



## Phylogenetic exploration and haemato-biochemical significance of *Anaplasma marginale* infection in water buffalo

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### ABSTRACT

Buffalo blood samples (8,352) were screened microscopically during 2017-2020 for *Anaplasma* species and 104 samples were found positive. Later on, same samples were confirmed positive using genomic DNA PCR technique. For efficiency of PCR technique, 335 blood samples were randomly chosen. Microscopy revealed *Anaplasma* species in erythrocytes of 1.19% (4 samples) of the total blood samples (n=335). Initially PCR assay was standardized targeting 16S rRNA for detection of genus *Anaplasma* with expected amplicon size of 781 bp. Thereafter, *msp1β* gene was targeted for species level identification of *Anaplasma* (*A. marginale*) with expected amplicon size of 407 bp. A total of 14 samples out of 335 samples (4.17%) were positive by PCR. Randomly, two confirmed positive samples were sent for bidirectional sequencing and the sequencing results were submitted in GenBank (MW269530, MW269531), to further confirm the *Anaplasma marginale* infection in buffalo. The study showed higher specificity and sensitivity of PCR test in comparison to blood smear examination. Erythron of infected animals showed significantly low level of mean Hb, PCV, RBC counts, MCH, MCHC and high level of mean MCV, indicating regenerative hypochromic macrocytic anaemia. Infected animals had a significantly low mean level of total protein, albumin and globulin and significantly high level of ALT, AST, total bilirubin, direct and indirect bilirubin. The current study provides indication of the association between *A. marginale* infection and probable liver damage or hepatic fatty degeneration. Nevertheless, advanced studies are warranted to explain the precise pathophysiological mechanism of this association.

**Keywords:** *Anaplasma marginale*, Haemato-biochemical, Haemoparasite, Molecular, PCR, Phylogenetic, *Rickettsia*

*Anaplasma marginale*, an obligatory intracellular rickettsial organism, is the main causative agent of anaplasmosis in ruminants. It is transmitted either biologically by infected ticks or mechanically by biting flies, blood-contaminated fomites, etc (Grau *et al.* 2013). It mainly affects cattle, but it can also infect ruminants such as bison, buffalo, and African antelopes (Aubry and Geale 2011). Giemsa-stained blood smears are commonly used to detect *A. marginale* in animals clinically suspected of acute illnesses, but they are ineffective in detecting pre-symptomatic or carrier animals. As a result, many serological tests have been developed; however, due to antigen cross-reactivity, these techniques are unable to differentiate between *Anaplasma* species (Jimenez *et al.* 2008).

Molecular methods have been developed with a high level of sensitivity and specificity (Molad *et al.* 2006, Carelli *et al.* 2007, Ganguly *et al.* 2018, Maharana *et al.* 2019a, b; Ganguly *et al.* 2020). The major surface protein 1 (MSP1) of *A. marginale* is a heterodimer and consists of two structurally unrelated proteins viz. *msp1α* which is encoded by a single gene *msp1α*, and *msp1β* which is encoded by

members of the *msp1β* multigene family (Camacho-Nuez *et al.* 2000). The *msp1β* gene being a sensitive and specific target has been used for detection of *A. marginale* in nested PCR (nPCR) (Molad *et al.* 2006).

Few studies reported a low prevalence rate of *A. marginale* in buffalo herds, even in endemic areas (Ashuma *et al.* 2013, da Silva *et al.* 2014a, Kumar *et al.* 2019). Regardless of the fact that this rickettsia has been reported in buffalo herds (Ashuma *et al.* 2013, da Silva *et al.* 2014a, b, 2015; Lima *et al.* 2019), little is known about its effect on hematobiochemical profile in these populations. Baring a few reports (Patel *et al.* 2018, Kumar *et al.* 2019), there is paucity of literature pertaining to molecular characterization of *A. marginale* buffalo isolates from Indian context. The present study was therefore intended to identify, characterize and elucidate the molecular phylogenetic profile of buffalo *A. marginale* and its effect on the blood biochemical and cellular profile.

### MATERIALS AND METHODS

**Sample collection and microscopic examination:** Lactating buffaloes (n=8352, 4-7 years of age) brought to outpatient department (OPD) of Veterinary Unit, LUVAS, Uchani, Karnal during July 2017 to June 2020 and showing clinical signs, viz. fever, pale mucous membrane,

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anaemia, jaundice, brownish urine, inappetance, dullness, constipation, etc. similar to anaplasmosis were included in the present study (Maharana *et al.* 2016a, Ganguly *et al.* 2017, 2018). For haemato-biochemical profile, buffaloes (n=30) showing  $\geq 5\%$  parasitaemia (infected group) and apparently healthy buffaloes (n=10) free from *A. marginale* (negative both by microscopy and direct blood PCR assay) were included. The blood samples from both the groups were collected in vials with or without anticoagulant (EDTA). Immediately after collection, thin blood smears were prepared, and Giemsa stained and examined microscopically for the presence of *A. marginale* inclusion bodies.

**Isolation and quantification of extracted DNA:** Genomic DNA was extracted from 200  $\mu\text{L}$  of blood using QIAamp DNA Kit (Qiagen, Germany) according to the manufacturer's instructions. Purity and concentration of DNA was determined using Biospectrometer (Eppendorf, Germany) and quality was assessed in 1.2% agarose gel electrophoresis. The DNA was stored at  $-20^\circ\text{C}$  until further use.

#### PCR amplification, purification and sequencing

**Polymerase chain reaction of 16S rRNA:** The *Anaplasma* spp. 16S rRNA gene was amplified by PCR. PCR was performed in a final 25  $\mu\text{L}$  reaction mixture containing 1  $\mu\text{L}$  of extracted DNA, 12.5  $\mu\text{L}$  of  $2 \times$  TopTaq PCR Master mix (TopTaq DNA Polymerase, PCR Buffer (with 3 mM  $\text{MgCl}_2$ ), and 400  $\mu\text{M}$  each dNTP), 0.5  $\mu\text{M}$  of each forward and reverse primers (P1/P2) (Table 1), and 0.5  $\mu\text{L}$  of TopTaq DNA Polymerase (Qiagen, USA) in automated thermocycler (Veriti™, Applied Biosystem, USA) for 32 cycles. After an initial denaturation step of 5 min at  $95^\circ\text{C}$ , each cycle consisted of denaturation at  $94^\circ\text{C}$  of 30 sec, annealing at  $58^\circ\text{C}$  for 30 sec and an extension step at  $72^\circ\text{C}$  of 30 sec. A final extension at  $72^\circ\text{C}$  for 5 min was carried out. The PCR products were analyzed on ethidium bromide stained 1.2% agarose gel and visualized under UV transilluminator.

**PCR amplification of *msp1 $\beta$*  gene of *A. marginale*:** The *msp1 $\beta$*  gene of *A. marginale* was PCR amplified in a 25  $\mu\text{L}$  final reaction mixture containing 1  $\mu\text{L}$  of extracted DNA, 12.5  $\mu\text{L}$  of  $2 \times$  TopTaq Master mix, 0.5  $\mu\text{M}$  of each sense and antisense primers (BAP-2/ AL34S) (Table 1), and 0.5  $\mu\text{L}$  of TopTaq DNA Polymerase (Qiagen, USA) in automated thermocycler (Veriti™, Applied Biosystem, USA). The PCR conditions were: initial denaturation at  $95^\circ\text{C}$  for 5 min, followed by 32 cycles of denaturation at  $94^\circ\text{C}$  for 1 min, annealing at  $60^\circ\text{C}$  for 45 sec and extension at  $72^\circ\text{C}$

for 30 sec with a final extension at  $72^\circ\text{C}$  for 5 min. The amplified PCR products were separated electrophoretically in 1.2% agarose gel and visualized using ethidium bromide and UV transilluminator.

**Purification and sequencing of PCR:** For molecular characterization of the *msp1 $\beta$*  gene of *A. marginale*, the PCR products were purified using QIA quick gel extraction kit (Qiagen, Germany) following the manufacturer's instructions. Two purified fragments/samples were sent for custom DNA sequencing in both directions using respective primers.

**Comparative sequence analysis of *msp1 $\beta$*  gene of *A. marginale*:** The purified PCR products of *msp1 $\beta$*  gene of *A. marginale* were sequenced in an automated DNA sequencer at commercial DNA sequencing facility. Chromatograms were analysed and verified in BioEdit program in order to check the quality of nucleotide sequences. All the sequence data generated in the current study were deposited to GenBank. The sequence analysis and similarity searches were performed with the basic local alignment search tool of NCBI. The default matrix (www.ncbi.nlm.nih.gov/BLAST) was used to obtain the corresponding sequences of other strains, isolates and species. Pairwise/Multiple sequence alignment of the resulting sequences was generated using ClustalW programme (Thompson *et al.* 1997). The phylogenetic tree was constructed based on the lowest Bayesian Information Criterion (BIC) score in MEGA X software. A multiple sequence alignment was commuted by using a gap opening and gap extension penalty for both pairwise and multiple alignments with the ClustalW program within MEGA X (Tamura *et al.* 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor 1969) with 500 bootstrap samplings (Tamura *et al.* 2013). The pair wise sequence distance was calculated with p-distance matrix. The GenBank sequences KT222946.1 (*T. annulata*, cattle isolate, India), and AF356509.1 (*Ehrlichia phagocytophila*, human isolate, USA) were used as outgroup species to root the tree for *msp1 $\beta$*  gene of *A. marginale*.

**Haematological examination:** Various haematological parameters like haemoglobin (Hb), packed cell volume (PCV%), total erythrocyte count, total leukocyte count (TLC), differential leukocyte count (DLC) and red blood cell indices like mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were analyzed in haematology analyzer (MS4Se, MeletSchloesing, Laboratories, France).

Table 1. Primers used in PCR for genus and species level identification of *Anaplasma*

Primer	Nucleotide sequence	Target gene	Target organism	PCR product (bp)	Reference
P1	5'agagtttgatcctggctcag3'	16S rRNA	<i>Anaplasma</i> spp.	781	Yousefi <i>et al.</i> 2017
P2	5'agcactcatcgtttacagcg3'				
BAP-2	5'gtatggcacgtactgtgggatca 3'	<i>msp1<math>\beta</math></i>	<i>Anaplasma</i>	407	Stich <i>et al.</i> 1993
AL34S	5'cagcagcagcaagacctca3'		<i>marginale</i>		

**Biochemical estimation:** The sera from blood samples were collected for estimation of various biochemical parameters, viz. calcium (Ca), phosphorus (P), total serum protein (TSP), albumin (Alb), glucose (Glu), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), blood urea nitrogen (BUN), total bilirubin (TBIL), indirect bilirubin (IBIL) and direct bilirubin (DBIL) in fully automatic biochemistry analyzer (EM Destiny 180, Erba Mannheim).

**Statistical analysis:** Independent Student's t-test was used for comparison of differences in mean values of various parameters among *A. marginale* infected and healthy control groups (Snedecor and Cochran 1994).

**RESULTS AND DISCUSSION**

Bovine anaplasmosis is accountable for substantial economic losses all around the world, especially tropics, including India (Maharana *et al.* 2016b). The current study systemically investigates the genetic evidence of *A. marginale* in buffalo. Buffalo blood samples (8352) from OPD were screened microscopically during 2017-2020 for *Anaplasma* species and 104 samples were found positive. Microscopy revealed *Anaplasma* species in erythrocytes of 1.19% (4 samples) of the total blood samples (n=335). Initially PCR assay was standardized targeting 16S rRNA for detection of genus *Anaplasma* with expected amplicon size of 781 bp (Fig. 1). Thereafter, *msp1β* gene was targeted for species level identification of *Anaplasma* (*Anaplasma marginale*) with expected amplicon size of approx. 407 bp (Fig. 1). A total of 14 samples out of 335 samples (4.17%) were found positive by PCR. The PCR results for both the 16S rRNA and *msp1β* genes were identical. These findings indicate the higher accuracy and sensitivity of PCR test

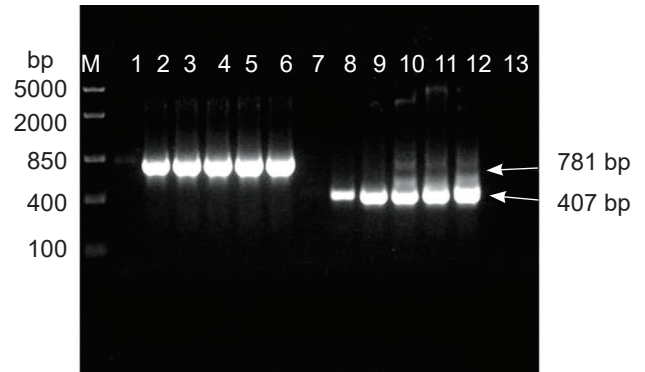


Fig. 1. Amplified DNA products of 781 bp at genus level and 407 bp size at species level were resolved in a 1.5% agarose gel. Lane M: Middle Range DNA ladder (Thermo Fisher Scientific, USA); Lane 1, 7, 13: Negative control; Lane 2-6: Field samples positive for *Anaplasma*; Lanes 8-12 : Field samples positive for *A. marginale*.

for *A. marginale* detection over blood smear examination. Similar findings were observed by earlier researchers all around the world (Ashuma *et al.* 2013, Ganguly *et al.* 2018, Kumar *et al.* 2019).

**Sequence analysis and reconstruction of phylogenetic relationships of *msp1β* gene:** Specific primers (BAP-2 and AL34S) directed amplification of *msp1β* gene resulted in a product of about 407 bp (Fig.1). Thereafter randomly two confirmed positive samples were sequenced and analyzed with similarity search using the BLASTn program of NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>). The 5' and 3' ends of the *msp1β* gene sequence of *A. marginale* were initially determined by comparing them with earlier published sequences. Following the homology/NCBI BLAST study, the obtained *msp1β* DNA sequence was submitted to NCBI

		Percent identity													
		1	2	3	4	5	6	7	8	9	10	11	12		
Divergence	1		98.7	47.7	96.4	89.6	88.6	86.3	91.4	35.0	33.8	91.9	91.6	1	AF112480.1A. <i>marginale</i> Mexico.seq
	2	1.3		47.5	95.7	89.6	88.1	85.8	92.1	35.8	33.5	92.1	92.4	2	AF221693.1A. <i>marginale</i> USA.seq
	3	94.3	95.4		17.3	16.7	16.5	16.4	16.4	24.2	6.6	16.5	16.5	3	AF356509.1E. <i>phagocytophila</i> USA.seq
	4	3.6	4.4	91.9		91.4	89.9	88.4	89.9	35.7	32.8	90.4	90.1	4	AY841153.1A. <i>marginale</i> Israel.seq
	5	11.2	11.2	103.1	9.2		96.3	93.9	91.4	36.2	28.6	91.7	91.2	5	CP006847.1A. <i>marginale</i> Mexico.seq
	6	11.1	11.7	102.1	9.6	2.5		93.4	89.7	36.2	28.1	90.0	90.0	6	CP023730.1A. <i>marginale</i> Brazil.seq
	7	11.9	12.5	100.0	9.5	3.3	2.6		88.5	34.5	24.9	88.3	88.3	7	CP023731.1A. <i>marginale</i> Brazil.seq
	8	7.3	6.5	97.7	7.2	4.8	5.4	4.9		37.0	30.1	99.0	99.5	8	KF696857.1A. <i>marginale</i> India.seq
	9	158.5	151.9	141.9	152.7	146.0	144.5	153.6	144.0		7.5	20.1	20.2	9	KT222946.1T. <i>annulata</i> India.seq
	10	0.0	0.8	66.6	0.0	13.1	14.9	18.5	12.2	117.7		88.7	88.7	10	KU647720.1A. <i>marginale</i> South Africa.seq
	11	7.3	7.0	97.8	7.2	5.0	5.6	5.7	1.0	144.9	12.2		99.2	11	MW269530.1A. <i>marginale</i> Karnal1 India.seq
	12	7.3	6.5	97.1	7.2	5.3	5.4	5.5	0.5	142.8	12.2	0.5		12	MW269531.1A. <i>marginale</i> Karnal2 India.seq
		1	2	3	4	5	6	7	8	9	10	11	12		

Fig. 2. Sequence pair-wise distance of *msp1β* gene using ClustalW.

GenBank for the assignment of the accession number and corresponding accession numbers were obtained for the individual isolates (MW269530, MW269531). The BLAST analysis identified 12 related nucleotide sequences (10 ingroup and 2 outgroup) from different species. The nucleotide sequence homology was recorded in the range of 88.3-99.5% in BLAST analysis. Very limited intra-specific nucleotide differences were observed between individuals of Indian buffalo isolates. Different buffalo isolates from Karnal (MW269530, MW269531) showed 99 to 99.5% sequence identity with that of Punjab isolates (KJ780079) in India. *msp1β* sequence of *A. marginale* Karnal (India) isolate revealed variable nucleotide homologies with a range of 88.3-92.4% when compared with sequences of related species across the globe. When the pair-wise sequence distance of *msp1β* gene was calculated, nucleotide sequence of Indian isolates showed maximum divergence from *E. phagocytophila* (97.8%) followed by *T. annulata* (142-144%) and very limited from that of buffalo isolates Punjab (0.5-1%) (Fig. 2).

The phylogeny based on *msp1β* region revealed that all the taxa beside outgroup (*T. annulata* and *E. phagocytophila*) were included in a big branch tree of two major clades. The topologies also showed that the *A. marginale* buffalo isolates from Karnal, India form a major clade in which *A. marginale* remained in the same cluster with other isolates reported from other parts of India, Brazil and Mexico. This is sister to the other clade which consists of *A. marginale* cattle isolates from USA, Mexico, Israel and South Africa (Fig. 3). Phylogenetic tree also revealed that the *A. marginale* buffalo isolates from Karnal, India forms a major clade in which *A. marginale* is positioned in the same cluster with other isolates reported elsewhere around the globe. There is very much close proximity of *A. marginale* Karnal isolate with

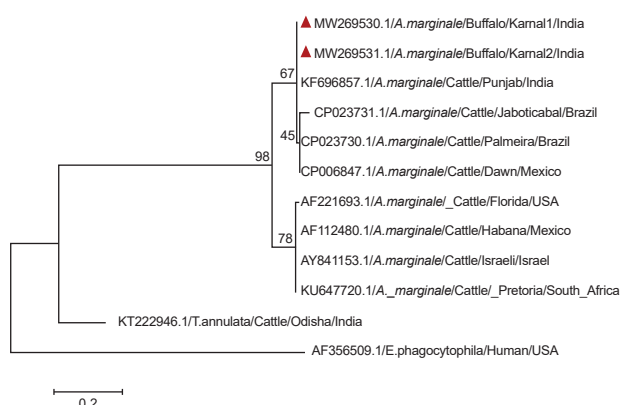


Fig. 3. Sequences of *msp1β* gene of different individual rickettsia were aligned separately using ClustalW in MEGA X. The percentage of replicating trees (500 replicates) in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The sequence generated in the present study is marked as red triangle.

that of Punjab isolate with minor genetic variations. The results are indicative of phylogenetically close relatedness between cattle and buffalo strains of *A. marginale*. In general, Indian *A. marginale* strains were heterogeneous showing similarity with strains reported from different parts of the world. This indicates evolutionary pressure within the parasite or may be due to migration of strains (George *et al.* 2017).

**Haemato-biochemical changes:** In microscopic and PCR positive buffalo samples, leucocytosis suggests stimulation of lymphoid organ and system due to parasites and toxins. The drop in TEC may be associated with the reduced systemic lifetime of red blood cells owing to extravascular erythrophagocytosis (Massard *et al.* 1998). Erythron of infected animals showed significantly low level of mean Hb, PCV, RBC count, MCHC and high level of mean MCV (Table 2), indicating regenerative hypochromic macrocytic anemia. The decrease in all the hematological indices except MCV could be due to decrease in lifespan of RBC and extravascular phagocytosis by bovine reticuloendothelial system. Immature RBCs are released from the bone marrow, after rapid degradation of RBCs by phagocytosis because of increased demand. Immature RBCs are bigger than mature red blood cells, leading to higher MCV levels. The high rise in MCV value is usually an indication of regenerative anaemia (Subramanian *et al.* 2019). When compared to apparently healthy animals, the total mixed cells (eosinophil and monocyte) count in infected animals was significantly ( $P < 0.05$ ) high. Infected animals had a significantly low mean level of total protein, albumin and globulin (Table 3). This is attributed to their reduced production as a result of liver damage. The findings in our study are in accordance with the previous reports (Allen *et al.* 1981, Abdullah *et al.* 2020). The characteristic signs of anaplasmosis include

Table 2. Mean values (Mean±SE) of haematological parameters of buffaloes infected with *A. marginale*

Parameter	Infected buffaloes (n=30)	Healthy buffaloes (n=10)	P value
Hb (gm/dl)	4.66±0.43**	9.49±0.27	<0.0001
PCV (%)	15.17±1.16**	25.95±0.76	<0.0001
TEC ( $\times 10^6/\mu\text{L}$ )	2.51±0.25**	4.32±0.08	<0.0001
TLC ( $\times 10^3/\mu\text{L}$ )	12.11±0.61 <sup>NS</sup>	10.19±0.62	0.3901
Lymphocyte (%)	34.93±2.38 <sup>NS</sup>	40.96±3.79	0.0700
Neutrophil (%)	59.77±2.50 <sup>NS</sup>	56.63±3.99	0.2101
Mixed (%)	5.29±0.70*	2.4±0.29	0.0421
MCV (fl)	66.3±1.51*	60.06±1.27	0.0344
MCH (pg)	19.6±0.81 <sup>NS</sup>	21.86±0.40	0.9262
MCHC (%)	30.78±1.48*	36.52±0.24	0.0119
THR ( $\times 10^3/\mu\text{L}$ )	204±38.87 <sup>NS</sup>	186.4±12.47	0.3519

\*, Infected and healthy buffaloes differ significantly at  $<0.05$ ; \*\*, significant at  $<0.001$ . Hb, Haemoglobin; PCV, packed cell volume; TEC, total erythrocyte count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; TLC, total leukocyte count.

fever, anaemia, jaundice, and sudden death. The parasitized erythrocytes are eliminated by phagocytosis in the reticular endothelial system with subsequent release of acute phase inflammatory reactants. This results in development of pyrexia. Mononuclear phagocytic system is responsible for continuous destruction of *A. marginale* infected or uninfected RBCs resulting in development of anemia and icterus (Ashuma *et al.* 2013). Infected animals showed significantly high level of total bilirubin, direct and indirect bilirubin. This could be attributed to excessive lysis of RBCs and hepatic dysfunction or indirect hepatocellular damage (Omer *et al.* 2003, Khan *et al.* 2011). AST and ALT of infected animals showed significant difference from that of their respective control values. The activities of serum liver enzymes are indicative of liver function and integrity (De *et al.* 2012). Similar findings are previously reported in dairy cows naturally infected with *A. marginale* (Coskun *et al.* 2012, Ganguly *et al.* 2018). Elevated AST is usually associated with muscle damage due to recumbency in acute anaplasmosis, followed by a substantial rise in serum creatinine kinase levels (De *et al.* 2012). However, this increase in AST and ALT levels is presumably due to hypoxic hepatic cellular disruption in chronically affected buffaloes. Furthermore, myocardial or erythrocytic damage may also be attributed to elevated serum levels of AST and ALT (Ganguly *et al.* 2018). Allen *et al.* (1981) observed that the augmented enzyme activity may be attributed to severe anaemia which leads to hypoxic condition and liver damage. In the present investigation, low PCV and higher values of TBIL, AST and ALT indicate liver dysfunction and supported the earlier findings.

Table 3. Mean values (Mean±SE) of biochemical parameters of buffaloes infected with *A. marginale*

Parameter	Infected buffaloes (n=30)	Healthy buffaloes (n=10)	P value
Ca (mg/dl)	10.81±0.48 <sup>NS</sup>	11.6±0.31	0.2383
P (mg/dl)	4.54±0.34*	6.5±0.23	0.0023
ALT (U/L)	68.11±1.93*	57.3±1.14	0.0057
AST (U/L)	263.4±17.07*	163.7±11.47	0.0243
Glucose (mg/dl)	69.94±7.95*	53.6±2.61	0.0013
TSP (g/dl)	5.8±0.21**	7.9±0.39	<0.0001
Albumin (g/dl)	2.6±0.12**	3.4±0.075	0.0006
Globulin (g/dl)	3.20±0.18**	4.5±0.16	.0002
BUN (mg/dl)	22.7±1.8 <sup>NS</sup>	18.45±1.04	0.0995
Cr (mg/dl)	1.49±0.20 <sup>NS</sup>	1.3±0.07	0.5897
TBIL (mg/dl)	1.79±0.32*	0.2±0.04	0.0264
DBIL (mg/dl)	0.52±0.12*	0.1±0.006	0.0444
IBIL (mg/dl)	1.27±0.24*	0.1±0.003	0.0275

\*, Infected and healthy buffaloes differ significantly at <0.05; \*\*, Significant at <0.001. Ca, Calcium; P, phosphorus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TSP, total serum protein; BUN, blood urea nitrogen; Cr, creatinine; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin.

The present study reported the molecular and phylogenetic characterization of *A. marginale* in buffalo.

The in-house standardized PCR assay targeting *msslβ* gene can be employed for molecular detection and surveillance studies of *A. marginale*. In addition, the current study also highlights the pathogenicity induced by *A. marginale* infection in buffalo through analysis of various hematobiochemical parameters. Anaemia, pale mucous membrane, and fever were the principal clinical findings of anaplasmosis in buffalo. Major haematology change observed is of hypochromic, macrocytic and regenerative anaemia. The outcomes of the present investigation indicate the possible association between *A. marginale* infection and hepatic damage. Further studies, nevertheless, are warranted to make clear the precise pathophysiological mechanism of this association.

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