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## Prevalence and haemato–biochemical profile of *Anaplasma marginale* infection in dairy animals of Punjab (India)

Ashuma<sup>1</sup>, Amrita Sharma<sup>1</sup>, Lachhman Das Singla<sup>1\*</sup>, Paramjit Kaur<sup>1</sup>, Mandeep Singh Bal<sup>2</sup>, Balwinder Kaur Batth<sup>1</sup>, Prayag Dutt Juyal<sup>1</sup>

<sup>1</sup>Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana–141004, India

<sup>2</sup>Animal Disease Research Centre, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana–141004, India

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### ABSTRACT

**Objective:** To do the systematic comparison of prevalence of anaplasmosis by PCR and Giemsa stained thin blood smear (GSTBS) based parasitological assays in dairy cattle of Punjab, which has not been reported yet. To analyse the haematobiochemical alterations in infected animals to arrive at the conclusion regarding the pathogenicity induced by *Anaplasma marginale* (*A. marginale*) in latent and patent infection. **Methods:** Study was conducted on 320 animals (236 cows, 62 calves and 22 buffaloes) of Punjab, India. PCR on genome of *A. marginale* was performed by targeting *msp1 β* gene using specific primers BAP–2/AL34S, amplifies products of size 407 bp. Questionnaires based data on the characteristics of the infected animals and management strategies of the farm were collected and correlated. **Results:** Higher prevalence and more significant association was observed in the PCR based molecular diagnosis ( $P=0.00012$ ) as compared to that in GSTBS ( $P=0.0288$ ) based diagnosis with various regions under study. With respect to the regions, highest prevalence was recorded in Ferozepur by PCR based diagnosis, while that in Jalandhar by GSTBS examination. Similar marked significant association of the PCR based diagnosis with the age of the animals under study ( $P=0.00013$ ) was observed elucidating no inverse age resistance to *A. marginale* in cow calves. Haematobiochemical profile of infected animals revealed marked anemia, liver dysfunction and increase globulin concentrate indicating rise in immunoglobulin level to counteract infection. **Conclusions:** PCR is far more sensitive in detecting the disease even in latent infection which may act as nidus for spread of anaplasmosis to susceptible animals in endemic areas. Severity of anaemia and liver dysfunction were comparable both in patent as well as latent infection indicating pathogenicity of both.

## 1. Introduction

Anaplasmosis, the tick–borne disease, is caused by an obligate intraerythrocytic rickettsial microorganism, *Anaplasma marginale* (*A. marginale*), of the order Rickettsiales, family anaplasmataceae. Sir Arnold Theiler named this disease as gall sickness or yellow bag in 1910[1–3]. Following Theiler's work, anaplasmosis was soon widely recognized as disease of ruminants in tropical,

subtropical and many temperate zones throughout the old world. A conservative estimate of the annual loss due to anaplasmosis in the US alone amounts to \$100 million and includes 50 000 to 100 000 cattle deaths[4]. *Anaplasma* is one of the most important organisms transmitted by at least 20 ticks of various species[5,6] but *Rhipicephalus (Boophilus) microplus* is found to be major transmitting agent[7]. Besides, it is also being transmitted mechanically by biting flies or blood–contaminated fomites. This rickettsial organism is more related to protozoa due to the lack of a traditional cell wall and not capable of synthesizing lipopolysaccharide and peptidoglycan[8]. Cattle is found to be more susceptible to *Anaplasma* infection than the buffaloes[9]. Disease is characterized by progressive haemolytic anaemia associated with fever, jaundice, decreased milk production, abortions,

\*Corresponding author: Dr. L. D. Singla, Professor–cum Head, Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana–141004, India.

Tel: +91 161 2500941

Fax: +91 161 2400822

E–mail: [ldsingla@gmail.com](mailto:ldsingla@gmail.com)

hyperexcitability and in some cases sudden death<sup>[10,11]</sup>.

The conventional parasitological techniques like Giemsa-stained thin blood smear always remains gold standard for diagnosis of bovine anaplasmosis for clinically suspected animals, but it is not applicable for the detection of subclinical infection in carrier animals, which serve as a reservoir for the spread of *A. marginale* since the parasites are seldom detected microscopically in chronic infections<sup>[12]</sup>. Therefore, several serological tests have been established<sup>[13]</sup>. Unfortunately, because of antigen cross reactivity<sup>[14]</sup>, these tests do not discriminate between different *Anaplasma* species. Thus molecular based PCR assays with a high degree of sensitivity and specificity have been developed to identify *A. marginale*<sup>[15–18]</sup>. The present study was undertaken with the aim to compare the prevalence of anaplasmosis by PCR and classical Giemsa stained thin blood smear (GSTBS) parasitological method. Further haematobiochemical alterations in infected animals as compare to healthy control were analyzed to arrive at the conclusions regarding the pathogenicity induced by *A. marginale*.

## 2. Materials and methods

### 2.1. Samples and data collection

Total 320 blood samples were collected in month of May to October, 2011 from ruminants (cattle 236, cow calf 62 and buffalo 22) primarily having history of tick infestation, fever, jaundice and anemia from 7 districts of Punjab viz. Ludhiana, Jalandhar, Patiala, Bathinda, Moga, Mansa and Ferozepur in order to study the prevalence of *A. marginale* based on molecular and parasitological studies. Blood samples were collected from the jugular vein of the animals. Questionnaire based information regarding characteristics of animals (species, gender, age and presence of ticks) and herds (location, size, management) was collected to exemplify their correlation with the study. Five mL of blood sample each was collected in EDTA coated vials and glass vials for evaluating haematological and biochemical parameters, respectively.

### 2.2. Blood smear preparation

Thin blood smears were prepared immediately after each blood collection. The blood smears were air dried, fixed in methanol, stained with Giemsa and examined microscopically for the presence of *A. marginale* in erythrocytes. The percent parasitized erythrocytes was calculated according to the method described by Coetzee et al<sup>[19]</sup>. Ticks were morphologically identified based on keys described by Miranpuri<sup>[20]</sup>.

### 2.3. DNA extraction

DNA was extracted using DNA isolation kit (HiPura) as per the protocol of the manufacturer. 20  $\mu$  L of reconstituted proteinase K solution was added into 2.0 mL collection tubes containing 200  $\mu$  L of fresh whole blood sample and was vortex for 10–15 s. 20  $\mu$  L of RNase A solution was added to the collection tubes, vortex for 10–15 s and incubated for 2 min at room temperature. Sample was then lysed with 200  $\mu$  L of lysis solution and thoroughly vortex for few seconds followed by 10 min of incubation at 55 °C. 200  $\mu$  L of ethanol was added with gentle pipeting and lysate was transferred into the column. The column was first centrifuged and then washed with 500  $\mu$  L of pre-wash and wash buffer with subsequent centrifugation. Finally, DNA was eluted with 100  $\mu$  L elution buffer. Amount of extracted DNA and its purity was measured at OD<sub>260</sub> and ratio of OD<sub>260</sub> to OD<sub>280</sub> respectively.

### 2.4. PCR amplification

The BAP-2 and AL34S set of oligonucleotide primer was used to amplify msp1  $\beta$  gene of *A. marginale*<sup>[16,18]</sup>. The nucleotide sequence of the primer was BAP-2 5' GTA TGG CAC GTA GTC TTG GGA TCA 3' and AL34S -5' CAG CAG CAA GAC CTT CA 3'. The PCR reaction mixture (25  $\mu$  L) was constituting 12.5  $\mu$  L of KAPA 2G TM Fast Hot Start Ready Mix (2X containing KAPA2G fast hot start DNA polymerase, KAPA 2G fast hot start PCR buffer, 0.2 mM dNTP each, 1.5 mM MgCl<sub>2</sub>) with 1.5  $\mu$  L of BAP-2/ AL34S 10 pmol primers. Reaction was conducted in automated Thermocycler (Eppendorf, master cycler personal) with the following programme: initial denaturation at 95 °C (5 min), 30 cycles of denaturation at 95 °C (1 min), annealing at 60 °C (1 min), extension at 72 °C (1.5 min) with final extension at 72 °C for 5 min. The amplified PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV Transilluminator for detection of 407 bp amplified product.

### 2.5. Hematological and biochemical analyses

The haematological parameters of the whole blood viz. white blood cells (WBC), haemoglobin (HB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (mchc) and platelets (Plt) were done with fully automated analyzed ADVIA 2120 Haematology System (Siemens Health Care Diagnostic Inc. Deerfield, IL, U.S.A). The sera from blood were separated after centrifugation at 5 000 rpm for 10 min and further stored at -20 °C until use for estimation of biochemical parameters. The different serum biochemical parameters viz. aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), glucose (GLU), blood

urea nitrogen (BUN), total protein (TP), albumin (ALB), creatinine (CRSC) and total bilirubin (TBIL) were estimated on VTROS DT 6011 System Chemistry using Ortho–Clinical Diagnostics Kit (Johnson & Johnson company) as per the instruction of the manufacturer.

### 2.6. Statistical analysis

The associations of prevalence by two diagnostic methods viz. PCR and GSTBS, with different districts of Punjab state were determined by  $\chi^2$  test. On the basis of age, animals were grouped as calves (less than 1 year of age) and adults (more than 1 year of age). The association of prevalence of the infection among the animals of different age groups was determined also by  $\chi^2$  test. One-way Analysis of Variance (ANOVA) was applied to various haematological and biochemical parameters of animals by using CPSC1 software to determine the variance in these parameters among GSTBS positive, PCR positive and healthy reference samples ( $P < 0.05$ ). All the values are expressed as means  $\pm$  standard deviations.

### 3. Results

Out of total 320 blood samples, PCR based diagnosis revealed 156 animals (48.75%) positive samples for *Anaplasma* including 107 cows, 45 cow calves and 4 buffalo (Table 1). The relative prevalence of *A. marginale* by PCR amplifying 407 bp fragment (Figure 1) showed significant association with the areas under study ( $\chi^2 = 27.43$ ,  $P = 0.00012$ ), prevalence being recorded highest in Ferozpur (80%). However, GSTBS (Figure 2) of these animals revealed 11.25% positive samples for *Anaplasma* including only 23 cows, 13 cow calves and no buffalo (Table 1). The association of GSTBS diagnosis (Figure 2) was also found to be significant with the areas under study ( $\chi^2 = 14.07$ ,  $P = 0.0288$ ), with prevalence being highest in Jalandhar (22.5%).

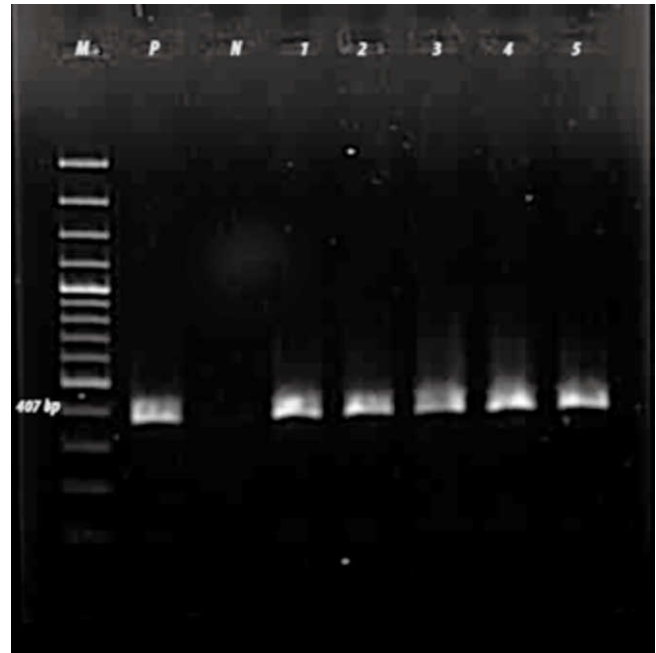
**Table 1**

Prevalence of *A. marginale* by GSTBS and PCR targeted on *msp1*  $\beta$  gene.

Sampling districts	<i>n</i>	GSTBS positive (%)	PCR positive (%)
Ludhiana	89	8 (8.98)	34 (38.20)
Jalandhar	40	9 (22.50)	25 (62.50)
Patiala	33	5 (15.15)	22 (66.66)
Bathinda	66	3 (4.55)	24 (36.36)
Moga	16	4 (25.00)	10 (62.50)
Mansa	51	3 (5.88)	21 (41.18)
Ferozpur	25	4 (16.00)	20 (80.00)
Cow (>1 yrs)	236	23 (9.66)	107 (45.33)
Cow calf (<1 yrs)	62	13 (20.96)	45 (72.58)

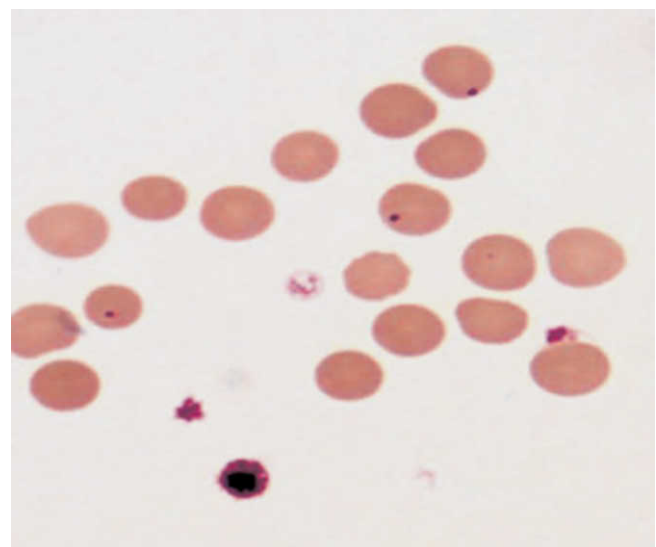
The PCR based molecular diagnosis ( $\chi^2 = 14.64$ ,  $P = 0.00013$ ) and GSTBS based conventional diagnosis ( $\chi^2 = 5.91$ ,  $P = 0.015$ ) had association with the age of

the animals under study. The cow calf showed higher level of both clinical and subclinical infection of *A. marginale* as compared to the adult. Statistical analysis also indicated that the presence of ticks on animal ( $P = 0.0014$ ) may play a significant role in transmission of the disease in the animal of less than 1 year of age. Questionnaire based data collected depicted that the management conditions including overcrowding of farm, lacunae in management conditions and use of acaricides and repeated use of contaminated needles in the herd also have impact on prevalence.



**Figure 1.** Agarose gel (1.5%) electrophoresis showing amplified DNA (407 bp) from *A. marginale* targeting *msp1*  $\beta$  gene of *A. marginale* using primer BAP-2/AL34S.

Lane M, molecular size marker 100 bp plus; Lane P, positive control and N, negative controls, Lane 1–5, amplified *A. marginale* genomic DNA from the blood of the infected animals.



**Figure 2.** Giemsa stained thin blood smear showing *A. marginale* at

margins of erythrocytes.

In the clinically positive (30) cases,  $(5.90 \pm 2.70)\%$  parasitaemia of *Anaplasma* was recorded. The clinical signs recorded in positive animals included high fever (103–105 °F) anemia, anorexia, pale mucous membrane, icterus, abortion, tachycardia and weight loss. Death was recorded in one of the adult (4 years) cow.

Haematological parameters showed (Table 2) significant decrease in the RBC count, Hb and PCV levels of the animals categorized under the group positive by PCR (a) and group positive by GSTBS (b) as compared to the health control group (c) ( $P=0.05$ ). Serum biochemical evaluation (Table 3) revealed significant increase the level of total protein, total bilirubin and ALT in the animals belonging to group a and b as compared to group c ( $P=0.05$ ).

#### 4. Discussion

Besides tropical and sub tropical regions anaplasmosis has been also recorded in some temperate areas<sup>[21]</sup>. The disease is responsible for substantial significant economic losses in endemic areas<sup>[22]</sup>. In the present study 37.5% more prevalence of *A. marginale* was recorded by PCR method targeting *msp1 β* gene of *A. marginale* as compared to the GSTBS technique. The prevalence range was wide and may be correlated with the managemental practices, animal's breed reared in region, drugs used by the field veterinarians, acaricides used to control the ticks in the different regions of the study. We were able to get lesser prevalence of the disease where either oxytetracycline drugs or acaricides were being used in the organized farms. Where there was little population of *R. (Boophilus) microplus* ticks, less prevalence of anaplasmosis was seen. In the area with highest prevalence (Ferozpur) only 16% animals were found positive for the infection by GSTBS examination while 80% were found positive by PCR based diagnosis, this may be attributed to

the fact that the cattle population was mainly constituted by the local breed, *Bos indicus* which are usually considered to be resistant for *Anaplasma* and other haemoprotozoan infections, but, at the same time, may act as the carrier of the infection for susceptible *Bos taurus* cattle. Out of 236 adult cows studied 23 (9.66%) were found positive by GSTBS and 107 (45.33%) were positive by PCR. Out of 62 cattle calves, 20.96% were positive by GSTBS and 72.58% by PCR technique. The studies indicates that there is no inverse age resistance in cow calves to *A. marginale* as reported earlier<sup>[23]</sup>. Only 22 buffaloes were encountered in the study that showed a prevalence of anaplasmosis to be 18.18% by PCR based diagnosis and no animal showed parasitaemia by GSTBS examination. Study explicates the role of buffalo as the potent carrier animal by not exhibiting any clinical symptoms but still harbouring the infection. Results opened the elements that the traditional Giemsa staining method is not applicable for determination of persistently infected cattle and buffalo. The results also explore the facts that young animals may also act as persistent carrier and subsequently source of infection for themselves and even for others in the herd. Noaman *et al*<sup>[24]</sup> considered molecular diagnostic of *A. marginale* in carrier cattle to be determinant for persistently infected cattle and reported 50% *A. marginale* positive cases out of 150 total blood samples in Iran. Torioni *et al*<sup>[25]</sup> quoted that PCR assay to be more sensitive for detection and quantification of rickettsemia in carrier, pre-symptomatic and symptomatic cattle.

High percent parasitemia recorded in clinically positive cases is the indicative of acute form of disease. The clinical signs recorded *viz* anemia, 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 haemoprotic and thermoregulatory centers of the body. Bundza and Samagh<sup>[26]</sup> also recorded similar finding in imported cattle in Canada affected with

**Table 2**

Comparison of haematological values in *A. marginale* infected and healthy control animals.

Group	WBC ( $10^3 / \mu\text{L}$ )	RBC ( $10^6 / \mu\text{L}$ )	HB (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Plt ( $10^3 / \mu\text{L}$ )
Samples positive by GSTBS(a, n=30)	13.38 ±5.67	5.38± 1.62*	7.74±2.39*	23.75±5.79*	45.61±7.79*	14.57±2.57	32.07±3.28*	319.96±154.45
Samples positive by PCR(b, n=89)	12.07 ±4.19	5.83±1.52*	8.66±1.95*	25.99±5.39*	45.75±7.54*	15.14±2.41	33.22±2.96*	284.35±171.74
Healthy control (c, n=30)	3011.06 ±1.25	6.68±1.29	10.09±1.39	28.76±4.12	43.99±6.04	15.42±2.55	35.12±3.18	336.83±112.62

Values are shown as Mean ± standard deviation;  $P<0.05$ .

**Table 3**

Comparison of biochemical values in *A. marginale* infected and healthy control animals(U/L).

Group	ALKP	GLU	AST	ALB	BUN	TP	TBIL	ALT	CRSC	GLO
Samples positive by GSTBS (a, n=17)	71.88±29.91	44.53±28.54	147.40±108.74	2.60±0.50	13.17±6.71	7.44±1.84	0.97±0.66	33.52±11.61	0.92±0.98	4.84±1.49*
Samples positive by PCR (b, n=40)	71.75±38.91	39.25±14.87	205.70±417.30	2.54±0.72	12.20±8.91	7.00±1.49	0.98±1.45	40.95±24.79	0.82±0.46	4.46±1.12*
Healthy control (c, n=8)	62.75±22.15	43.25±5.92	70.87±23.21	2.40±0.46	13.75±2.60	6.76±0.66	0.50±0.18	26.75±6.61	0.84±0.17	4.36±0.83

Values are shown as Mean ± standard deviation. \* $P<0.05$ .



anaplasmosis.

Ticks were observed on 83.4% of the affected cattle and 11.2% of the affected buffaloes. Major population of the ticks was constituted by *R. (Boophilus) microplus* followed by *Hyalomma annatolicum annatolicum*, correlating the fact that incidence of *A. marginale* is tick borne pathogenesis. Above all the major fraction of sampling is done from cattle and they are more infested with *R. microplus* probably because it preferred dense hair coat<sup>[27]</sup>. Epidemiological findings of Haque *et al*<sup>[28]</sup> also revealed *R. microplus* as the predominant tick in cattle population (85.28%), in Punjab India. Sampling was done from farms having the herd size ranging from 20–150, thus the overcrowding of the farm can be corroborated higher and rapid spread of infection due to various direct and indirect factors including management strategies.

A peculiar observation made in the farms of Patiala and Ludhiana revealing their lacunae in management system. The repeated use of infected needles for medication with in the herd by the labour attributed to the higher incidence of *A. marginale* leading to factors for its iatrogenic transmission.

The values of TLC were apparently higher in group a and b animals as compared to healthy controls. 19 GSTBS positive animals (63.33%) showed leucocytosis indicating stimulation of lymphoid organs and system due to parasite and its toxins. 51 PCR positive animals lied in normal range of total leucocytes count depicting the fact that low parasitaemia don't trigger the lymphatic system. However 38 PCR positive animals (43.82%) also revealed leucocytosis, these cases may be in the incubation period and progressing towards the clinical form of diseases. The results are in agreement with that reported by Middleton<sup>[29]</sup>.

Haemogram of the infected animals revealed anaemia as indicated by significant decrease ( $P<0.05$ ) in RBC, Hb and PCV in both the infected groups positive by GSTBS and PCR as compared to the health control group. On an average, there was significant increase in MCV and decrease in MCHC in infected groups as compared to healthy control group indicating macrocytic hypochromic anaemia, however, the maximum number of animals were lying in normal range of MCV and MCHC indicating normochromic normocytic anaemia in most of the infected animals. It reflects that animals' haemopoietic system was activated in response to erythrophagocytosis, the results are in agreement of Mohammed *et al*<sup>[30]</sup> who studied same changes in camels suffering from anaplasmosis. 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. Anaemia is considered to be a major component in anaplasmosis<sup>[31]</sup>. Platelet count was within normal range in most of the infected animals, while few animals showed

thrombocytosis.

In serum biochemistry, though the values of AST, ALKP and total bilirubin showed no significant difference ( $P<0.05$ ) among the group a, b and c animals, however noteworthy proportion of the infected animals tested showed the level of ALT, AST and TBIL above normal. Lower PCV and higher TBIL, AST and ALT indicate liver dysfunction. These findings were similar to Kataria and Bhatia<sup>[32]</sup> and Boid and Luckins<sup>[33]</sup>. They stated that damage to the skeletal or heart muscles, hepatic tissues and erythrocytes may resulted in considerable increase in the level of AST and ALT. Manna<sup>[34]</sup> reported hyperbilirubinemia in camels infected with anaplasmosis due to excessive destruction of RBCs and the indirect hepatocellular damage. Globulin showed significant ( $P<0.05$ ) increase in both the infected groups (a and b) as compared to group c animals, which signifies that the defensive immune system may be activated leading to elevation of circulating immunoglobulin in the serum to counter the infection in the system.

### Conflict of interest statement

We declare that we have no conflict of interest.

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