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Full Length Article

Prevalence and Molecular Characterization of *Anaplasma marginale* in Cattle Population of Khyber Pakhtunkhwa Province, Pakistan

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Abstract

Anaplasmosis is a hemo-rickettsial disease of cattle and is most prevalent in tropical and subtropical regions of the world including Pakistan. This disease has been placed as one of the most economically important haemoparasitic diseases. The aim of the current study was to determine the molecular characterization and to assess the prevalence of *Anaplasma marginale (A. marginale)* infection in cattle and associated risk factors in three districts of Khyber Pakhtunkhwa (KP) province of Pakistan *viz*., Mardan, Kohat and Swat. The blood samples were collected conveniently from 434 tick-infested animals keeping the aseptic measures. *A. marginale* was identified from blood samples by microscopy and PCR. Sequencing and phylogenetic analysis of the sequenced isolates of this study showed close sequence similarity with the reported strains of USA, Thailand, Uganda, Uruguay, Zimbabwe, Philippines and China. Moreover, multiple sequence alignment of the 16S ribosomal RNA gene sequences of 5 different clones of the *A. marginale* depicts substantial variation in the genotypes of *A. marginale* found in different locations of KP. The prevalence of *A. marginale* infection was non-significantly associated ($P > 0.05$) with districts, season, breed, age and sex of cattle. The highest prevalence of *A. marginale* infection was recorded in district Swat (20.30%) followed by Kohat (16.81%) and Mardan (15.00%) districts of KP. The prevalence of infection was highest in exotic breeds and their crosses, adults and female cattle. 10.70, 16.11, 46.70 and 26.70% were the prevalence of infection recorded for winter, spring, summer and autumn season, respectively. This study concludes that *A. marginale* infection is dominant in district Swat followed by Kohat and Mardan districts of KP province of Pakistan, respectively.

Key words: Prevalence; Anaplasmosis; Cattle; Sequencing; Phylogeny; Risk factors

Introduction

Anaplasma belongs to the rickettsial group of parasites which is an intraerythrocytic obligate bacteria responsible for bovine anaplasmosis and is transmitted by ticks (Inokuma 2007). Important species of the genus Anaplasma include *Anaplasma centrale*, *A. marginale*, *A. phagocytophilum*, *A. ovis*, *A. platys* and *A. bovis*. *A. bovis* is found both in wild and domestic animals in different parts of the world (Liu *et al*. 2012). *A. centrale* and *A. marginale* cause bovine anaplasmosis (Minjauw and McLeod 2003; Kocan *et al*. 2004). Gall sickness is another name of the disease. Almost all domesticated animals like buffaloes, cattle, goats, sheep as well as wild ruminants are affected by this disease. Bovine anaplasmosis is most commonly caused by *A. marginale.* It is a highly pathogenic disease characterized by weakness, anorexia, weight loss, depression, fever, jaundice, hemolytic anemia, decreased milk production, abortion and death. Cattle are more susceptible to infection than buffaloes and the disease causes high mortality in livestock (Rajput *et al*. 2005; Kocan *et al*. 2010). Transmission of the disease occurs mostly by ticks, about 20 ticks species are involved in the transmission of the disease (Marchette and Stiller 2018). Notable ticks species are *Hyalomma species*, *Rhipicephalus species*, *Ixodes species*, *Boophilus species* and *Dermacentor species* (Jongejan and Uilenberg 2004). Anaplasmosis occurs mainly in hot, humid and rainy seasons due to an abundance of ticks (El-Metenawy 2000). Surgical blades and contaminated needles are the mechanical sources of

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transmission of the disease. It is estimated that globally tick-borne diseases produce losses ranging from 13.9–18.7 billion US$ per year affecting 80% population of cattle (Ghosh *et al*. 2007). Anaplasmosis is one of the global importance diseases and is prevalent in developing countries like Zambia where livestock faces some serious challenges from tick-borne diseases especially anaplasmosis (Makala *et al*. 2003; Minjauw and McLeod 2003). Bovine anaplasmosis is highly prevalent in Africa and Asia due to the vast tick's movement and global warming (Jonsson and Reid 2000).

Anaplasmosis is a hemo-rickettsial disease of cattle and is highly prevalent in tropical and subtropical regions of the world including Pakistan (Dumler *et al*. 2001; Atif *et al*. 2013; Iqbal *et al*. 2019). It is one of the most prevalent hemoparasitic infections in Pakistan affecting bovines and its prevalence is 4–75.5% (Khan *et al*. 2004). It is a major health issue for livestock and cattle population in particular in Khyber Pakhtunkhwa (Nieto *et al*. 2012; Nasreen *et al*. 2016; Shah *et al*. 2017; Farooqi *et al*. 2018; Khan *et al*. 2019; Turi *et al*. 2019). This project aimed to find out the prevalence, molecular diagnosis, and characterization of *A. marginale* in the KP province of Pakistan.

Materials and Methods

Study area

This study was carried out from January 2018 to March 2019. The blood samples were collected from different cattle breeds of three districts of KP province *viz*., Mardan, Kohat and Swat as shown by Fig. 1. 434 blood samples were collected by the convenient method of sampling (Fanzana and Srunv*et al*. 2001). A total of 160, 131 and 143 blood samples were collected from Mardan, Kohat and Swat districts, respectively. A pre-tested data collection form was used having information about the data regarding date of sample collection, details about the animal (age, breed and sex) and place of the collection (Thrusfield 2007). About 5 mL of blood was collected from the jugular vein of cattle into vacutainers containing EDTA as an anticoagulant for the preservation of blood samples. Then these samples were shifted to parasitology laboratory UVAS, Lahore and were stored at −20ºC. GPS data was processed in MS Excel and then imported into ArcGIS 10.2. The sampling sites were geo-visualized in the form of point map. Then same points were populated on the map of Pakistan to present the spatial distribution of samples.

Microscopic examination

Thin smears were prepared on the spot for better results. The smears were fixed with absolute ethanol and stained with Giemsa in the laboratory of Parasitology, University of Veterinary and Animal Sciences Lahore. The smears were examined at 100x magnification under a compound microscope for the presence of *A. marginale* (Kumar *et al*. 2015).

Molecular examination

Molecular identification of *A. marginale* was carried out through polymerase chain reaction (Roy *et al*. 2018). The DNA was extracted by using a DNA extraction kit (cat. No: FABGK001-2) by the method used by (d'Oliveira *et al*. 1995). The purity and concentration of extracted DNA were checked by Nanodrop and was stored at −20ºC. The Polymerase chain reaction (PCR) was performed as described by (Gubbels *et al*. 1999). The extracted DNA samples were subjected to PCR which amplified the 16S rRNA gene of Anaplasma by using general primer EHR. This general primer consists of a forward primer EHR-16SD (5'- GGTACCTACAGAAGAAGTCC–3') and a reverse primer EHR-16SR (5'-TAGCACTCATCGTTTACAGC–3') and this set of primers targeted the 16S ribosomal RNA gene of *Anaplasma* (Tay *et al*. 2014). Master mix solution for PCR was prepared, 20 μL reaction mixture having 1.5 U of Taq DNA polymerase was taken and 2 μL of extracted DNA, 25 pmol of each primer, 200 m*M* of each dNTP, 5 μL of 10X PCR buffer and 1.5 m*M* $MgCl_2$ (Promega, Madison, W.I., U.S.A.) were added. An initial denaturation step was the first step of the PCR reaction cycle which was set at 94ºC for 5 min followed by a second cycle of denaturation (40 cycles) at 94ºC for 30 s, then annealing for 30 s at 55ºC and then extension for one min at 72ºC. The final extension was done for 5 min at 72ºC which was followed by a hold step at 4ºC. In each PCR experiment, a control positive (Agricultural Research Service, Animal Disease Research Unit, Department of Agriculture, Pullman, W.A., U.S.A.) and control negative were included. Gel electrophoresis was used for checking of positive bands against a standard molecular ladder of 100 bp on ethidium bromide stained 1.5% agarose gel at 200 amperes, 120 V for 30 min (Fig. 3). The bands for *A. marginale* were observed at 345 bp level. The positive bands were then cut and were considered for sequencing for further confirmation.

Sequencing

The PCR bands of haemoparasites were cut on 1.5% agarose gel using a cutter. The gel extraction kit (WizPrep^{TM} Gel/PCR purification kit, Ref. W70150-300) was then used for the extraction and purification of DNA bands from gel following the directions of the manufacturer. By using gel electrophoresis DNA concentration was checked and the DNA samples were sent to 1st base DNA sequencing services, Singapore for sequencing. The phylogenetic tree was constructed for the isolates identical to *A. marginale* by using MEGA 7 at maximum likelihood algorithm and with bootstrapping at 1000 replications (Fig. 4).

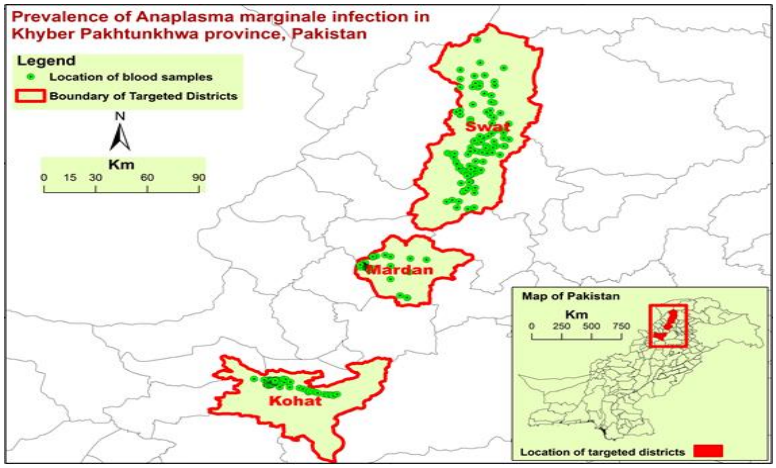

Fig. 1: Map of Pakistan showing sampling sites in targeted districts of KP

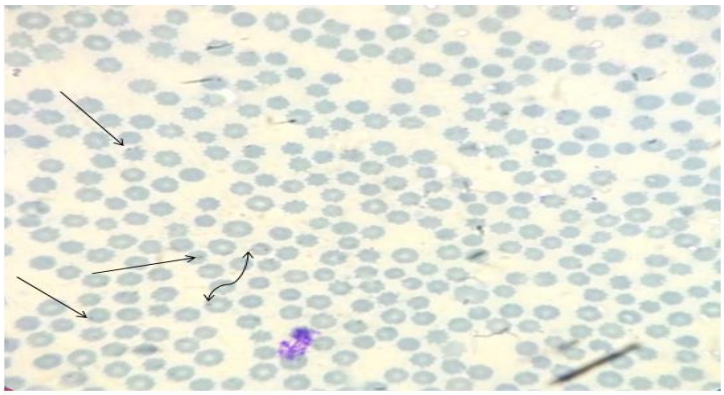

Fig. 2: Figure shows *A. marginale* like intracellular bodies in Giemsa's stained thin blood smear (indicated by arrows)

Statistical analysis

Chi-square test was used to analyze the prevalence of *Anaplasma species* data using SPSS version 20. *P*-value < 0.05 was taken as level of significance for the achievement of a 95% confidence interval (Farooqi *et al*. 2018).

Results

Microscopically thin blood smears were prepared and checked for the presence of intraerythrocytic inclusions bodies resembling *A. marginali* (Fig. 2). Microscopic examination showed that there were 10.00, 12.98 and 17.48% positive cases for *A. marginale* infection in cattle of Mardan, Kohat and Swat districts, respectively (Table 1). PCR showed 15.00, 16.79 and 20.28% positive cases in Mardan, Kohat and Swat districts, respectively (Table 2). It was clear from the results that PCR is a more sensitive and accurate method for the diagnosis of *A. marginale* infection as shown by Table 3. The PCR products of *A. marginale* were subjected to sequencing. BLAST and CLUSTAL W alignments were used for the analysis of these sequences. The resulted sequenced nucleotide after BLAST indicated the sequence similarity with the 16S ribosomal RNA gene of *A. marginale*. For comparison, the nucleotide sequences of these organisms were aligned from the NCBI database. The amplicons showed 96–99% similarity with the sequences of nucleotide for this gene which was deposited in GenBank. Five sequence products of *A. marginale* from KP with the allotted accession numbers from NCBI *i.e.*, MT893360.1 *A. marginale* (KP-1 Pak), MT893363.1 *A. marginale* (KP-2 Pak), MT893366.1 *A. marginale* (KP-3 Pak), MT893368.1 *A. marginale* (KP-4 Pak) and MT893370.1 *A. marginale* (KP-5 Pak) were used for the construction of phylogenetic tree as shown by Fig. 4 (Tamura 1992; Kumar *et al*. 2016).

It was observed from the results that breed, age and sex of cattle were non-significantly associated ($P > 0.05$) with *A. marginale* infection among the studied districts. The district wise prevalence of infection was recorded highest in district Swat followed by Kohat and Mardan districts, respectively. The results showed that the rate of infection was higher in exotic breeds (Friesians and crossbred) as compared to local breeds (Achi and Sahiwal) of cattle. The adult cattle were

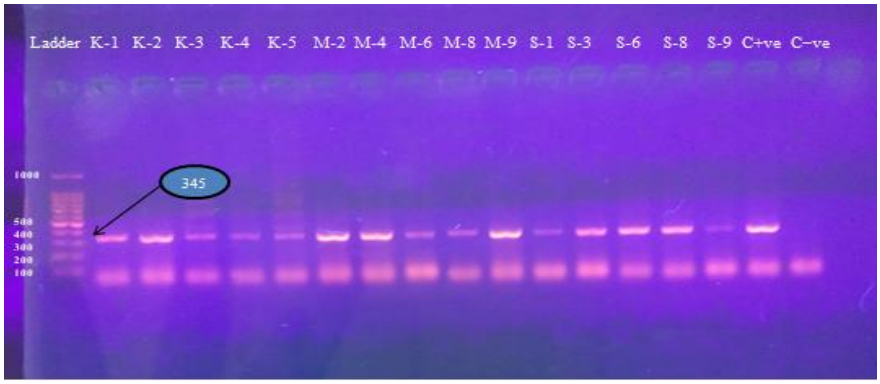

Fig. 3: PCR results for *A. marginale*. It shows gel electrophoresis after PCR having clear bands of an amplified 345 base pair DNA fragment of *A. marginale* against a marker of known molecular weight of 100 base pair. The lane Ladder shows a molecular weight marker. Positive samples of *A. marginale* are shown in lane just after the lane ladder for Kohat, Mardan and Swat districts, respectively. Lane C +ve shows control positive (*A. marginale*) while lane C−ve shows control negative

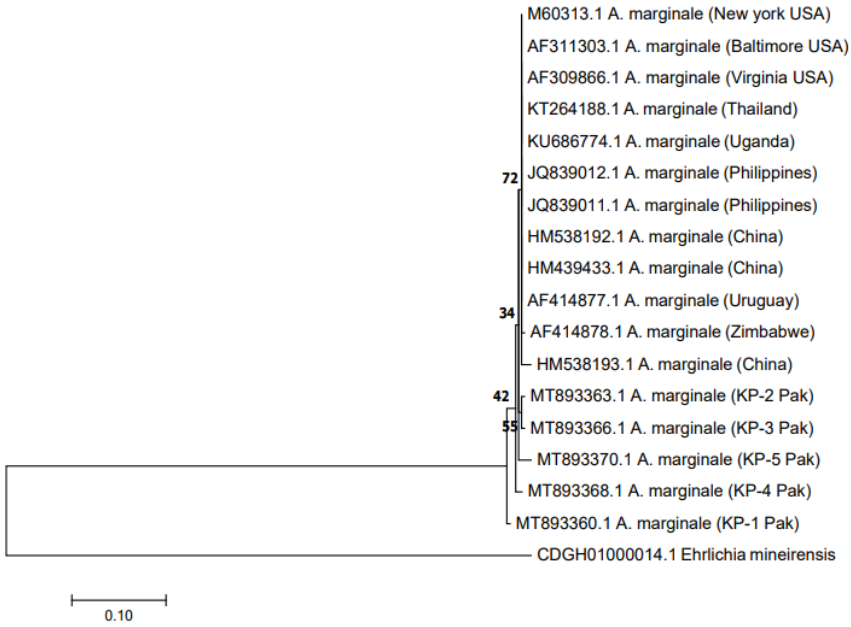

Fig. 4: It shows dendrogram representing the phylogenetic locations of genotypes of *A. marginale* based on the partial sequencing of 16S ribosomal RNA gene. MT893360.1 *A. marginale* (KP-1 Pak), MT893363.1 *A. marginale* (KP-2 Pak), MT893366.1 *A. marginale* (KP-3 Pak), MT893368.1 *A. marginale* (KP-4 Pak) and MT893370.1 *A. marginale* (KP-5 Pak) represent the gene sequences from this study with their allotted accession numbers from Genbank. The published sequences of *A. marginale* from the Genbank database were used in the analysis process. *Ehrlichia mineirensis* (CDGH01000014.1) was used as an outgroup for this study

affected more by the *A. marginale* infection as compared to young animals. Similarly, the prevalence of infection was recorded higher in female animals as compared to males as shown by Table 4. 10.70, 16.11, 46.70 and 26.70% were the prevalence of infection recorded for winter, spring, summer and autumn season, respectively. It was observed from the phylogenetic tree of *A. marginale* (Fig. 4) that the sequence isolates of this study were closely associated with each other and they showed sequence similarities with the reported strains of USA (M60313.1, AF311303.1, AF309866.1), Thailand (KT264188.1), Uganda (KU686774.1), Philippines (JQ839012.1, JQ839011.1), China (HM538192.1, HM439433.1, HM538193.1), Uruguay (AF414877.1) and Zimbabwe (AF414878.1). Multiple sequence alignment of the 16S ribosomal RNA gene sequences of 5 different clones of *A. marginale* depicts substantial variation in the genotypes of *A. marginale* found in different locations of KP (Fig. 5).

Discussion

Anaplasmosis is distributed throughout the world affecting

cattle populations especially in developing countries where the disease is highly endemic resulting in huge economic losses (Futse *et al*. 2003; Rodríguez *et al*. 2009). There is insufficient data of tick-borne diseases especially anaplasmosis in the KP province of Pakistan, even though livestock faces major challenges from these tick-borne diseases (Khan *et al*. 2004). The current study relates to the seasonal prevalence and molecular characterization of *A. marginale* in the KP province of Pakistan. The blood was screened by microscopy for *A. marginale*. The slides showed intraerythrocytic inclusion bodies resembling *A. marginale* under a light microscope. It correlates with the findings of Ahmad and Hasmi (2007), Atif *et al*. (2012) and Maharana *et al*. (2016). It was clear from the results that polymerase chain reaction gave better results in the identification of *A. marginale* than microscopy, so it was a more accurate and sensitive method than microscopy. It is in accordance with the findings of Khattak *et al*. (2012) and Saad *et al*. (2015) who confirmed PCR as a more sensitive technique for the diagnosis of haemoparasitic infection.

The prevalence of *A. marginale* infection in three districts of KP was recorded by using the Chi-Square test. It was observed that the difference in the prevalence of infections was non-significant ($P > 0.05$) in the studied districts. The highest prevalence was recorded in district Swat followed by Kohat and Mardan districts respectively. These findings correlate with the work done by Rajput *et al*. (2005); Atif *et al*. (2013); Farooqi *et al*. (2018).

The breeds of the cattle were checked for the presence of *A. marginale* infection and it was observed that the prevalence of infection was non-significantly ($P > 0.05$) associated with breeds of cattle in the studied districts. The results showed a higher prevalence of infection in exotic breeds and their crosses as compared to local breeds of cattle. The findings of this study are in accordance with the studies done by Chowdhury *et al*. (2006); Atif *et al*. (2012); Farooqi *et al*. (2018); Khan *et al*. (2019) who have also reported a higher prevalence of Anaplasma infection in exotic and crossbred animals as compared to local breeds of animals. This is because the exotic breeds and their crosses are in a state of more danger to tick infestation (Bock *et al*. 1997).

A. marginale infection was checked in cattle of KP province according to their age groups. It was observed that there was a non-significant ($P > 0.05$) association between the different age groups of cattle and the prevalence of infection in the studied districts. The results showed a higher *A. marginale* infection in adults than young cattle. Khan *et al*. (2004) also reported a non-significant relationship ($P > 0.05$) between the prevalence of blood parasites of bovine and age groups and they observed higher prevalence in adults (30.76%) than young animals (23.07%) which are in accordance with this study. Atif *et al*. (2013) also reported higher anaplasmosis in adults than in young cattle. The reason for higher parasitic infection in adults than young animals is the higher immunity of young animals due to the presence of foetal haemoglobin in their circulatory blood system (Ristic and Levy 1981). On the other hand, Nazar *et al*. (2018) and Khan *et al*. (2019) reported a higher prevalence of anaplasmosis in younger stock as compared to adult cattle which mismatches with the results of this study.

Table 1: Microscopic examination of blood for *A. marginale*

District	Positive n(%)	Negative n(%)	Total
Mardan	16 (10.00)	144 (90.00)	160
Kohat	17 (12.98)	114 (87.02)	131
Swat	25 (17.48)	118 (82.52)	143

Table 2: Molecular (PCR) examination of blood for *A. marginale*

S. No.	District	Positive n(%)	Negative n(%)	Total
1	Mardan	24 (15.00)	136 (85.00)	160
2	Kohat	22 (16.79)	109 (83.21)	131
3	Swat	29 (20.28)	114 (79.72)	143

Table 3: Comparison between results of microscopy and PCR for *A. marginale*

S. No.	District	Microscopy n(%)	PCR n(%)
1	Mardan	16 (10.00)	24 (15.00)
2	Kohat	17 (12.98)	22 (16.79)
3	Swat	25 (17.48)	29 (20.28)

The sex of cattle was checked against the prevalence of *A. marginale* infection in the study districts. It was observed from the results that there was a non-significant ($P > 0.05$) association between the prevalence of infection and sex of the cattle. It was clear from the results that the prevalence of infection was higher in females than male cattle. This study matches with the findings of Rajput *et al*. (2005) and Atif *et al*. (2012) who also reported higher anaplasmosis in females than male animals in their studies. Hormonal imbalances and immunosuppression in female animals are some of the reasons for the higher prevalence of haemoparasitic infections in females than male animals (Kocan *et al*. 2003). The results of this study showed that *A. marginale* infection was prevalent in different seasons of the year in KP province of Pakistan. Similar studies about the seasonal prevalence of anaplasmosis in KP province were conducted by Nasreen *et al*. (2016) and Khan *et al*. (2019)

Sequencing and phylogenetic analysis of *A. marginale* was studied in the KP province. It was observed from the phylogenetic tree of *A. marginale* (Fig. 4) that the sequence isolates of this study were closely associated with each other and they showed sequence similarity with the reported strains of USA, Thailand, Uganda, Philippines, China, Uruguay and Zimbabwe. Moreover, multiple sequence alignment of the 16S ribosomal RNA gene sequences of 5 different clones of the *A. marginale* depicts substantial variation in the genotypes of *A. marginale* found in different locations of KP (Khan *et al*. 2020). Liu *et al*. (2005); Ferrolho *et al*. (2016); Byaruhanga *et al*. (2018) have conducted similar studies for the sequencing and phylogenetic characterization of *A. marginale* which correlate with this study. The importation

Table 4: Prevalence of *A. marginale* infection according to breed, age and sex of cattle

Parameter		Mardan n(%)	Kohat n(%)	Swat n(%)	Total (n%)	Chi-square value	*P*-value
District wise		24 (15.00)	22 (16.81)	29 (20.30)	75 (17.30)	1.504	0.472
Breed	Friesian	06 (17.10)	03 (17.60)	08 (25.00)	17 (20.21)	0.728	0.695
	Crossbreed	12 (17.41)	14 (21.90)	12 (17.41)	38 (18.80)	0.576	.750
	Achai	06 (13.30)	05 (12.81)	06 (18.20)	17 (14.51)	0.498	0.780
	Sahiwal	00	00	03 (33.30)	03 (09.70)	8.119	0.017
Age	Young	06 (17.60)	02 (5.91)	06 (18.80)	14 (14.0)	2.836	0.242
	Adult	18 (14.30)	20 (20.61)	23 (20.70)	61 (18.31)	2.145	0.342
Sex	Male	05 (10.40)	04 (09.31)	04 (10.30)	13 (10.00)	0.035	0.982
	Female	19 (17.00)	18 (20.51)	25 (24.00)	62 (20.41)	1.662	0.436

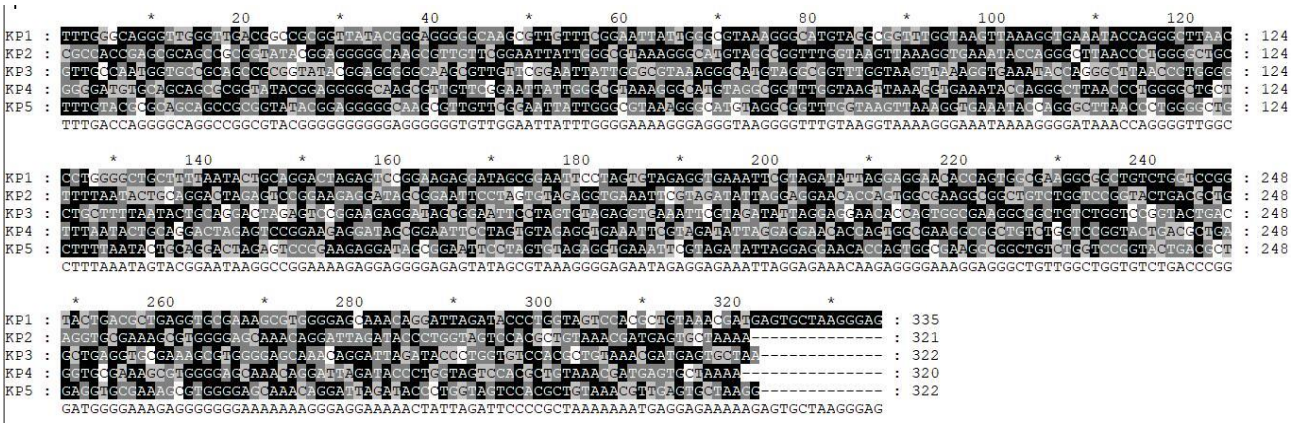

Fig. 5: The multiple sequence alignment of sequences of 5 clones of *A. marginale viz*., MT893360.1 *A. marginale* (KP-1 Pak), MT893363.1 *A. marginale* (KP-2 Pak), MT893366.1 *A. marginale* (KP-3 Pak), MT893368.1 *A. marginale* (KP-4 Pak) and MT893370.1 *A. marginale* (KP-5 Pak) based on the partial sequencing of 16S ribosomal RNA gene. The conservation level is denoted by background shading of the sequences "black" shows 100% conservation, the "gray with black" shows 80% conservation "grey with white" shows 60% conservation and "white" reflects no conservation

of livestock especially the live cattle from different regions of the world is the main reason for sequence similarities of the local and globally found haemoparasites (Rjeibi *et al*. 2018).

Conclusion

This study concludes that *A. marginale* infection was most prevalent in district Swat followed by Kohat and Mardan districts of KP province respectively. Prevalence of anaplasmosis was non-significantly ($P > 0.05$) associated with districts, season, breed, age and sex of cattle. The prevalence of infection was highest in exotic breeds and their crosses, adults and female animals. Phylogenetic analysis of *A. marginale* showed close sequence homology with the reported strains of different countries of the world like USA, Thailand, Uganda, Uruguay, Zimbabwe, Philippines and China. 5 different clones of the *A. marginale* found in different locations of KP showed genetic variations in the target sequence.

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Author Contributions

Manuscript write-up, data analysis and most of the experiments were performed by MS. MAK and FAK helped in the collection of blood samples from targeted districts. SHF helped in microscopy and PCR examinations in the laboratory. RW helped at review stage of article. MIR, HA and AAS helped in drafting of the research article and supervised the work.

Conflicts of Interest

All other authors declare no conflicts of interest

Data availability

Data presented in this study will be available on fair request to the corresponding author.

Ethics Approval

The experiments were carried out in accordance with the guidelines issued by the Animal Ethics Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan.

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