-10 herd-sized animals showed the highest prevalence of mix infection (18.38%) with a highly significant association. In different farm types, dairy animals were more prone to *A. marginale* and *T. annulata* mix infection (15.44%). Avermectin showed the highest resistance against tick-borne mix infection (18.90%) and by using Pyrethroids the lowest resistance was observed against tick-borne mix infection. Among different seasons, the incidence of tick and tick-borne pathogens was found significantly higher (P=0.0001) in the summer (18.03%) season while the lowest infection rate was observed in the winter (5.48%) season. However, statistical analysis revealed that there was a non-significant association with the farm type of animals.

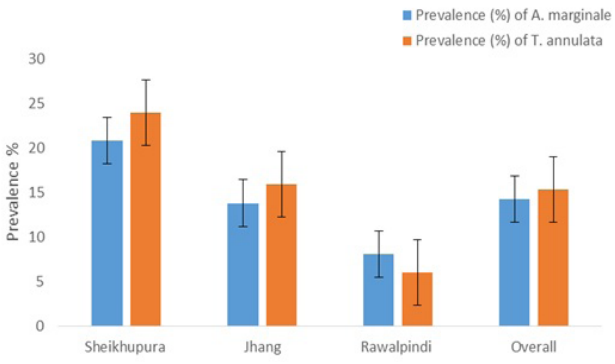

Fig. 1. District wise prevalence of *Anaplasma marginale* and *Theileria annulate* in study districts.

To confirm and validate the PCR results, sequencing was performed. Phylogenetic analysis of our obtained sequences was performed using the maximum likelihood method on megaX software to observe the evolutionary relationships among our sequences and previously published sequences on NCBI (Fig. 2). It was observed that the sequence Pak-3* was distantly similar to *A. marginale* isolated from Horse in Pakistan (MK792344.1)

Table I. Molecular distribution of *Anaplasma marginale* and *Theileria annulata* in buffaloes.

Risk factors/ Category	Total no. of samples	*A. marginale*					*T. annulata*				
		No. of positive	P (%)	C.I	OR	P value	No. of positive	P (%)	C.I	OR	P value
Age											
< 1 year	246	17	6.91	Ref.	Ref.	Ref.	64	24.33	1.68-4.02	2.60	0.0001
1-3 years	263	27	10.27	0.82-2.90	1.54	0.1808	36	14.63	0.72-2.78	0.94	0.8649
3-5 years	288	35	12.15	1.02-3.42	1.86	0.0442	37	12.85	2.17-8.55	3.78	0.9876
>5 years	355	85	23.94	2.44-7.34	4.24	0.0001	39	10.99	Ref.	Ref.	Ref.
Breed											
Kundi	343	59	17.20	1.05-2.30	1.55	0.0285	59	17.20	1.05-2.30	1.55	0.7086
Nili-Ravi	326	48	14.72	0.85-1.95	1.29	0.2259	48	14.72	0.85-1.95	1.29	Ref.
Non-descript	483	57	11.80	Ref.	Ref.	Ref.	57	11.80	Ref.	Ref.	0.6987
Gender											
Male	617	42	6.81	Ref.	Ref.	Ref.	42	6.81	Ref.	Ref.	Ref.
Female	535	122	22.80	2.55-5.41	3.71	0.0001	122	22.80	2.55-5.41	3.71	0.9874
Tick infestation											
Yes	598	113	18.90	1.61-3.27	2.29	0.0001	113	18.90	1.61-3.27	2.29	0.0095
No	554	51	9.21	Ref.	Ref.	Ref.	51	9.21	Ref.	Ref.	Ref.
History of TBDs											
Yes	613	121	19.74	1.96-4.11	2.83	0.0001	121	19.74	1.96-4.11	2.83	0.0065
No	539	43	7.98	Ref.	Ref.	Ref.	43	7.98	Ref.	Ref.	Ref.
Body condition score											
1	295	49	16.61	1.14-3.76	2.06	0.0173	49	16.61	1.14-3.76	2.06	0.0449
2	200	33	16.50	1.09-3.87	2.05	0.0266	33	16.50	1.09-3.87	2.05	0.0967
3	182	16	8.79	Ref.	Ref.	Ref.	16	8.79	Ref.	Ref.	Ref.
4	300	42	14.00	0.92-3.10	1.68	0.0911	42	14.00	0.92-3.10	1.68	0.4390
5	175	24	13.71	0.84-3.22	1.64	0.1433	24	13.71	0.84-3.22	1.64	0.1947
Herd size											
1-5	149	20	13.42	0.62-2.18	1.16	0.6326	20	13.42	0.62-2.18	1.16	0.9876
6-10	251	38	15.14	0.69-1.94	1.15	0.5808	38	15.14	0.69-1.94	1.15	0.8835
11-20	325	49	15.08	0.79-2.23	1.33	0.2725	49	15.08	0.79-2.23	1.33	0.9208
21-40	234	32	13.68	0.68-2.08	1.19	0.5398	32	13.68	0.68-2.08	1.19	0.9876
>40	193	25	12.95	Ref.	Ref.	Ref.	25	12.95	Ref.	Ref.	Ref.
Farm type											
Dairy	530	75	14.15	0.66-1.52	1.00	0.9639	75	14.15	0.66-1.52	1.00	0.8110
Beef	285	40	14.04	Ref.	Ref.	Ref.	40	14.04	Ref.	Ref.	Ref.
Mix	337	49	14.54	0.76-1.72	1.01	0.9895	49	14.54	0.76-1.72	1.01	0.9565
Feeding pattern											
Grazing	378	28	7.41	Ref.	Ref.	Ref.	28	7.41	Ref.	Ref.	Ref.
Stall feeding	338	61	18.05	1.21-3.17	1.96	0.0089	61	18.05	1.21-3.17	1.96	0.0490
Mix	436	75	17.20	1.16-2.94	1.85	0.0057	75	17.20	1.16-2.94	1.85	0.5628

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Risk factors/ Category	Total no. of samples	*A. marginale*					*T. annulata*				
		No. of positive	P (%)	C.I	OR	P value	No. of positive	P (%)	C.I	OR	P value
Type of acaricide											
Organophosphates	284	43	15.14	1.07-2.71	1.71	0.0229	43	15.14	1.07-2.71	1.71	0.8987
Pyrethroids	413	39	9.44	Ref.	Ref.	Ref.	39	9.44	Ref.	Ref.	Ref.
Avermectin	455	82	18.02	1.40-3.16	2.10	0.0003	82	18.02	1.40-3.16	2.10	0.0448
Season											
Summer	287	56	19.51	1.73-4.80	2.88	0.0001	56	19.51	1.73-4.80	2.88	0.0417
Autumn	261	30	11.49	0.88-2.72	1.54	0.1291	30	11.49	0.88-2.72	1.54	0.2675
Winter	310	24	7.74	Ref.	Ref.	Ref.	24	7.74	Ref.	Ref.	Ref.
Spring	294	54	18.37	1.65-5.67	2.89	0.0269	54	18.37	1.65-5.67	2.89	0.0286

Body condition score 1, Emaciated animal; 2, The animal was thin; 3, The animal in average body condition; 4, The animal in heavy condition; 5, A fat animal; P, Prevalence; C.I, confidence interval; OR, odds ratio; Ref, reference value.

Table II. Overall molecular distribution of mixed infection in buffaloes.

Risk factors/ Category	No. of samples	No. of positive	P (%)	C.I	OR	P value
Age						
< 1 year	246	59	23.98	3.74-11.94	6.68	0.0001
1-3 years	263	47	17.87	2.54-8.33	4.61	0.0001
3-5 years	288	27	9.38	1.15-4.15	2.19	0.0161
>5 years	355	16	4.51	Ref.	Ref.	Ref.
Breed						
Nili-Ravi	483	37	7.66	Ref.	Ref.	Ref.
Kundi	343	64	18.66	1.79-4.25	2.76	0.0001
Non descript	326	48	14.72	1.32-3.27	2.08	0.0016
Gender						
Male	617	56	9.08	Ref.	Ref.	Ref.
Female	535	93	17.38	1.47-3.00	2.10	0.0001
Tick infestation						
Yes	598	95	15.89	1.22-2.49	1.74	0.0021
No	554	54	9.75	Ref.	Ref.	Ref.
History of TBDs						
Yes	613	101	16.48	1.40-2.90	2.01	0.0002
No	539	48	8.91	Ref.	Ref.	Ref.
Body condition score						
1	300	51	17.00	1.61-6.28	3.18	0.0008
2	295	46	15.59	1.44-5.70	2.87	0.0026
3	182	11	6.04	Ref.	Ref.	Ref.
4	200	29	14.50	1.27-5.44	2.63	0.0088
5	175	12	6.86	0.49-2.66	1.14	0.7545
Herd size						
1-5	149	12	8.05	0.47-2.29	1.03	0.9237
6-10	234	43	18.38	1.43-4.97	2.67	0.0088
11-20	325	45	13.85	1.03-3.52	1.90	0.0393
21-40	251	34	13.55	0.98-3.52	1.85	0.0571
>40	193	15	7.77	Ref.	Ref.	Ref.
Farm type						
Dairy	285	44	15.44	1.07-2.52	1.64	0.0232
Beef	530	53	10.00	Ref.	Ref.	Ref.
Mix	337	52	15.43	1.09-2.47	1.64	0.0177
Feeding pattern						
Grazing	338	28	8.28	Ref.	Ref.	Ref.
Stall feeding	441	67	15.19	1.24-3.16	1.98	0.0040
Mix	373	54	14.48	1.15-3.03	1.87	0.0107
Type of acaricide						
Organophosphates	284	43	15.14	2.01-6.10	3.50	0.0001
Pyrethroids	413	20	4.84	Ref.	Ref.	Ref.
Avermectin	455	86	18.90	2.75-7.60	4.57	0.0001
Season						
Summer	294	53	18.03	2.13-6.71	3.79	0.0001
Autumn	261	38	14.56	1.61-5.33	2.93	0.0004
Winter	310	17	5.48	Ref.	Ref.	Ref.
Spring	287	41	14.29	1.59-5.18	2.87	0.0005

Table continued on next column.....

For body condition scores and other statistical details see Table I.

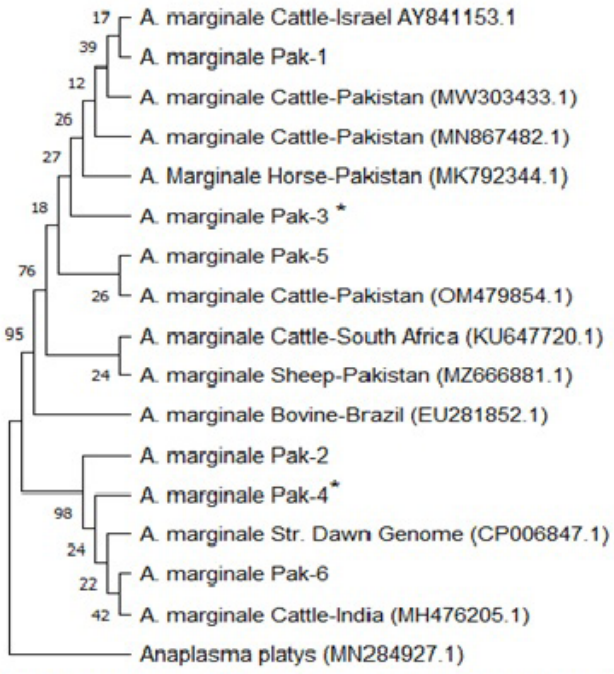

Fig. 2. Phylogenetic analysis of *Anaplasma marginale* based on *msp1β* gene. Our revealed sequences are indicated with Pak-3* and Pak-4*.

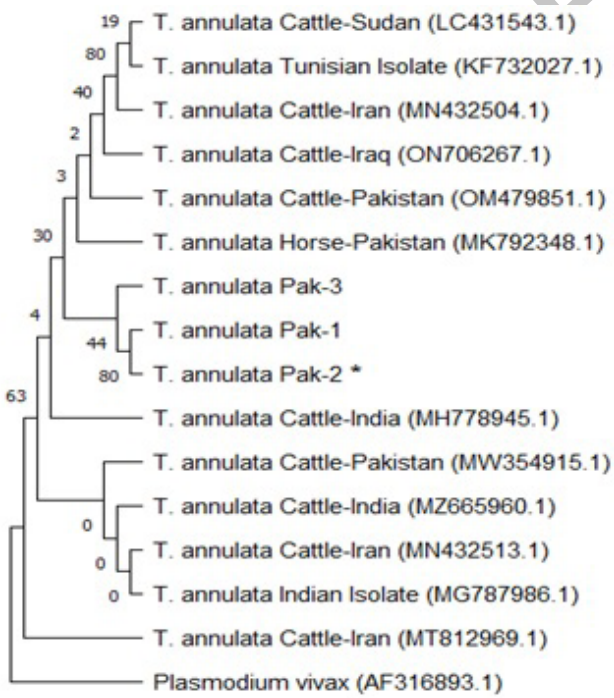

Fig. 3. Phylogenetic analysis of *Theileria annulate* based on cytochrome b gene. Our revealed sequences are indicated with Pak-2*.

and was a lineage of other Pakistani strains (MN867482.1, MW303433.1). It was also observed that sequence Pak-4* was closely related to Str. Dawn genome (CP006847.1). The sequences were deposited to the Genbank database for gene bank accession numbers and accession numbers OQ538218, and OQ538219 have been allotted to Pak-3* and Pak-4* sequences of *A. marginale*. However, for *T. annulata* one sequence was submitted and OQ549989 gene bank accession numbers have been allotted to Pak-2* sequences (Fig. 3). It was observed that sequence Pak-2* was ascendant of Indian isolate (MH778945.1) and distantly related to Pakistani isolate (MK792348.1).

DISCUSSION

In developing countries, more than 50% of production losses are due to TTBPs, mainly *Anaplasma* and *Theileria* (Ashraf *et al*., 2021). Various studies have been conducted to date for the estimation of tick-borne parasitic diseases through conventional methods but due to low sensitivity and specificity, none of them was declared as a gold standard for true estimation of the prevalence of TBPs in the animal population. Furthermore, simple PCR assay is not convenient in developing countries like Pakistan due to the high cost of detection of a single pathogen. Additionally, this method is not field friendly at the farm level in these countries. Therefore, the establishment of novel methods like duplex PCR assay, to detect multiple infections at once, is a need of the hour. In this study, duplex PCR has been employed for the simultaneous detection of *A. marginale* and *T. annulata*, which is also economical and field-friendly (Bilgic *et al*., 2013; Ganguly *et al*., 2020).

Our results showed that these blood parasites vary in different regions of the world because Sheikhupura and Jhang districts are warmer than the Rawalpindi district. Warmer areas favor the growth and production of tick and tick-borne pathogens (Garcia-Sanmartin *et al*., 2008). Mixed infection in the current study highlighted the importance of duplex PCR for the simultaneous detection of tick-borne pathogens to save time, budget, and personal efforts. In the current study, epidemiological data helps in the detection of risk factors that contribute to the effective control of TBDs (Simunnza *et al*., 2011).

The current study depicted an overall prevalence of mixed infection as 12.92% in the buffalo population of the studied districts. Previously, various studies have been conducted on the prevalence of *A. marginale* and *T. annulata* mixed infection in different regions of the world including Pakistan through various techniques. For example, the prevalence of *A. marginale* was reported as 14.08%, *T. annulata* as 15.49%, and mixed infection at

2.81% in Egypt (El-Ashker *et al*., 2015). In Pakistan, the prevalence of *A. marginale* and *T. annulata* was reported as 16.3 and 29.9%, respectively (Zeb *et al*., 2020). Our results were in accordance with these studies conducted in the world including Pakistan with a slight difference in the prevalence rate. The difference in the prevalence can be due to variations in temperature, humidity, farm practices, and vector population in different regions of the world (Zafar *et al*., 2022).

Among different studied districts, the highest prevalence of TBPs was reported in Sheikhupura, followed in decreasing order by Jhang and Rawalpindi. The higher prevalence in Sheikhupura is due to the humid environment as the most of rice crop is cultivated in that area which needs waterlogged irrigation, ultimately, providing a suitable humid environment that favors the growth and multiplication of ticks (Ashraf *et al*., 2013). It has been reported that the maximum number of egg-laying and hatching *Hyalomma* ticks occur at 32-34°C temperature with 85% humidity (Durrani and Shakoori, 2009).

Various risk factors i.e., age, breed, gender, tick infestation, history of tick-borne diseases, body condition score, herd size, type of acaricide use, and season are predisposing factors for the buffalo population during the present study. Our findings indicate that the mixed infection was significant ($p<0.05$) in the < 1-year-aged group. Our results coincided with the studies of Simuunza *et al*. (2011) and Farooqi *et al*. (2018). Simuunza *et al*. (2011) stated that calves are at higher risk when they acquire an infection for the very first time. Our results showed a higher prevalence in the Kundi breed of buffalo. Similarly, Sajid *et al*. (2014) reported a higher prevalence of anaplasmosis in Kundi than in Nili-Ravi breeds of buffalo in Khanewal. In the case of *Theileria*, significantly higher ($p<0.05$) prevalence was observed in Kundi as compared to other breeds of buffalo. In contrast to our findings, a higher prevalence was reported in KPK where non-descript buffalo has more prevalence of *T. annulata* than Kundi and Nili-Ravi (Farooqi *et al*., 2017).

Our study showed that the prevalence of tick-transmitted pathogens was significantly higher ($p<0.05$) in tick-infested animals. Our results are in line with Bock *et al*. (2004) and Glass *et al*. (2003) who reported that the animals are more susceptible to disease due to higher infestation of ticks. In Southern Punjab, Jhang, and KPK, the prevalence of mix infection was higher in tick-infested animals as compared to non-tick-infested animals (Ashraf *et al*., 2013; Atif *et al*., 2021). Our results are also in line with the results done in Lodhran and Dera Ghazi Khan where TBPs were more prevalent in tick-infested animals than the animals with no tick infestation history (Zafar *et al*., 2022). Similar findings have also been reported by Farooqi *et al*. (2018), Parveen *et al*. (2021), and Inci *et al*. (2008).

In different body condition scoring, a higher prevalence was observed in Score-1. Similar results were reported in the Western Kenya study where TBPs have a high prevalence in animals with a body condition score of 1-2.5 and low in animals having a body condition score of 3-5 (Chiuya and Villinger, 2021). Hordofa *et al*. (2021) reported that animals with poor body condition score (1) have the highest infection than medium and good body condition score animals. This is due to the weaker immune system of weak animals as compared to healthy animals. Among different herd sizes, the highest prevalence was observed in herd sizes of 6-10 and 10-20 animals. The results are in accordance with the studies of Shaukat and Mehmood (2019) and Atif *et al*. (2012, 2013) in which higher prevalence was reported in smaller herd sizes as compared to larger herd sizes. It is due to the fact that the larger herd sizes are kept under commercial farming systems with modern managemental practices.

Among different acaricides, it was observed that the prevalence of TBPs was higher in animals that were being treated by avermectin. Similar results were found by Atif *et al*. (2022) and Kispotta *et al*. (2016) in Pakistan and Bangladesh, respectively. The higher prevalence in avermectin-treated animals was due to resistance against avermectin which is excessively used by farmers. However, in contrast to avermectin, a relatively lower prevalence was observed in organophosphates and pyrethroids. Our study showed a higher prevalence of parasites in the summer season as compared to autumn, winter, and spring. Similar results were reported by Atif *et al*. (2013), Khan *et al*. (2019), Ashraf *et al*. (2021), and Siddique *et al*. (2020) in Pakistan because the climatic conditions in the summer season are almost similar in different areas of Pakistan. Likewise, Simuunza *et al*. (2011) reported a higher incidence of ticks and TBPs in the wet season. Moreover, during monsoons, a higher incidence has been noticed due to higher moisture and higher tick load (Roy *et al*., 2018).

To support and confirm PCR results, sequencing was performed. Phylogenetic analysis confirmed that our Pak-3* sequence was more similar to Pakistani isolates (MW303431.1, MW303432.1, MT603500.1). However, showed lesser homology with Brazilian (CP023730.1) and Israeli (AY841153.1) isolates. Pak-4* sequence showed more homology with Pakistani isolates from horse and goat (MK792346.1, MN931649.1) and lesser homology with Indian (MH476205.1), American (M59845.1) Iraqi isolates (467524.1). Similarly, sequence Pak-2* of *T. annulata* showed homology with Tunisia (KF732028.1), Egypt (LC632662.1), India (MZ665960.1), and Iran

(MN432513.1). A recent study successfully depicted similar duplex techniques for the isolation of *A. marginale* and *T. annulata* for field epidemiological studies. Phylogenetic analysis revealed that our sequences are novel and they are grouped separately. All of our cytochrome b gene and MSP1β gene sequences are closely related to Pakistan, India, South Africa, and Iran but showed lesser homology with other countries. It can be concluded from the above discussion that simultaneous infection of *A. marginale* and *T. annulata* is widespread and duplex PCR is suitable for field surveillance studies.

CONCLUSION

It has been concluded that simultaneous infection of multiple pathogens in single host can complicate the epidemiology and pathology of the disease. However, concurrent infection can exacerbate the clinical picture. In addition to this, country wise projects should be launched to monitor clinical and pathological effects under controlled clinical trials for developing better control strategies.

ACKNOWLEDGMENT

The authors are thankful to laboratory assistants and laboratory fellows for their assistance in sample collection and transportation.

Funding

All the funding for this study was provided by Higher Education Commission Pakistan's Project No. 9041/ Punjab/NRPU/R&D/HEC/2017.

IRB approval

Not applicable.

Ethics statement

This study was carried out under institutional guidelines of the ethical review committee of University of Veterinary and Animal Sciences, Lahore, Pakistan (No. DR/1147; Dated 26th October 2017).

Statement of conflict of interest

The authors have declared no conflict of interest.

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