A Report on the Tick Burden, Molecular Detection and Phylogenetic Analysis of Anaplasma Marginale in the Blood Samples of Cattle Collected from District Layyah in Punjab (Pakistan)

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Research

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Abstract

Back ground: Anaplasmosis is known as yellow bag or yellow fever and it is a tick borne disease caused by obligate intercellular gram negative bacteria, *Anaplasma marginale*.

Aim: Present study is reporting seasonal prevalence, epidemiology and phylogeny of *Anaplasma marginale* in three cattle breeds from District Layyah, Southern Punjab, Pakistan. Methods and results: A total of 844 blood samples (Cross = 300, Holstein Friesian = 244, Sahiwal breed = 300) from apparently healthy cattle on seasonal basis were collected along with epidemiological data.

Results: Polymerase chain reaction generated 265 base pair amplicon specific for Major surface protein-1b encoding gene of *Anaplasma marginale* in 8.6% (73/844) of enrolled cattle. Highest prevalence was observed during autumn (18.3%) followed by summer (9.7%) and winter season (7.1%). Holstein Friesian breed was most susceptible to *A. marginale* infection (13.1%) followed by Sahiwal (7.6%) and cross breed (6%). Representative amplified partial gene sequences of *A. marginale* were submitted to GenBank (Accession numbers MK032842 and MK032843). Its phylogenetic analysis had shown similarities with sequences submitted by other countries. 37/844 (4.3%) Giemsa stained blood smears were found positive for *Anaplasma* spp. Analysis of epidemiological factors revealed that female cattle and farm with water supply from pool, farms where other dairy animals and dogs were living with cattle and dogs having ticks load on them had significant association with *A. marginale* prevalence. It was observed that white blood cell, lymphocytes (%), Monocytes (%) heamatocrit, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were significantly disturbed in *A. marginale* positive than negative cattle.

Conclusion: We recommend that this PCR protocol should be used for the detection of *A. marginale* in livestock for their proper diagnosis and treatment.

Introduction

Livestock is our natural resource for the conversion of roughage into good quality-foods like milk and meat. The other livestock products like hides are raw material for industries [1]. In a country like Pakistan with rain-fed agricultural production system, livestock provides security against crop-failure [2]. According to a recent report, livestock is contributing 53.2% in agricultural sector and has about 11.4% share in Pakistan's GDP that is greater than the crop growing part [47.4% of the agriculture and 10.3% of entire GDP] [3]. Cattle constitute 43% of the total livestock population in Pakistan including exotic and crossbreed cattle in addition to 15 indigenous breeds of cattle. [1, 4]. Despite good genetic potential most dairy animals in Pakistan have considerably low quantity of milk due to lack of disease control. Climatic conditions of Pakistan (being in tropics) are favorable for growth and development of tick species that are considered as major vector for several pathogens causing a variety of diseases in cattle [5].

Anaplasmosis is a tick-borne disease of cattle and is caused by *Anaplasma marginale* (*A. marginale*), an obligate intracellular Gram-negative bacteria from order Rickettsiales [6]. *A. marginale* are erythrocytic parasites that are transmitted by *Ixodes* sp., *Dermacentor* sp., *Rhipicephalus* sp. and *Haemaphysalis* sp. ticks, from infected to susceptible cattle by biting flies or by blood-contaminated fomites including needles, ear tagging and dehorning and castration equipment [7]. *A. marginale* are known to cause hemolytic anemia in host cattle and more than 70% erythrocytes are reported to be invaded by them in acute disease conditions [8]. Common clinical signs of *A. marginale* infection are fever, dyspnoea, lethargy, icterus, hyperexcitability, abortion due to hypoxia [9]. Recovered animals from the acute stage of anaplasmosis will develop lifelong low level of bacteraemia. These carrier animals act as a reservoir of infection for the susceptible host [6].

Present study was designed for the season based detection of *Anaplasma marginale* in three breed of cattle from District Layyah through conventional (smear screening) and modern tool (Polymerase chain reaction) in three different cattle breeds (cross, Holstein Friesian and Sahiwal) and to report the association of parasite prevalence with epidemiological factors and complete blood count parameters, if any.

Materials And Methods

Sample and Data Collection

Blood samples of 844 apparently cattle (Holstein Friesian, Sahiwal and cross breed) were collected from District Layyah, Southern Punjab, Pakistan on seasonal basis [summer (May till July), autumn (August till October), winter (November till January) and spring (February till April)]. After the informed consent of owners all the animals were examined by the veterinarian on the sampling sites and blood sample (approximately 10 ml) was collected from Jugular vein of each cattle and immediately preserved in a sterile tube containing 0.5M EDTA as an anticoagulant to be used for DNA extraction and for complete blood count analysis. A questionnaire was filled on the sampling site in order to collect data regarding epidemiological factors (gender, water source, tick load, other animals associated with herd, dog associated with herd and tick load on dog) associated with the prevalence of *Anaplasma marginale* in cattle.

Blood smear formation and screening

Geimsa stained blood smear of each animal was prepared and examined under the oil immersion lens of microscope for the microscopic detection of *Anaplasma marginale* following Takihi and Sandes [10].

Tick identification

Ticks were collected from the body of each animal during the collection of blood sample. Collected ticks were preserved in 70% ethanol and identified at the Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan using stereomicroscope and tick identification key following Madder et al. [11].

DNA Extraction from blood and tick samples

Inorganic method of DNA extraction was used following Saeed et al. [3]. The quality of the DNA extracted was assessed by measuring the optical density at 260/280 nm (O.R.I. Reinbeker, Hamburg) and by using submerged gel electrophoresis. DNA from ticks was extracted by Ammonium hydroxide method following Ammazalorso et al. [12].

PCR amplification

A set of oligonucleotide primers, Fwd 5'GCT CTA GCA GGT TAT GCG TC3' and Rev 5'CTG CTT GGG AGA ATG CACCT 3' was used to amplify 265 base pairs of major surface protein-1b encoding gene of *Anaplasma marginale* in blood and tick sample following Asif et al. [13]. Polymerase chain reaction (PCR) was performed in a final volume of 50 µl containing 13 mM Tris-HCl (pH8.3), 65 mM KCl, 2 mM MgCl₂, 0.0013% gelatin, 300 µM of each dNTP, 1U of AmpliTaq DNA polymerase, 0.5 µM of eachcytob1primer, 0.2 µM of each MAR1bB2 primer and 0.4 µM of each bovar2A primer and 2 µl of template DNA *Anaplasma marginale* positive sample, kindly provided by Prof. Kelly A. Brayton from Washington State University, USA, and negative samples (reaction mixture without DNA) were also amplified during each PCR reaction as positive and negative controls respectively.

Reaction conditions comprised of initial denaturation step of 94°C for 5 min followed by 30 cycles of denaturation 95°C for 50 s, primer annealing 56°C for 50 s and extension 65°C for 50 s. A final extension at 65°C for 5 min was carried out following Asif et al. [13]. PCR products were held at 4°C until gel electrophoresis was carried out by using 2% agarose gel and visualized under a UV Trans illuminator (Biostep, Germany).

DNA sequencing and phylogenetic analysis

To confirm the PCR results of *A. marginale*, five representative PCR products were randomly selected for DNA sequencing. PCR products were purified from agarose gel slices with NZYGelpure® (Nzytech, Portugal), and subsequently sequenced (First base Sequencing Service, Malaysia) with the same primers used for DNA amplification. The obtained sequences were analyzed by a BLAST search in Gene Bank for determining the accuracy of the PCR and submitted to NCBI Gene Bank.

Phylogenetic analysis was conducted using MSP1b sequences based on a 216 nucleotides aligned, and tree was created in MEGA 7.0 [14]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood (-549.04) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 20 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 155 positions in the final dataset.

Hematological Analysis

Complete blood count parameters in all cattle blood samples were analyzed by using automated complete blood count analyzer following Saeed et al. [3].

Statistical Analysis

All the data with quantitative values is expressed as Mean $\pm$ Standard error of mean. Statistical package Minitab (version 17, USA) was used for the statistical analysis of the results. Association between the presence of *Anaplasma marginale* and various risk factors, i.e. gender, water source, tick load, other animals associated with herd, dog associated with herd and tick load on dog was assessed by contingency table analysis using the Fisher's exact test (for 2×2 tables). 2 sample t-test was calculated to compare various studied hematological parameters between *Anaplasma marginale* positive and negative blood samples.

Results

Blood smear screening based prevalence of *Anaplasma marginale*

Following Giemsa's staining, blood smears from 844 cattle were examined microscopically and 37 (4.3%) cattle were found positive for *Anaplasma* spp. Upon microscopic examination, *Anaplasma* spp. appeared as dense, homogeneously stained blue-purple inclusions located toward the margin of the infected erythrocyte. Breed wise analysis indicated that 6.5% Holstein Friesian, 4% Sahiwal and 3% cross breed cattle blood smears were positive for *Anaplasma* (data not shown here). When data was analysed on season basis, it was observed that maximum *Anaplasma* prevalence was observed in blood smears prepared during autumn (43%) followed by autumn (29%), winter (21%) and spring (5.4%) (data not shown here).

PCR based prevalence of *Anaplasma marginale*

Polymerase chain reaction (PCR) amplified a 265 base pair amplicon specific for Major surface protein-1b gene in 73 out of 844 (8.6%) analyzed cattle blood samples. Highest prevalence of *Anaplasma marginale* was observed in blood samples collected during autumn (18.3%) followed by samples collected in summer (9.7%), winter (7.1%) and spring season (1.7%) (Table 1).

Table 1

Over all and seasonal prevalence of *Anaplasma marginale* among Sahiwal, Holstein Friesian and cross breed blood samples collected during present study through PCR amplification of *msp1b* gene.

Season (Number of collected samples)	<i>Anaplasma marginale</i> positive cross breed samples	<i>Anaplasma marginale</i> positive Holstein Friesian breed samples	<i>Anaplasma marginale</i> positive Sahiwal breed samples	<i>Anaplasma marginale</i> positive samples (% Prevalence)
Spring (N = 225)	0	2	2	4 (1.7%)
Summer (N = 225)	2	18	2	22 (9.7%)
Autumn (N = 169)	11	6	14	31 (18.3%)
Winter (N = 225)	5	6	5	16 (7.1%)
<i>Anaplasma marginale</i> positive samples (% Prevalence)	18/300 (6%)	32/244 (13.1%)	23/300 (7.6%)	73/844 (Over all prevalence 8.6%)

Table 2

Association of studied epidemiological parameters of Holstein Friesian breed cattle collected from District Layyah with the prevalence of *Anaplasma marginale*. Prevalence (%) of *Anaplasma marginale* is given in parenthesis. P –values indicate the results of Fischer test calculated for each studied parameter

Parameters		<i>A. marginale</i> + ive samples from spring	P value	<i>A. marginale</i> + ive samples from summer	P value	<i>A. marginale</i> + ive samples from autumn	P value	<i>A. marginale</i> + ive samples from winter	P value
Sex	Male	0/0 (0%)	#	2/8 (25%)	1	1/2 (50%)	1	0/9 (0%)	1
	Female	2/75 (3%)		16/ 67 (24%)		4/17 (23%)		6/66 (9%)	
Water resource	Pump	2/75 (3%)	#	7/55 (13%)	0.0003**	19/19 (100%)	#	#	#
	Pool	0/0 (0%)		11/20 (55%)		0/0 (0%)		#	
Tick load	Yes	1/25 (4%)	1	7/30 (23%)	1	0/8 (0%)	0.3	5/56 (9%)	1
	No	1/50 (2%)		11/45 (24%)		5/11 (45%)		1/19 (5%)	
Other dairy animals at farm	Yes	0/5 (0%)	1	15/59 (25%)	0.7	0/3 (0%)	0.01*	6/52 (12%)	0.1
	No	2/70 (3%)		3/16 (19%)		5/16 (31%)		0/23 (0%)	
Dogs at farm	Yes	0/30 (0%)	0.5	14/52 (27%)	0.5	5/13 (46%)	0.3	6/66 (9%)	1
	No	2/45 (4%)		4/23 (17%)		0/6 (0%)		0/9 (0%)	
Tick load on dogs	Yes	0/5 (0%)	1	14/52 (27%)	0.5	4/4 (100%)	0.02*	6/59 (10%)	0.3
	No	2/70 (3%)		4/23 (17%)		1/15 (6%)		0/16 (0%)	
P > 0.05 = Non significant; P < 0.05 = Least significant (*); P < 0.01 = Significant (**).									
# Statistical analysis was not possible									

Among the breeds, prevalence of *Anaplasma marginale* was highest in Holstein Friesian breed (13.1%) followed by Sahiwal (7.6%) and cross breed cattle (6%) (Table.1). When prevalence data from each breed was analyzed on seasonal basis, it was observed that highest prevalence of *Anaplasma marginale* in Holstein Friesian breed was detected in blood samples collected during summer (24%) followed by autumn (8%), winter (8%) and spring season (2.6%). In Cross breed, the higher prevalence of *Anaplasma marginale* was observed in blood samples collected in autumn (14.6%) followed by winter (6.6%) and summer season (2.6%). *Anaplasma marginale* was not detected in blood sample of cross breed cattle collected in spring season. Similarly Higher prevalence of *Anaplasma marginale* in Sahiwal breed was observed in blood samples collected during autumn (18%) followed by winter (6.6%), summer (2.6%) and spring season (2.6%) (Table 1).

Number of tick samples collected during present study was limited and *Anaplasma marginale* was not detected in any of them through PCR amplification.

DNA sequencing and Phylogenetic Analysis

Two represented amplicon of 265 bp from Major surface protein-1b gene sequence were confirmed by DNA sequencing and were submitted to the GenBank database under the Accession numbers MK032842 and MK032843. A BLAST analysis revealed nucleotide sequence identities of 97–99% with homologous sequences of *Anaplasma marginale* isolates registered in GenBank. The phylogenetic analysis of the obtained DNA sequence from *Anaplasma marginale* placed it in stable monophyletic cluster along with major surface protein 1b gene of *Anaplasma marginale* downloaded from public databases (Fig. 1).

Morpho-Taxonomic identification of ticks

In present study, *Rhipicephalus microplus* and *Haemaphysalis punctata* ticks were identified. The morphological structures analyzed included the capitulum, Haller's organ, adanal plates, spiracles, festoons and other aspects of general morphology (data not shown here).

Risk factor analysis

Analysis of studied epidemiological factors for Holstein Friesian breed revealed that prevalence of *Anaplasma marginale* was significantly higher during summer in farms supplied by water pools ($P = 0.003$) and in autumns in farms where only cattle were reared ($P = 0.01$) and having dogs present in farms with tick burden (Table. 2). For Sahiwal breed during autumn season, it was observed that prevalence of *Anaplasma marginale* was significantly higher in farms without dogs (data not shown here). For cross breed, none of the studied epidemiological factors were found associated with prevalence of *Anaplasma marginale* (data not shown here).

Complete blood count analysis

Analysis of results indicated that cattle of Holstein Friesian breed positive for *Anaplasma marginale* infection during summer had significantly reduced Mean cell hemoglobin ($P = 0.017$) mean corpuscular hemoglobin concentration ($P = 0.016$) and Platelet count ($P = 0.016$) than *Anaplasma marginale* negative animals (Table 3). For Sahiwal breed, White blood cell count during summer ($P = 0.01$) and autumn ($P = 0.02$), Lymphocytes % in winter ($P = 0.02$) and Platelet count in winter ($P = 0.05$) were significantly reduced in *Anaplasma marginale* positive than negative cattle (data not shown here). For cross breed, it was observed that *Anaplasma marginale* positive animals had significantly reduced Monocytes (%) during autumn ($P = 0.03$), mean corpuscular hemoglobin concentration ($P = 0.002$) and platelet count ($P < 0.001$) during summer than parasite negative animals. All other studied parameters varied non-significantly ($P > 0.05$) when compared between *Anaplasma marginale* negative and positive cattle blood samples of all three breeds (data not shown here).

Table 3

Comparison of complete blood count parameters between *Anaplasma marginale* positive and negative blood samples of Holstein Friesian cattle collected from District Layyah during seasons in 2017–2018. Data is expressed as mean $\pm$ standard error of mean (SEM). P-value indicates the result of two sample t –test calculated for each studied parameter when compared between parasite positive and negative blood samples collected during a particular season.

Parameters	Spring		Summer		Autumn		Winter	
	<i>A. marginale</i> (+ ive) N = 2	<i>A. marginale</i> (- ive) N = 72	<i>A. marginale</i> (+ ive) N = 18	<i>A. marginale</i> (- ive) N = 57	<i>A. marginale</i> (+ ive) N = 6	<i>A. marginale</i> (- ive) N = 13	<i>A. marginale</i> (+ ive) N = 6	<i>A. marginale</i> (- ive) N = 69
White blood cell ($\times 10^3 \mu\text{L}^{-1}$)	7.6 $\pm$ 1.8	9.71 $\pm$ 0.3	8.3 $\pm$ 0.4	8.4 $\pm$ 0.3	11.7 $\pm$ 1.1	11.12 $\pm$ 1.1	8.2 $\pm$ 0.32	8 $\pm$ 0.1
Lymphocytes (%)	49.6 $\pm$ 1.4	46.2 $\pm$ 1.6	51.3 $\pm$ 2.1	54.4 $\pm$ 1.6	22.3 $\pm$ 4.6	25.6 $\pm$ 3.2	24.1 $\pm$ 5.4	35.1 $\pm$ 1.5
Monocytes (%)	5.2 $\pm$ 0.3	4.7 $\pm$ 0.2	0.32 $\pm$ 0.01	0.31 $\pm$ 0.004	3.5 $\pm$ 0.2	3.2 $\pm$ 0.1	8.2 $\pm$ 4.4	5.7 $\pm$ 0.6
Red blood cells ($\times 10^6 \mu\text{L}^{-1}$)	5.4 $\pm$ 0.5	5.8 $\pm$ 0.1	5.17 $\pm$ 0.16	5.11 $\pm$ 0.12	3.8 $\pm$ 0.03	3.7 $\pm$ 0.08	4.7 $\pm$ 0.3	4.6 $\pm$ 0.08
Hemoglobin (gdL^{-1})	8.5 $\pm$ 1.3	9.6 $\pm$ 0.1	8 $\pm$ 0.2	7.9 $\pm$ 0.1	9.4 $\pm$ 0.37	9.3 $\pm$ 0.34	8.76 $\pm$ 0.2	8.74 $\pm$ 0.1
Mean cell volume (f L)	45.1 $\pm$ 0.45	45.2 $\pm$ 0.2	47.5 $\pm$ 1.8	46.4 $\pm$ 0.8	79 $\pm$ 1.6	79.6 $\pm$ 2.2	68.7 $\pm$ 6	77.4 $\pm$ 0.8
Hematocrit (%)	24.5 $\pm$ 2.8	26 $\pm$ 0.4	24.5 $\pm$ 1	23.6 $\pm$ 0.6	28.6 $\pm$ 1.4	29.4 $\pm$ 1.2	34.1 $\pm$ 3.9	39.8 $\pm$ 1
Mean corpuscular hemoglobin concentration (g/dl)	34.7 $\pm$ 1.1	37 $\pm$ 0.4	34.7 $\pm$ 1.1*	37 $\pm$ 0.4	33.6 $\pm$ 1.3	35.3 $\pm$ 1.6	31 $\pm$ 1.3	32 $\pm$ 0.2
Platelets ($\times 10^3 \mu\text{L}^{-1}$)	258 $\pm$ 78	318 $\pm$ 14	265.8 $\pm$ 16*	323 $\pm$ 17	190 $\pm$ 31	209 $\pm$ 17	28.2 $\pm$ 9.8	27.2 $\pm$ 6.5
P > 0.05 = Non significant P < 0.001 = Significant (*)								

Discussion

Tick-borne rickettsial diseases are considered as one of the major constraints to livestock improvement programmes as they are responsible for serious health problems resulting in reduced animal productivity and economic losses [15]. It has been documented that ticks of *Rhipicephalus* sp. and *Haemaphysalis* sp. are responsible to transmit *Anaplasma marginale* to a variety of vertebrate host due to elevated temperature and favorable climate, in tropical countries [16]. Our results are in line with these established facts as in present study, ticks belonging to, *Rhipicephalus* and *Haemaphysalis* were identified from the cattle under investigation during morpho-taxonomic identification (data not shown here).

Diagnosis of *A. marginale* is performed by both modern and conventional methods. The most common but less reliable method is the Giemsa stained blood smear screening for the detection of these bacteria within the erythrocytes [17]. However, this technique is unable to detect pre-symptomatic, and carrier animals having low parasitemia [18]. PCR is usually considered as gold standard for the detection of tick borne blood parasites and it is more sensitive and reliable than parasite

detection by blood smear screening [6]. The sensitivity of PCR for detection of *Anaplasma marginale* (8.6% prevalence) was higher than the blood smears screening (4.3% prevalence) during present study. Ybanez et al. [19] had also documented that PCR based detection of *Anaplasma marginale* is more sensitive tool than through Giemsa stained blood smear screening. Several studies regarding the detection of *Anaplasma marginale* infection in cattle have been reported from Africa {25.4% in Tunisia [20]}; 21.9% in Morocco [21], 6.1% in Sudan [22] and 3.7% in Egypt[23]}, 2.3% from Turkey [24] and 5.4% from Brazil [25]. There are few studies from various parts of Pakistan reporting the prevalence of *Anaplasma marginale* in cattle. Atif et al. [26] had reported 9% prevalence of *Anaplasma marginale* in Giemsa stained blood smear of cattle collected from Sargodha District in Punjab. Sajid et al. [27] had reported 4.07% (34/836) prevalence of *Anaplasma marginale* in cattle samples collected from Khanewal District in Punjab through blood smear screening. In a recent study, Turi et al. [28] has reported 41.6% prevalence of *Anaplasma marginale* in cattle blood samples collected from District Peshawar and Lakki Marwat through real time PCR technique. Similarly, Farooqi et al. [29] has reported PCR based prevalence to be 18.33% (165/900) in cattle and buffalo blood samples collected from various parts of Khyber Pukhtoon Khwa. The difference in infection rates of *Anaplasma marginale* may be due to differences in tick control programmes, habitat suitability for ticks, farm management, husbandry practices, wildlife reservoir hosts and/or abiotic factors as reported by others [20, 30].

The data on genetic diversity of *Anaplasma marginale* is very rare from Pakistan. To investigate the genetic background of *Anaplasma marginale* present in Pakistan, we performed phylogenetic analysis based on a 265 base pair amplicon from major surface protein–1b gene that revealed greater genetic diversity as several diverse genetic groups can be seen in phylogenetic tree based on partial sequence homology (Fig. 2). Analysis revealed that sequences obtained during present study are clustered with the previously reported sequences from United States of America (AF348137 and CP000030) indicating that these are closely related genetic variants (Fig. 1).

Prevalence of *Anaplasma marginale* varied with sampling season during present study and highest parasite prevalence was observed during autumn (Table 1). High parasite prevalence in autumn during present study is probably due to abundance of vector ticks that were developed during summer months. Our result is contradictory to the findings of Vohra et al. [31] and Roy et al. [32] who had reported high prevalence of *Anaplasma marginale* in summer season. These differences observed in the prevalence between these studies are due to different geographical locations, abundance of tick vector and breeding microenvironment of ticks (temperature and humidity)[33].

According to our result Holstein Friesian breed had highest infection rate of *Anaplasma marginale* as compared to cross and Sahiwal breed (Table 1). Our result is in line with result of Tay et al. [34] who had reported that *Anaplasma* spp. prevalence was higher in Holstien cattle as compared to local breeds. This result may be due to some kind of resistance of the local breed that is developed against rickettsial infection. Indeed, local breeds are known to be more resistant to tick-borne pathogens [3]. Longer and thicker hairs of Holstein Friesian breed may be the factor for their higher infestation of ticks as compared to other breed. It has been reported that there is a positive association of hair length and coat thickness with the number of ticks [35]. However, our result is contradictory to findings of Belkahia et al. [20] who had showed Holstein cattle were significantly less infected by *Anaplasma marginale* ($p < 0.001$) than other breeds.

Epidemiological data analysis for gender, although remained non significant, revealed that female cattle were more prone to *Anaplasma marginale* infection than male. This is in accordance with Kamani et al. [36] who revealed prevalence of *Anaplasma* infection was higher in female cattle possibly due to the fact that they were kept longer for breeding and milk production purpose, supplied insufficient feed against their high demand. The immune suppression in advanced pregnancy and or lactation in high producing study animals are the possible reasons for the higher prevalence of *Anaplasma marginale* in female cattle [8]. The dog associated with herd of cattle is another parameter that has showed significant association with the *Anaplasma* infection indicating that dogs are acting as tick carrier and bring the infection to the herd Our results are contradictory to Ashraf et al. [6] which showed higher *Anaplasma marginale* prevalence in buffalo herds without dog. Significantly higher prevalence of *Anaplasma marginale* was observed in traditionally managed farms where the animals were not regularly vaccinated against tick borne diseases and water was supplied through pools with contaminated water instead of pumps providing fresh ground water. Our results is in accordance with Swai et al. [37] and Atif et al. [38] who had

reported significantly higher prevalence of *Anaplasma marginale* in traditionally managed farms when compared to modern ones. Poor management, lack of tick control practices and inadequate economic sustainability of poor resource smallholders may contribute to the higher prevalence of *Anaplasma marginale* [38].

Analysis of the complete blood count parameters indicated significant decrease in hematocrit, mean cell hemoglobin and mean corpuscular hemoglobin concentration in parasite positive cattle. These observations are in line with Gannguly et al. [39] who had reported that in severely infected cases, cattle had hemoglobin level as low as 3 g/dl and packed cell volume was decreased to $1.99 \times 10^6/\mu\text{l}$. This might be due to damage caused by *Anaplasma marginale* inside the RBCs during their multiplication. Our result is also in accordance with Riond et al. [40] who had reported decreased mean corpuscular hemoglobin concentration and increased mean corpuscular volume in *Anaplasma marginale* infected as compared to uninfected cattle. They have reported that fast destruction of RBCs by phagocytosis leads to their increased demand, therefore bone marrow cells start to release immature RBCs. The immature RBCs are bigger in size than mature RBCs having more corpuscular volume. In present study, the mean corpuscular volume was non-significantly increased in infected animals. It reflects that animal's haemopoietic system was activated in response to erythrophagocytosis initiated by parasitic damage to erythrocytes and that leads to decrease in RBC, this may be also due to increased level of activated complement products and removal of destroyed cells by bovine reticuloendothelial system [41]. Analysis of hematological parameters indicated that white blood cells, platelet count and lymphocytes (%) were significantly decreased in *Anaplasma marginale* positive cattle blood samples than in blood samples where parasite was not detected (Table 3). Our result coincides with Khan et al. [42] who recorded significant decrease of WBCs in prepatent phase and non-significant decrease of WBCs in early and late stages of the disease. Our results are in agreement with the finding of Riond et al. [40] who had reported that anemic cattle showed thrombocytopenia i.e a condition in which there is a low blood platelet count. The increased platelet consumption may be attributed to an immune-mediated process or result from intravascular disseminated coagulation [43].

In conclusion, we are reporting the prevalence of *Anaplasma marginale* infection in three cattle breeds from Pakistan with Holstein Friesian breed being most susceptible to this infection. We have documented that PCR is more reliable and sensitive technique for *Anaplasma marginale* detection than blood smear screening. We are also reporting that parasite prevalence varied with the sampling season and presence of *Anaplasma marginale* had significant effects on the hematological profile of all cattle breeds under investigation. Data generated through this study will pave the way for the control of anaplasmosis in Pakistan. We recommend this technique to the livestock owners for prophylactic detection of *Anaplasma marginale* in cattle blood in order to improve their economic output.

Declarations

Ethics approval and consent to participate

All the animal handling procedures and lab protocols were approved by the ethical committee of Institute of Pure and Applied Biology following the instructions of Institutional Review Board (IBR) of Bahauddin Zakariya University Multan, Pakistan. Informed consent was obtained from each cattle owner before and blood, tick and data collection.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this submitted article.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

FI has designed and supervised the study, SA, and AP has performed the smear screening, PCR and hematological analysis. MA and SO has done the DNA sequencing and phylogenetic analysis. MMA has carried out the sample and data collection. SA, has prepared and ADA, MSA FI have revised the manuscript.

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Figures

Figure 1

Phylogenetic tree based on partial major surface protein-1b gene sequences from *Anaplasma marginale* isolates from cattle in Pakistan and cattle worldwide, available in GenBank. The evolutionary history was inferred using the maximum likelihood method based on the Tamura 3-parameter model. The three new sequences of *Anaplasma marginale* obtained in the present study are represented in bold.