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RESEARCH ARTICLE

Molecular prevalence of *Anaplasma marginale* in ruminants and *Rhipicephalus* ticks in northern Pakistan

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ABSTRACT

Anaplasma marginale is the most prevalent tick-borne haemoparasite of cattle and causes huge economic losses to the dairy industry worldwide. This study aimed to determine the occurrence of *A. marginale* infection in blood and tick samples collected from livestock animals in the districts located in Khyber Pakhtunkhwa (KPK), Pakistan. A total of 184 blood and 370 tick samples were included in this study. It has never been reported that sheep, goats, and cattle in Tank, Ghulam Khan, Birmil and Miran Shah areas were infected with *A. marginale*. All samples of blood and ticks were collected through random sampling from March 2021 to January 2022 from cattle, sheep and goats and screened through PCR for anaplasmosis by using primer pairs of *Anaplasma* spp. Three hundred and seventy ticks were collected from infested hosts (120/184, 64.21%). Among the four morphologically identified tick species, the highest occurrence was recorded for *Rhipicephalus sanguineus* (n=138, 37.29%), followed by *Rhipicephalus microplus* (n=131, 35.4%), *Rhipicephalus annulatus* (n=40, 10.81%), *Hyalomma anatolicum* (n=31, 8.37%), and *Hyalomma marginatum* (n=30, 8.1%). The occurrence of female tick was highest (n=160, 43.24%), followed by nymphs (n=140, 37.38%) and males ticks (n=70, 18.9%). Among these ticks, *A. marginale* was detected in female ticks of *R. microplus*, and *R. sanguineus.* Molecular identification of *A. marginale* was confirmed in 120 out of 184 blood samples and 6 out of 74 tick samples. Overall, occurrence of *A. marginale* in blood and tick samples was found to be 65.21% and 8.1% respectively. Species-wise occurrence in blood samples of goats were 71.11% followed by sheep 68.31% and cattle 50%. Specie-wise occurrence of *A. marginale* in tick samples of cattle were 12.5% followed by goats 6.89%. The obtained sequence showed similarity with *A. marginale* reported from Kenya and USA. We report the first PCR based detection of *A. marginale* infection in blood samples and in *R. sanguineus* ticks of goats simultaneously.

Keywords: *Anaplasma marginale*; goat; *Rhipicephalus sanguineus*; PCR; Pakistan.

INTRODUCTION

Anaplasma marginale is the causative agent of anaplasmosis in livestock animals which is the most common tick-borne infection in cattle worldwide (Mekonnen *et al*., 2002). The dairy business suffers massive economic losses as a result of this parasitic disease (Aubry & Geale, 2011). In Pakistan, *Rhipicephalus microplus* and *Rhipicephalus annulatus* tick species have been identified as possible vectors of *A. marginale* transmission to cattle and buffalos (Jabbar *et al*., 2015; Ali *et al*., 2019; Rehman *et al*., 2019). *Rhipicephalus microplus* is the most common vector of *A. marginale* in the tropical and subtropical areas. The transmission of *A. marginale* in the United States is mediated by *Dermacentor andersoni* and *Dermacentor variabilis* (Stiller *et al*., 1989).

Anaplasma marginale is the causative agent of anaplasmosis and mechanically transmitted by the biting flies such as *Stomoxys calcitrans* (Diptera:Muscidae) (Potgieter *et al*., 1981). Weight loss, fever, depression, progressive anemia, and icterus are symptoms of anaplasmosis (Fereig *et al*., 2017). When compared to the other species, *A. marginale* is the most pathogenic and causes outbreaks worldwide. Tick vectors are endemic in those regions where *A. marginale* is found. Despite its host specificity for cattle (Ma *et al*., 2016). *R. microplus* has been reported infesting small ruminants (Brito *et al*., 2005).

Rhipicephalus sanguineus is one of the most studied ticks due to its veterinary and public health importance (Dantas-Torres, 2008). *Rhipicephalus sanguineus* has been linked to the transmission of a number of pathogens that cause diseases in dogs, including babesiosis, ehrlichiosis, hepatozoonosis, and rickettsioses (Cabezas-Cruz *et al*., 2019). *Rhipicephalus sanguineus* is a common tick found on cattle in Africa and is thought to be the vector of *A. marginale* (Hoogstraal, 1956).

The focus of veterinary parasitology research in Pakistan with reference to tick-borne diseases persisted on large ruminants

and were least addressed in small ruminants (Saeed *et al*., 2015). However, that *R. sanguineus* ticks are the probable vectors of *A. marginale* in Pakistan is still unproven. The present investigation was designed to report the presence of *A. marginale* in *R. sanguineus* tick samples which were obtained from goats of different areas of Khyber Pakhtun khwa (KPK), Pakistan.

MATERIALS AND METHODS

Ethical approval

All the experimental procedures were performed according to the guidelines approved by Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan with No. DR/420, Dated: 13 October 2021.

Study design and sampling

Pakistan is divided into ten agroecological zones. These zones are based on climate, land use, soil type, and physiography (Khan, 2004). The selected areas of KPK (Miran Shah, Tank, Birmil and Ghulam Khan) are present in Barani agroecological zones of Pakistan. A random sampling was used to collect ticks (n= 370) and blood samples (n= 184) from cattle, sheep, and goats from March 2021 to January 2022 from different districts in the province of KPK including Miran Shah (33.0007°N, 70.0401°E), Tank (32.5810°N, 70.1638°E), Birmil (32.5423°N, 69.4440°E) and Ghulam Khan (33.0722°N, 70.0216°E) (Figure 1). In the present study, the sample size of farms was calculated from a large population of cattle, and small population of sheep and goat with a desired precision of 10% and a confidence interval of 95% (Cannon, 1982). In this method, one district was selected from each province. From each district, five villages were randomly selected, and five farms were visited in each village, half of the animals on a farm were randomly selected to be examined for tick infestation, resulting in a minimum of 5 and maximum of 10 animals per farm. The collections were randomly conducted from cattle, sheep, and goats at various collection sites in the four districts of KPK, Pakistan. During collection, blood samples were collected in EDTA vacutainers and tick specimens were collected in Eppendorf tubes and brought to the Department of Parasitology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan for further processing. After collection, every specimen of tick was preserved individually in 70% ethanol and blood samples were stored in 4°C freezer for further processing.

Morphological identification

Morphological identification of ticks was performed with stereomicroscope (Euromex StereoBlue, Holland) through guidelines of electronic keys of ticks (Walker *et al*., 2005).

Genomic DNA isolation

Individual tick was washed in distilled water three times for (30 minutes) before being ground with a mortar and pestle, and the ethanol was removed before the DNA extraction. For blood DNA extraction, 2-3 ml of blood was collected with the help of a disposable syringe from jugular vein and transferred into ethylene diamine tetraacetate (EDTA) vacutainers separately. The extraction procedure was performed by using a GeneJET Genomic DNA purification kit (Thermo Scientific, Van Allen Way, Carlsbad, California) following the company's protocol. For further analysis DNA was kept at -20°C.

Polymerase chain reaction amplification

Genomic DNA was extracted from 120 blood samples and 74 selected ticks (Table 1). PCR of ticks and blood samples were performed using G-STORM Thermocycler and Taq Polymerase (cat#k0171), with the primer pairs of *Anaplasma* spp., AF-5-GGAATTCAGAGTTGGATCATGGCTCAG-3, and AR-5-CGGGATCCCGAGTTTGCCGGGACTTCTTCT -3 were used (Aktas *et al*., 2009). The PCR product was prepared in a total volume of 25µl. Initial denaturation was given for 4 min at 95°C, and reaction was cycled for 35 times. Each cycle was started with final denaturation for 1 min at 95°C, annealing step for 1 min at 50°C, initial extension for 1 min at 68°C, and a final extension for 10 min at 68°C was given. After the completion of PCR reaction, the amplified PCR products were analyzed on 1.5% agarose gel. A positive control of *A. marginale* and a negative control (DEPC water) were included in the PCR run. The PCR amplicons were purified through the Gel/PCR Purification Mini Kit (WizPrep, Korea Ref no: W70150-300).

DNA sequencing and phylogenetic analysis

Purified PCR-amplified products were shipped for sequencing (1st BASE biological technology Singapore). Previously reported and current study isolates of *A. marginale* sequences were aligned and evaluated in BioEdit software version (7.2) using the alignment method (CLUSTAL W). A phylogenetic tree was constructed based on obtained sequences by using neighborhood joining method on MEGA 10 (Kumar *et al*., 2018). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Statistical analysis

Chi-square and descriptive analyses were performed in SPSS version 20.0 (SPSS Software, Inc., Chicago, Ill, USA). P-value of < 0.05 was set as significant.

RESULTS

Morphological identification of ticks

Based on the animal hosts, the highest occurrence of ticks was recorded in goats (32/45, 71.1%) followed by cattle (69/101, 68.31%), and sheep (19/38, 50%). The highest occurrence of tick was recorded in the Ghulam Khan district (n=102, 27.56%), followed by the Tank district (n=94, 25.4%), Miran Shah district (n=90, 24.32%) and Birmil district (n=84, 22.7%) (Table 1; Figure 1). The highest occurrence of tick species was observed for *R. sanguineus* (n=138, 37.29%), followed by *R. microplus* (n=131, 35.4%), *R. annulatus* (n=40, 10.81%), *Hy. anatolicum* (n=31, 8.37%), and *Hy. marginatum* (n=30, 8.1%). The most prevalent life stage of ticks was adult females (n=160, 43.24%), followed by nymphs (n=140, 37.38%) and adult males ticks (n=70, 18.9%) (Table 1).

Occurrence of ticks associated with different variables

Based on the gender and host, the highest tick occurrence was observed in female animals as compared to the male. Based on age, animals were categorized into three different age groups; the highest tick occurrence was recorded in more than 3 years of age group of animals, followed by the 1 to 3 years and lowest in less than or equal to 1-year of age. Based on season, highest occurrence was recorded in summer (June-Aug), followed by spring (March-May), fall (Sept-Nov), and winter (Dec-Jan). District-wise, the highest occurrence was recorded in the Miran Shah while the lowest was in the Tank district. All variables, including genders, age, host, and seasons associated with tick occurrence, were highly significant (Table 2).

Occurrence of *A. marginale* in tick samples

Out of 74 ticks, 6/74 (8.1%) (2/26 (7.6%) Miran Shah, 1/13 (7.6%) Tank, 2/16 (12.5%) Birmil, and 1/22 (4.54%) Ghulam Khan) were found positive for *Anaplasma* spp. (Figure 2). Among various tick species, *R. microplus*, and *R. sanguineus* were found positive for *Anaplasma* spp. *R. microplus* ticks showed the highest occurrence for *Anaplasma* spp. (n=4, 9.52%), followed by *R. sanguineus* (n=2, 5.71%). The highest occurrence of *Anaplasma* spp. was recorded in Birmil district (n=2/16, 12.5%), followed by Miran shah (n=2/26,

Table 1. Occurrence of *A. marginale* in blood and tick samples collected from livestock animals

Province	District locations	Animals Observed	Animals infested and positive for *A. marginale*	Tick Species	Tick stages	PCR based molecular screening	No. Of Ticks +ve for *A. marginale*
KPKKhyber Pakhtunkhwa	Miran shah (33.0007°N, 70.0401°E)	Cattle n=30	20 (66.67%)	*R. microplus*	45 (15N, 10M, 20F)	5N, 5F	2F
		Sheep n=10	6 (60%)	*Hy. anatolicum*	15 (8N, 2M, 5F)	3N, 2F	–
		Goat n=11	9 (81.81%)	*R. sanguineus*	30 (11N, 1M, 18F)	6N, 5F	–
	Tank (32.5810°N, 70.1638°E)	Cattle n=20	12 (60%)	*R. microplus*	37 (7N, 24M, 6F)	2N, 3F	–
		Sheep n=9	5 (55.5%)	*Hy. anatolicum*	16 (4N, 1M, 11F)	3F	–
		Goat n=13	8 (61.53)	*R. sanguineus*	41 (20N, 10M, 11F)	3N, 2F	1F
	Birmal (32.5423°N, 69.4440°E)	Cattle n=29	21 (72.4)	*R. microplus*	49 (20N, 15M, 14F)	3N, 5F	2F
		Sheep n=6	3 (50%)	*Hy. marginatum*	10 (6N, 4F)	2N, 1F	–
		Goat n=9	7 (77.7%)	*R. sanguineus*	25 (15N, 1M, 9F)	3N, 2F	–
	Ghulam khan (33.0722°N, 70.0216°E)	Cattle n=22	16 (72.7)	*R. annulatus*	40 (15N, 5M, 20F)	4N, 5F	–
		Sheep n=13	5 (38.46%)	*Hy. marginatum*	20 (13N, 1M, 6F)	3N, 2F	–
		Goat n=12	8 (66.66%)	*R. sanguineus*	42 (6N, 36F)	2N, 6F	1F
Total		**184**	**120 (65.21%)**		**370 (140N, 70M, 160F)**	**74 (34N, 40F)**	**6 (8.1%)**

N: Nymph. **F:** Female. **M:** Male.

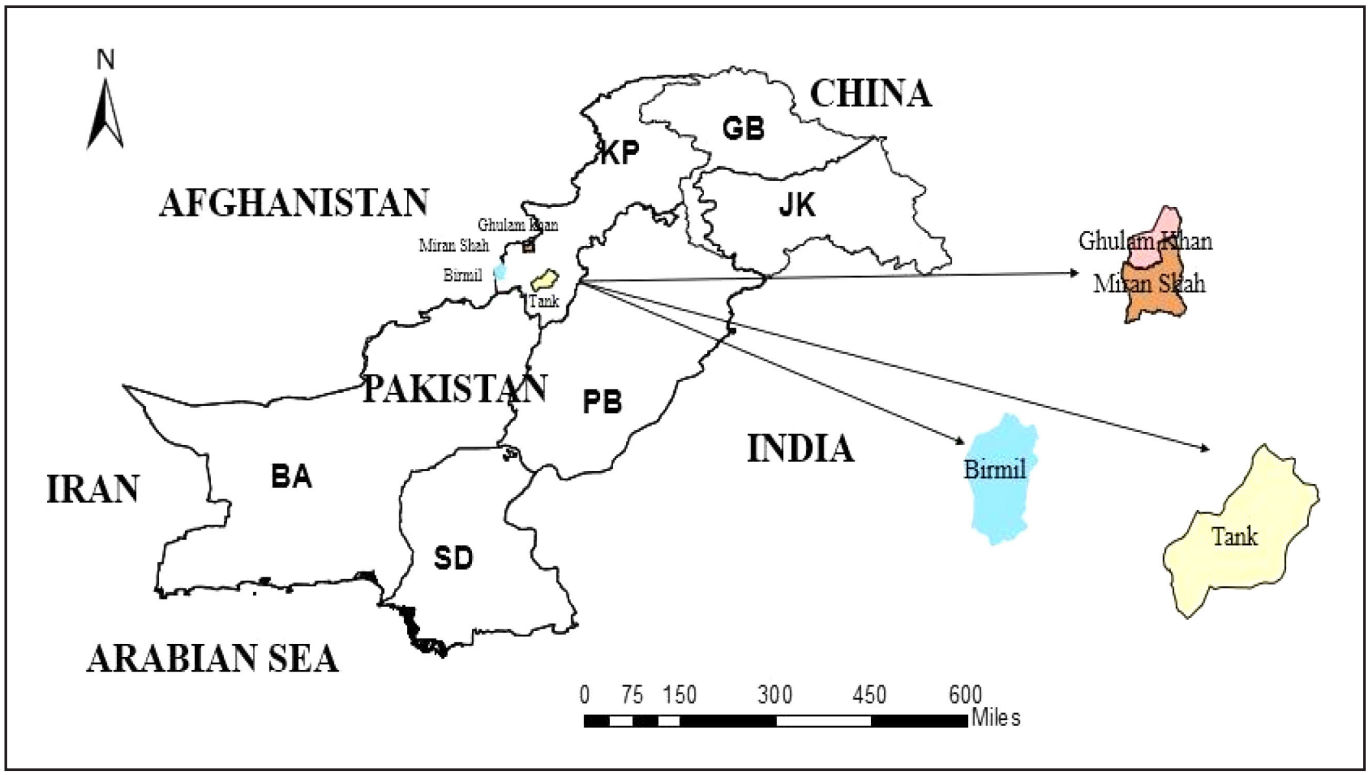

Figure 1. STUDY AREA OF KPK. Map is showing research areas of Khyber Pakhtunkhwa where blood and tick samples were collected, and this map is created by ArcGIS software version 10.3.1. Abbreviations: KPK, Khyber Pakhtunkhwa; BA, Balochistan; PB, Punjab; SD, Sindh; GB, Gilgit Baltistan; JK, Jammu, and Kashmir.

Table 2. Occurrence of ticks associated with different variables

Variables	Category	Total	Tick Infested animals	Chi-square	Degree of freedom	p-value > 0.05
Gender	Male	42	8	51.14	1	0.000
	Female	142	112			
Age	≤1 years	45	15	30.35	2	0.000
	1–3 years	60	40			
	>3 years	79	65			
Animal hosts	Cattle	101	58	10.17	2	0.006
	Sheep	38	24			
	Goats	45	38			
Season	Spring	65	44	14.2	3	0.03
	Summer	80	60			
	Fall	27	10			
	Winter	12	06			

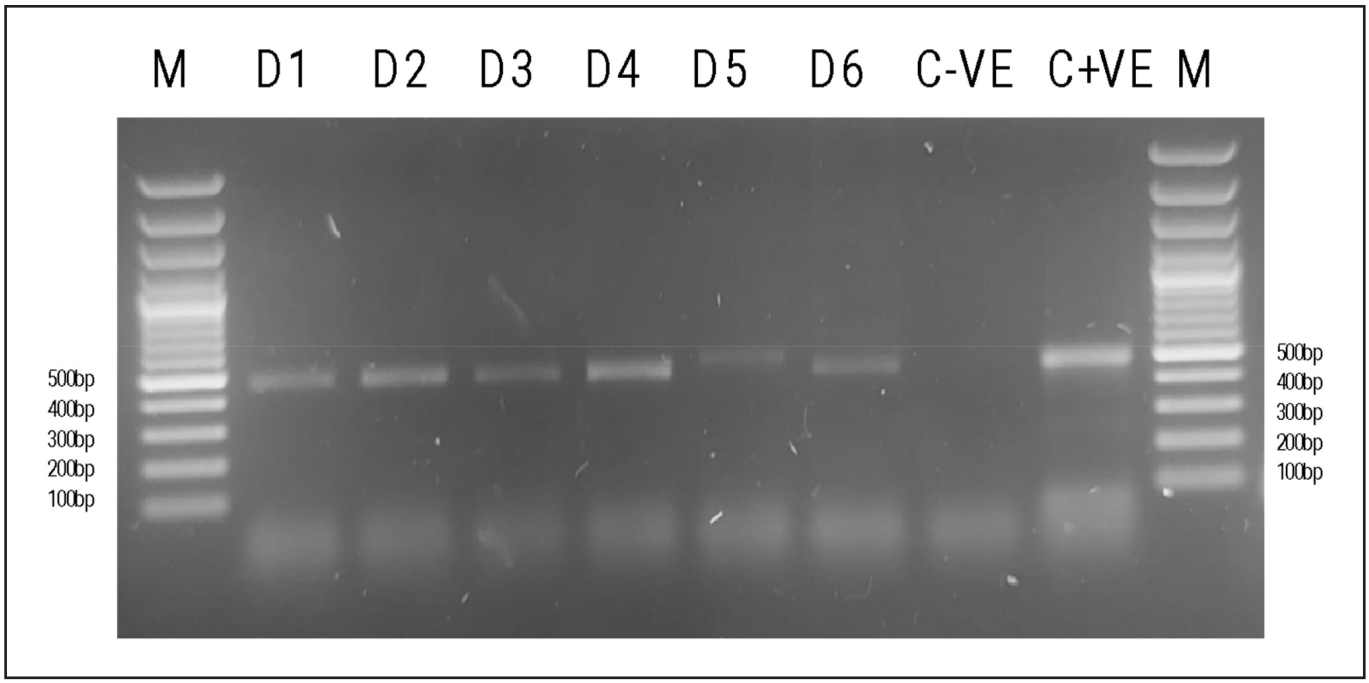

Figure 2. PCR detection of *A. marginale* in blood samples of goats and *R. sanguineus* ticks collected on goats. Lane M contains the DNA Ladder of 100 bp (Gene Ruler 100bp plus DNA ladder Ref: SM0323). Lane D1-D3 indicate positive blood samples of goat, Lane D4-D6 indicate positive samples of *R. sanguineus* ticks, Lane C-VE indicate negative control, Lane C+VE indicates positive control of *A. marginale* with 484bp band.

7.69%), Tank (n=1/13, 7.69%), and the lowest in Ghulam khan (n=1/22, 4.54%). Positive *R. microplus*, infesting cattle were reported from the Miran shah and Birmil districts, whereas positive *R. sanguineus* infesting goats were reported from the Tank and Ghulam khan districts (Table 1).

Occurrence of *A. marginale* in blood samples

The genomic DNA of blood samples of cattle, sheep and goats were subjected to PCR for *16S rRNA* amplification of *Anaplasma* spp. Out of 184 blood samples, 120 blood samples were positive and 60 samples were negative for *A. marginale* (Figure 2). An overall occurrence of *A. marginale* in blood samples of cattle, sheep and goats was 120/184 (65.21%). The highest occurrence of *A. marginale* in blood was recorded in Birmil district (31/44 (70.45%), followed by 35/51 (68.62%) Miran shah district, 29/47 (61.70%) Ghulam khan district, and the lowest in Tank district (25/41; 60.97%). The highest occurrence of *A. marginale* was recorded in goats 32/45 (71.11%) followed by cattle 69/101 (68.31%) and lowest in sheep 19/38 (50%) (Table 1).

Phylogenetic analysis of *A. marginale* in *R. sanguineus* tick

The BLAST results of the obtained 16S rDNA (484 bp) sequence showed 98–100% similarity with the *A. marginale* reported from USA (DQ00613 and DQ00617), Kenya (MW255052) and 62% similarity with the *A. marginale* sequences reported from Uruguay (AF414877), China (OM065781) and Thailand (KT264188). The

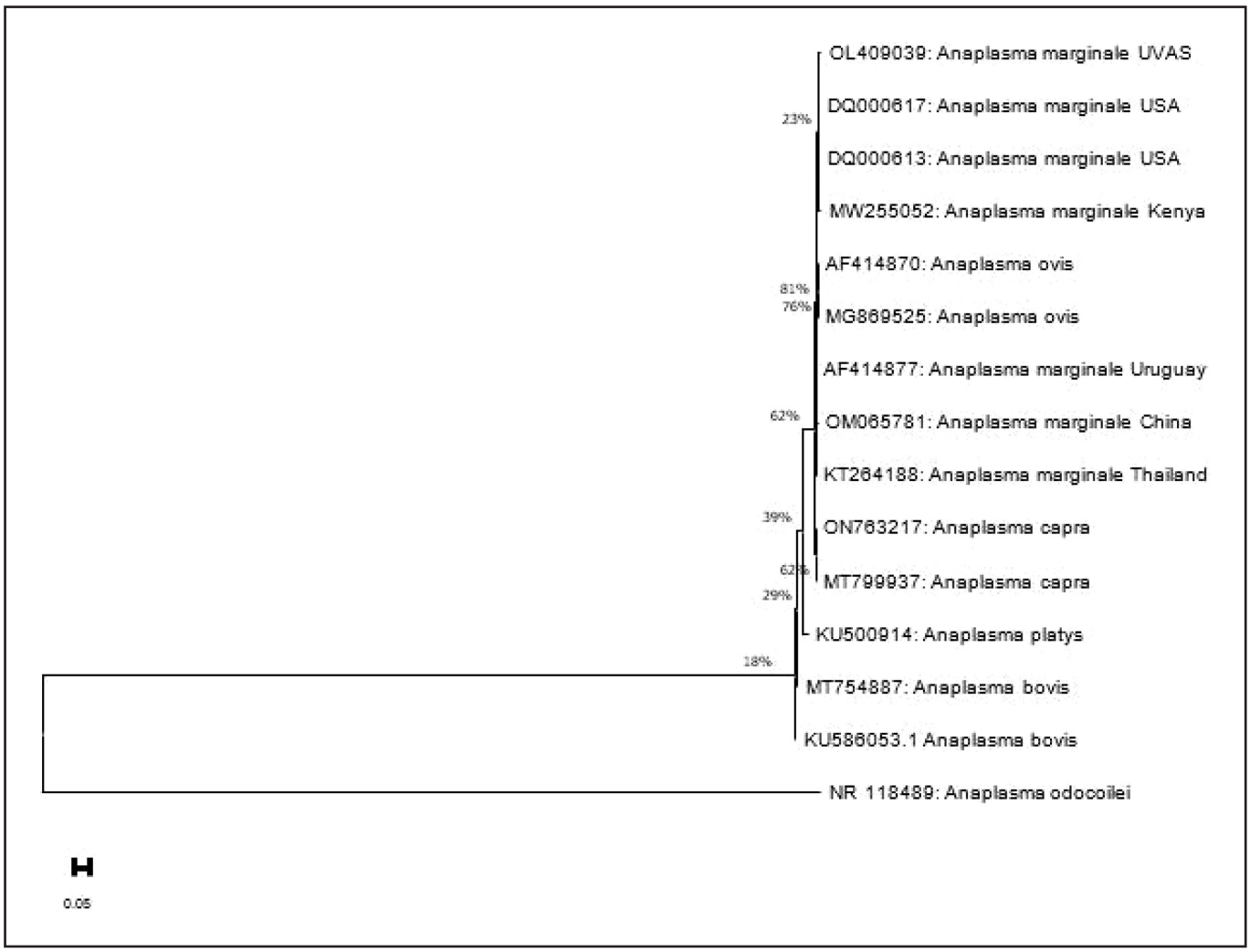

Figure 3. The maximum likelihood phylogenetic tree of *Anaplasma marginale* was created based on a partial 16S rRNA sequence. *Anaplasma odocoilei* sequence was used as an outgroup. The obtained sequence is represented with *Anaplasma marginale* UVAS (OL409039).

BLAST results of the obtained 16S rDNA (484 bp) sequence showed 81% similarity with the *A. ovis* (AF414870 and MG869525), 76% with *A. capra* (ON763217 and MT799937), 62% with *A. bovis* (MT754887 and KU586053) and 39% with *A. platys* (KU500914). The phylogenetic tree obtained a 16S rDNA sequence of *A. marginale* clustered with the identical species sequences reported from USA, Kenya, China, Uruguay, and Thailand. The 16S rDNA sequence of *A. marginale* was uploaded to NCBI under the accession number OL409039. *Anaplasma odocoilei* (GenBank accession no. NR118489) was used as an out-group in the tree (Figure 3).

DISCUSSION

Pakistan's climate is hot and humid (Average temp=23.43°C; Average humidity=37.9%) and this type of climate is ideal for ticks and their pathogens (Karim *et al*., 2017; Ali *et al*., 2020). According to previous studies, numerous tick species have been shown to infest a wide range of hosts in different regions of the country (Ali *et al*., 2019; Rehman *et al*., 2019; Ali *et al*., 2021). *Anaplasma marginale*, which causes bovine anaplasmosis, is biologically transmitted by more than twenty different types of hard ticks (Pothmann *et al*., 2016). In Pakistan, there are only few molecular studies for identifying *A. marginale* in ticks, since this pathogen is typically detected by microscopy and blood smear examination (Bhutto *et al*., 2012; Khan *et al*., 2017). The current study identified hard ticks comprised of four medically and veterinary important tick species infesting cattle, sheep, and goats. Among these four tick species, two species in the genus *Rhipicephalus* (*R. microplus* and *R. sanguineus*) were found positive for *A. marginale.* No study has been reported in literature for the simultaneous detection of *A. marginale* infection in the blood samples of goats and *R. sanguineus* ticks collected from goats.

Previous studies have reported that the occurrence of ticks was found to be significantly higher in female animals as compared to male animals (Rehman *et al*., 2019). Due to hormonal changes, female animals have a higher occurrence of ticks as compared to male animals because of the high level of progesterone and prolactin hormone which make them more susceptible to ticks infestation (Ghosh *et al*., 2019). Previous studies, also suggested that a higher occurrence of ticks in adult animals as compared to younger animals (Kamran *et al*., 2021a, 2021b). Adult animals are more susceptible to tick attachment due to free grazing practices and large surface areas, whereas younger animals are less susceptible due to less grazing, a smaller surface area of their bodies, and a strong immune system (Swai *et al*., 2005).

Different studies emphasized the occurrence of *A. marginale* infection in the livestock animals in the world. It was reported as 44% in cattle in China through a qPCR detection method (Qiu *et al*., 2016). Anaplasmosis in cattle has been reported as 14.04% through PCR in Egypt (El-Ashker *et al*., 2015). In Pakistan, 34.85% and 47.25% *A. marginale* infection has been detected through PCR in goats and sheep, respectively (Hussain *et al*., 2017). However, in cattle, 16.3% (Zeb *et al*., 2020) and 8.6% prevalence rates have been

reported previously in Pakistan (Ashraf *et al*., 2021). There were two hypotheses why the discrepancy was found compared with the current study. Firstly, the difference in occurrence of *A. marginale* infection is due to the involvement of *R. sanguineus* which has never been reported to be present on livestock animals specially in goats in Pakistan. Secondly, the difference might be due to the involvement of unattended area of Pakistan along the border of Afghanistan for the sampling in our study.

Rhipicephalus microplus is the known vector of *A. marginale* worldwide (Kocan *et al*., 2010). *Rhipicephalus microplus* has been reported as a vector of *A. marginale* in cattle and buffalo in Pakistan (Rehman *et al*., 2019; Adegoke *et al*., 2020; Ghafar *et al*., 2020). *Anaplasma marginale* detected in *Hy. anatolicum* ticks through PCR has been reported in Pakistan as well (Rehman *et al*., 2019). It is well known that *R. sanguineus* is the main vector of diseases in dogs, and it has been experimentally used for the transmission of *A. marginale* infection to cattle in Australia (Hoogstraal, 1956). In addition, *R. sanguineus* has been reported as a vector of *A. marginale* infection in cattle in Africa (Parker & Wilson, 1979). *Anaplasma marginale* has been detected in *Hy. anatolicum* ticks through PCR in cattle in Iraq (Al-Obaidi *et al*., 2020). Similarly, *A. marginale* has been detected in *Hy. schulzei* through a PCR in goat in Iran-Pakistan border (Choubdar *et al*., 2021). There are very less studies available for the sampling of *R. sanguineus* from livestock animals.

PCR-based 16S rRNA gene amplification for the detection of *Anaplasma* spp. was used in the current study which effectively diagnosed the organism in apparently healthy carrier animals. Similar findings have been reported that PCR and PCR-RFLP based on 16S rRNA gene as a preferred method for the identifying of anaplasmosis in carrier animals (Noaman *et al*., 2009). The sequence obtained in this study shared a 96-98% identity with previously deposited *A. marginale* sequences in GenBank from the USA and Kenya (Figure 3).

CONCLUSION

This study provides the first molecular detection of *Anaplasma marginale* in blood samples and *Rhipicephalus sanguineus* ticks collected from goats in Pakistan. *Anaplasma marginale* infection was not reported before in sheep, goats and cattle in Tank, Ghulam khan, Birmil and Miran shah areas of KPK, Pakistan. Future research is needed to find the role of *R. sanguineus* and the presence of dogs to attract these ticks in mixed livestock farming system.

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Conflict of interest

The authors declare no competing interests.

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