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Molecular diagnosis and haemato-biochemical changes in *Anaplasma marginale* infected dairy cattle

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ABSTRACT

The present study was a undertaken to diagnose *Anaplasma marginale* in naturally infected crossbred cows and to determine its effect on haemato-biochemical profile. Blood samples were collected from animals (200) for detection of the rickettsial organism by direct smear and direct blood PCR based techniques targeting the major surface protein 5 (MSP-5). Direct blood PCR revealed a 382-bp amplified fragment in positive control samples. When random blood samples were screened under light microscope and direct blood PCR method, 7.5% of samples were positive under microscopic examination whereas PCR analysis revealed 10.5% samples positive for *A. marginale*. The infected group (25) showed significantly decreased levels of TEC, Hb and PCV than healthy control animals. However, differences in the red blood cell indices were non-significant indicating normocytic normochromic anaemia in affected crossbred cattle. Serum samples (25) of infected cows showed significantly higher values of ALT, AST, BUN, creatinine and TBIL than that of healthy control. A significant decrease of TSP and albumin was also recorded in the infected cows compared to healthy control. The standardized PCR method of the present investigation may be useful for rapid and accurate diagnosis of *A. marginale* in subclinical/carrier animals as the whole blood could be directly used. Haemato-biochemical studies concluded that anaemia and erythrophagocytosis are considered to be the major components of this disease and adversely affect liver and soft tissue of the affected animals.

Key words: *Anaplasma marginale*, Cattle, Diagnosis, Haemato-biochemical, Molecular, PCR

Anaplasma marginale is a rickettsial organism causing bovine anaplasmosis in cattle with significant economic losses in tropical, subtropical regions and many temperate countries. It invades the erythrocyte and leads to extravascular hemolysis. Ticks (most commonly Rhipicephalus (*Boophilus microplus*) are biological vectors of *A. marginale* but the pathogen is often transmitted mechanically to susceptible cattle by blood-contaminated mouth parts of biting flies or fomites. In the acute phase of the disease, when rickettsemia is high, initial bodies are easily detected in bovine erythrocytes by microscopy of stained blood smears (Maharana *et al.* 2014). However, the rickettsemia of chronically infected animals is generally low, hindering its diagnosis. Microscopic examination by Giemsa stained blood smears, which is used to confirm acute anaplasmosis, can only detect levels of $>10^6$ infected erythrocytes/ml (Gale *et al*. 1996). Subinoculation of *A. marginale*-infected erythrocytes into susceptible, splenectomized calves is considered the gold standard for detection of persistently infected cattle, but the procedure is not practical for routine testing (Luther *et al.* 1980). Serological tests have been the most commonly used methods to detect *A. marginale*-infected cattle in the field but may not be able to reveal the current status of infection (Jaswal *et al.* 2014). Nucleic acid based detection techniques like PCR assays allow diagnosis of parasite at levels far below the detection limit of the frequently used parasitological techniques and has advantage in separating clinical and subclinical forms of parasitic infection (Almeria *et al*. 2001). The present study was intended at molecular diagnosis of *Anaplasma marginale* directly from blood in naturally infected crossbred cows and to determine its effect on haemato-biochemical profile of host animals.

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MATERIALS AND METHODS

Sample collection: Lactating crossbred cows (3–6 years; 200) brought to outpatient department (OPD) of Referral Veterinary Diagnostic and Extension Centre (RVDEC), LUVAS, Uchani, Karnal during July 2015 to June 2016 and showing clinical signs, viz. fever, severe anemia, jaundice, brownish urine, loss of appetite, dullness, muscular tremors, constipation, pale mucous membrane and

labored breathing etc. similar to anaplsmosis were included in the present study. Crossbred cows (25) showing ≥5% parasitemia constituted the infected group; whereas, 10 healthy crossbred cows, free from *Anaplasma* (negative both by microscopy and direct blood PCR assay) constituted the healthy control group. The blood samples from both the groups were collected in vials with or without anticoagulant (EDTA). Immediately after collection, blood smears were prepared, stained with Giemsa and the presence of *A. marginale* inclusion bodies was examined microscopically.

Direct blood PCR: Oligonucleotide primers were designed using online Primer3 software (http://primer3.ut.ee/) and genomic specificity of the primers was tested online using Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Direct blood PCR assay was performed by taking 2 µl of whole blood, targeting the targeting MSP-5 of *A. marginale*. The assay revealed a product size of 382 bp in 1.5% agarose gel specific for *A. marginale*, using specific primer pairs (AMF: ACAGGCGAAGAAGCAGACAT; AMR: ATAAATGGG-AACACGGTGGA).

Initially, PCR assay was standardized on positive samples (blood with ≥ 10% parasitemia) using Phusion blood direct PCR kit (Thermo Fisher Scientific, India) following manufacturer's instructions. Briefly, PCR was carried out in a thermal cycler (Veriti^{TM}, Applied Biosystem, USA) with a final reaction volume of 20 µl containing 2 µl of whole blood, 1 × Phusion blood PCR buffer, 0.5 µM of each primer and 0.4 µl Phusion blood II DNA polymerase. A negative control without template (whole blood) was always included to rule out any PCR carryover. The PCR conditions were initial denaturation at 98°C for 5 min; followed by 35 cycles of 98°C for 2 sec, 53°C for 30 sec and 72°C for 30 sec; with a final extension step of 72°C for 1 min. After completion of PCR, tubes were centrifuged at 1000 × g for 2 min to collect the clear supernatant. Aliquots of 5 µl of PCR products (supernatant) were visualized on 1.5% agarose gel (stained with ethidium bromide) and documented under gel documentation system (Gel Doc XR+, Bio-Rad, USA) to confirm the fragment size. Two representative samples of amplified products were purified and sequenced. Samples were considered positive when amplification of a fragment corresponding to the expected amplicon size was visualized under ultraviolet light. All the 25 animals of infected group with varying degree of parasitemia were screened through standardized direct blood PCR assay. In order to validate the PCR assay and to check its sensitivity, 200 random blood samples of crossbred cattle were screened microscopically as well as through direct blood PCR method. The coagulated blood samples were centrifuged (5000 rpm, 15 min) and supernatant (serum) was collected for biochemical estimations.

Haematological examination: Approximately 1.5 ml of blood sample was collected in anticoagulant vials for haematological profile. Haematological parameters, viz. 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), mean corpuscular haemoglobin concentration (MCHC) and thrombocytes were analysed using blood cell counter (MS4Se, HD consortium).

Table 1. Mean values[#] of haematological parameters of crossbred cattle infected with *Anaplasma marginale*

Parameter	Infected cattle (n=25)		Healthy cattle (n=10)		P value
	Mean± SEM	Range	Mean± SEM	Range	
Hb (g/dl)	3.396± 0.192	2–6.5	12.37± 0.17	11. 5–12.9	<0.00001**
PCV (%)	10.6± 0.65	6–21	37.9± 0.70	35–41	<0.00001**
TEC ($\times 10^6$/µl)	2.17± 0.13	1.36–3.96	7.109± 0.146	6.25–7.56	<0.00001**
TLC ($\times 10^3$/µl)	8.87± 0.49	5.9–14.4	6.07± 0.306	4.1–7.6	0.001478**
Lymphocyte (%)	49.8± 3.54	16–78	51.1± 1.95	42–60	0.823^{NS}
Monocyte (%)	1.24± 0.13	0–2	1.7± 0.33	1–4	0.129^{NS}
Neutrophil (%)	47.16± 3.5	20–80	45.8± 1.89	37–56	0.813^{NS}
Eosinophil (%)	1.8± 0.8	1–4	1.4± 0.22	1–3	0.2225^{NS}
MCV (fl)	49.74± 1.91	35.29–70.92	53.54± 1.6	48.16–60.51	0.245^{NS}
MCH (pg)	16.038± 0.57	11.76± 23.76	17.47± 0.47	15.71–20.64	0.144^{NS}
MCHC (%)	32.62± 0.915	25.38± 40	32.67± 0.31	31.21–34.59	0.973^{NS}
THR ($\times 10^3$/µl)	147.76± 22.49	35–346	313.2± 13.59	226–360	0.000049**

[#]Mean±SE; *Infected and healthy cattle differ significantly at P<0.05; **Significant at P<0.001. Hb, Haemoglobin; PCV, packed cell volume; TEC, total erythrocyte count; THR, thrombocytes; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; TLC, total leukocyte count.

Biochemical estimation: Calcium (Ca), phosphorus (P), total serum protein (TSP), glucose (Glu), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), blood urea nitrogen (BUN), triglycerides (TG), cholesterol, high density lipoproteins (HDL), total bilirubin (TBIL) and direct bilirubin (DBIL) were measured by fully automatic biochemistry analyzer (EM Destiny 180, Erba). Low density lipoproteins (LDL) were estimated by the Friedewald equation (Friedewald 1972): LDL = TC – HDL - TG/5.0 (mg/dl).

Statistical analysis: The differences of means of estimated parameters between *A. marginale* infected and healthy control groups were compared using Student's t-test (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

The direct blood PCR assay in the present investigation

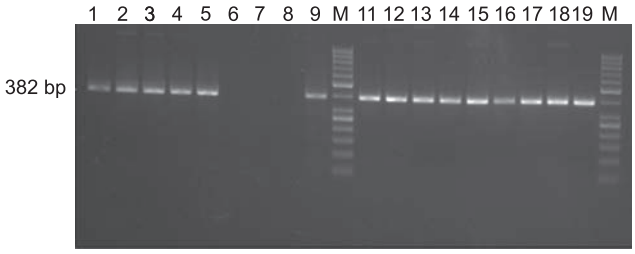

Fig. 1. Detection *of Anaplasma marginale* from whole blood samples using Direct blood PCR method. Lane M, 50 bp DNA ladder; lane 6, negative control; lanes 7–8, negative blood samples; lanes 1–5, 9, 11–19, samples with various degree of parasetemia.

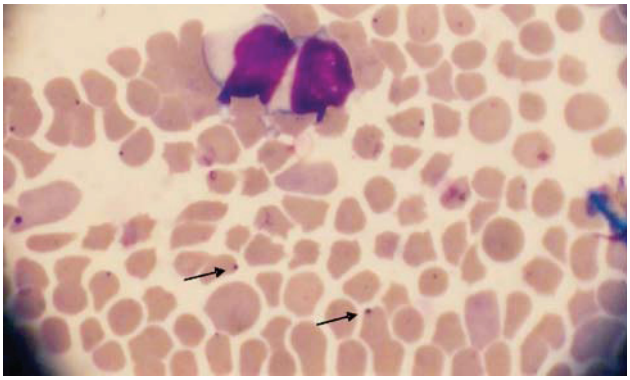

Fig. 2. Microscopic examination of Giemsa stain blood film showing *Anaplasma marginale* inside the erythrocytes.

produced 382-bp fragment as expected, pertaining to MSP-5 gene of *A. marginale* (Fig.1). Sequencing result further confirmed MSP-5 gene specific amplicon. All the animals of the infected groups, confirmed positive by stained blood smears, were found to be positive with direct blood PCR assay. When, random blood samples were screened under light microscope (Fig. 2) and direct blood PCR method, 7.5% (15/200) of samples were positive under microscopic examination, whereas PCR analysis revealed 10.5% (21/200) positive for *A. marginale*.

The result revealed that the PCR assay is 100% sensitive (true positive (TP)/true positive (TP) + false negative (FN) × 100) and 96.75% specific (true negative (TN)/true negative (TN) + false positive (FP) × 100) when compared with microscopy based detection method with a positive (TP/TP + FP) and negative predictive value (TN/TN + FN) of 71.42% and 100%, respectively. This further affirms its utility in rapid diagnosis of *A. marginale* with high specificity and sensitivity especially in carrier animals/subclinical infections with very low parasitemia in the fields because in this method whole blood can be directly used, especially for identifying carrier animals. No need for DNA isolation. The PCR generally detects DNA traces from previous infections, however, this is not true in case of bovine anaplasmosis because once *A. marginale* infects an animal, it becomes infected for years allowing us to infer that a positive PCR animal is still infected with *A. marginale* (Tana-Hernández *et al.* 2017).

The infected group showed significantly (P<0.001) lowered values of TEC, Hb and PCV than healthy control animals. There were no differences (P>0.05) between infected and healthy crossbred cattle pertaining to red blood cell (RBC) indices, viz. MCV, MCH, and MCHC (Table 1). Moreover, they were within normal range (Jain 1993) indicating normocytic normochromic anaemia (Table 1). However, the type of anaemia largely depends on the disease severity and accordingly various types of anaemia in anaplsamosis, babesiosis and theileriosis has been reported (Ashuma *et al.* 2013, Ganguly *et al*. 2015, 2017a). In the

Table 2. Mean values[#] of biochemical parameters of crossbred cattle infected with *Anaplasma marginale*

Parameter	Infected cattle (n=25)		Healthy control (n=10)		P value
	Mean± SEM	Range	Mean± SEM	Range	
Ca (mg/dl)	8.7± 0.16	7.1–10.5	10.1± 0.236	9.1–11.5	0.000041**
P (mg/dl)	5.172± 0.187	4–7	4.83± 0.15	4.2–5.6	0.2838NS
ALT (U/l)	50.28± 2.77	34.75	35.3± 2.02	23-42	0.0026*
AST (U/l)	113.56± 7.61	35–196	79± 2.35	69–89	0.00799*
Glucose (mg/dl)	55.776± 1.41	34–67	60.6± 1.99	52–65	0.039*
TSP (g/dl)	6.02± 0.126	4–7	6.99± 0.182	6.1–7.8	0.00018**
Albumin (g/dl)	2.496± 0.10	1.1–3.4	3.19± 0.06	3–3.5	0.000354**
Globulin (g/dl)	3.5± 0.158	2.1–4.6	3.8± 0.206	3.1–4.7	0.336NS
BUN (mg/dl)	22.072± 0.61	167± 27.2	20.93± 0.67	18.2–23.5	0.292NS
Cr (mg/dl)	2.2± 0.16	1.0–2.7	0.99± 0.16	0.4–1.5	0.235NS
TBIL (mg/dl)	1.688± 0.077	1–2.2	0.7± 0.116	0.2–1.2	0.00001**
DBIL (mg/dl)	0.432± 0.059	0.1–1.1	0.31± 0.04	0.1–0.5	0.227NS
IBIL (mg/dl)	1.256± 0.078	0.5–1.8	0.39± 0.076	01–0.7	<0.00001**
TG (mg/dl)	57.96± 4.21	32–95	50.8± 2.71	40–65	0.308NS
Chol (mg/dl)	136.2± 11.55	60–370	136.5± 4.24	112–157	0.987NS
HDL (mg/dl)	21.84± 2.20	17–45	45.9± 2.53	37–56	<0.00001**
LDL (mg/dl)	102.768± 11.93	39–335	80.44± 6.38	44.4–104.0	0.258NS
A:G	0.76± 0.057	0.41–1.36	0.868± 0.061	0.73–1.21	0.305NS

[#]Mean±SE; *Infected and healthy cattle differ significantly at P<0.05; **Significant at P<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; TG, triglycrides; Chol, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein.

present study, normocytic normochromic anaemia observed in the *A. marginale* infected crossbred cattle may be attributed to intravascular haemolysis of red blood cells.

Significant increase of TLC (P<0.01) was observed in infected crossbred animals compared to healthy control group (Table 1). Marked thrombocytopenia (P<0.001) was also observed in infected animals. The clinical signs recorded, viz. anaemia, icterus, high fever, weakness, weight loss and sometimes the death of the affected animals, may be due to the endogenous pyrogens liberated by *A. marginale* causing the destruction of erythrocytes and triggering various haemopiotic and thermoregutory centers of the body. It reflects that animals' haemopiotic system was activated in response to erythrophagocytosis. The erythrophagocytosis has been 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.

Serum samples of *A.marginale* infected crossbred cows showed significantly higher (P<0.01) values of ALT, AST, TBIL and indirect bilirubin than that of healthy control (Table 2). Similar findings had also been reported in *A. marginale* infected cattle by Jaseem and Agaa (2015).

Comparison of lipid profile of healthy and infected groups revealed significant decrease in the level of HDL (P<0.001) and nonsignificant increase in LDL level (Table 2). In the present study, a significant decrease of calcium, TSP (P<0.001) and serum albumin level (P<0.001) was monitored in the infected group compared to healthy control (Table 2). Lower PCV and higher TBIL, AST and ALT indicate liver dysfunction. Anaemia and icterus in this study may result from the massive phagocytosis of infected erythrocytes by the bovine reticuloendothelial system. These findings were similar to earlier reports (Ashuma *et al*. 2013, Ganguly *et al.* 2017b, Maharana *et al.* 2016). Damage to the skeletal or heart muscles, hepatic tissues and erythrocytes may result in a considerable increase in the level of AST and ALT. Manna (1990) reported hyperbilirubinemia in camels infected with anaplasmosis due to excessive destruction of RBCs and the indirect hepatocellular damage.

The decrease of serum albumin value has been found to be associated with the acute phase of many infectious diseases (Allen and Kuttler 1981). In addition, albumin level may have decreased due to decreased protein synthesis capacity of the affected liver and its excretion in urine as albuminuria in addition to the malnutrition status occurs during the disease (Henley and Judith 1985). These observed changes in haematological and biochemical parameters of *A. marginale* infected crossbred cows may be useful in understanding the disease pathogenesis, undertaking appropriate corrective measures and subsequent disease eradication.

Direct blood PCR method can be adapted for rapid and accurate diagnosis of bovine anaplasmosis in the fields as the whole blood could be directly used, especially for identifying subclinical/carrier animals with very low parasitemia. Normocytic normochromic anaemia observed in the *A. marginale* infected crossbred cattle may be attributed to intravascular haemolysis of red blood cells. Anaplasmosis adversely affect liver and soft tissue of the affected animals which was also evident in the present investigation through disturbance in serum protein fractions, hepatic dysfunction and enzymes activities.

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REFERENCES

Almeria S, Castella J, Ferrer D, Ortuno A, Estrada-Pena A and Gutierrez J F. 2001. Ovine piroplasms in Minorca (Balearic Islands, Spain): a comparison of PCR-based and light microscopy detection. *Veterinary Parasitology* **99**: 249–59.

Ashuma, Sharma A, Singla L D, Kaur P, Bal M S, Batth B K and Juyal P D. 2013. Prevalence and haemato-biochemical profile of *Anaplasma marginale* infection in dairy animals of Punjab (India). *Asian Pacific Journal of Tropical Medicine* **6**(2): 139–44.

Friedewald W T, Levy R I and Fredrickson D S. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry* **18**: 499–502.

Gale K R, Dimmock C M, Gartside M and Leatch G. 1996. *Anaplasma marginale*: detection of carrier cattle by PCR ELISA. *International Journal of Parasitology* **26**: 1103–09.

Ganguly A, Bhanot V, Bisla R S, Ganguly I, Singh H and Chaudhri S S. 2015. Haemato-biochemical alterations and direct blood polymerase chain reaction detection of *Theileria annulata* in naturally infected crossbred cows. *Veterinary World* **8**: 24–28.

Ganguly A, Bisla R S, Ganguly I, Singh H, Bhanot V and Chaudhri S S. 2017a. Direct blood PCR detection of *Babesia bigemina* and its effect on haematological and biochemical profile in crossbred cattle of eastern Haryana. *Indian Journal of Animal Research* **51**(1): 141–45.

Ganguly A, Bisla R S, Singh H, Bhanot V, Kumar A, Kumari S, Maharana B R and Ganguly I. 2017b. Prevalence and haemato-biochemical changes of tick borne haemoparasitic diseases in crossbred cattle of Haryana, India. *Indian Journal of Animal Sciences* **87**(5): 552–57.

Jain N C. 1993. *Essentials of Veterinary Hematology*. Lea and Febiger, Philadelphia, USA.

Jaseem Ghaidaa Abbas and Agaa Olaa A. 2015. Molecular and biochemical study of *Anaplasma marginale* in cattle in Wassit Province of Iraq. *Journal of Bacteriology Research* **7**(4): 36–41.

Jaswal H, Bals M S, Singla L D, Sharma A, Kaur P, Mukhopadhyay S and Juyal P D. 2014. Application of msp 1 b PCR and 16S rRNA semi nested PCR-RFLP for detection of persistent anaplasmosis in tick infested cattle. *International Journal of Advanced Research* **2**(8): 188–96.

Luther D G, Cox H U and Nelson W O. 1980. Comparison of sero tests with calf inoculations for detection of carriers in anaplasmosis-vaccinated cattle. *American Journal of Veterinary Research* **41**: 2085–86.

Manna A M M. 1990. 'Clinical and some bio-chemical changes in healthy and diseased camels.' Ph.D Thesis, Faculty Veterinary Medicine, Assui University.

Maharana B R, Vala K B, Panigrahi P N and Behra S K. 2014. Therapeutic management of anaplasmosis in a Jaffrabadi buffalo. *Indian Journal of Veterinary Medicine* **34**(2): 158–59.

Maharana B R, Kumar B, Prasad A, Patbandha T K, Sudhakar N R, Joseph J P and Patel B R. 2016. Prevalence and assessment of risk factors for haemoprotozoan infections in cattle and buffaloes of South-West Gujarat, India. *Indian Journal of Animal Research* **50**(5): 733–39.

Snedecor G W and Cochran W G. 1994. *Statistical Methods*. 9th edn. Oxford and IBH Publishing Co., New Delhi.

Tana-Hernández Leandro, Navarrete-Arroyo Katherine, Ron-Román Jorge, Reyna-Bello Armando and Chávez-Larrea María Augusta. 2017. PCR diagnosis of *Anaplasma marginale* in cattle populations of Ecuador and its molecular identification through sequencing of ribosomal 16S fragments. *BMC Veterinary Research* **13**: 392.