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Molecular characterisation of *Anaplasma marginale* and associated haematobiochemical alterations in naturally affected bovines of central Kerala

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Abstract

Anaplasma spp. are obligate intracellular organisms responsible for significant tick-borne diseases affecting both human and animal health. Microscopic examination of blood smears (n=139) from symptomatic cattle in the present study revealed Anaplasma marginale in 15.82 per cent of the samples. Further, species specific PCR targeting msp4 gene of Anaplasma marginale yielded positive results in 86 samples (61.87 per cent). Haematological analysis of the infected animals revealed a significant reduction in total leucocyte count, granulocyte count, total erythrocyte count, haemoglobin, volume of packed red cells and a significant increase in mean corpuscular haemoglobin compared to the healthy animals. Biochemical analysis revealed a significant increase in total bilirubin in the infected group of animals. Phylogenetic analysis of the msp4 sequence of A. marginale revealed close relationship of the isolate A. marginale Mannuthy to the isolates from Turkey and Malaysia, though with moderate divergence indicating a common lineage with these isolates. Thus, this study records the molecular characterisation of A. marginale along with the haematobiochemical alterations in the infected cattle that would pave the way for adopting appropriate treatment regimen and control strategies.

Keywords: *Anaplasma, cattle, msp4, haematobiochemical*

Bovine anaplasmosis is an economically important tick-borne haemo-rickettsial disease of ruminants caused by *Anaplasma* sp., an obligate intracellular organism parasitising mammalian blood cells. *Anaplasma marginale* is the most common species causing the disease in cattle in Kerala, although other species such as *A. centrale, A. bovis, A. ovis, A. phagocytophilum* and *A. platys* have also been recognised in animals. Primarily spread through ticks, the disease is also transmitted by biting flies, blood-contaminated instruments such as needles, ear-tagging devices, dehorning tools, castration equipment and vertically from mother to calf (Aubry and Geale, 2011). Clinical signs exhibited by affected cattle include fever, jaundice, decreased milk production, abortion and in some cases, sudden death. During the acute phase of the disease, characterised by high levels of rickettsemia, initial bodies can be readily observed in

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bovine erythrocytes through microscopy of stained blood smears (Maharana *et al.,* 2014). However, blood smears are not reliable in the detection of subclinical and chronic infections. Animals recovered from acute anaplasmosis act as potential carriers owing to spread of the disease. Although microscopy is a widely accepted and cost-effective technique for diagnosing haemoparasites, it lacks the higher sensitivity required for detecting low levels of infection (Nair *et al*., 2013). Moreover, it is challenging to diagnose anaplasmosis in cattle because of the difficulty in distinguishing between *Anaplasma* organisms and structures such as Heinz bodies, Howell-Jolly bodies or staining artefacts in Giemsa-stained blood smears (Subramanian *et al*., 2020). Hence advanced molecular techniques like Polymerase chain reaction (PCR), renowned for their exceptional sensitivity and specificity, had been developed to detect haemoparasites such as *A. marginale* (Carelli *et al*., 2007). This study aimed to the molecular characterisation of *A. marginale* and assess its impact on the haematological and biochemical profiles of the infected cattle.

Materials and methods

Sample collection

Blood samples (n=139) were collected from cattle showing clinical signs suggestive of anaplasmosis presented to different veterinary hospitals of Thrissur and Palakkad districts of Central Kerala during the period from January 2024 to August 2024. Blood smears were prepared using blood collected from peripheral ear vein. Whole blood samples aseptically collected from jugular vein of both infected animals and control group. Two millilitres of the collected blood was transferred to EDTA coated vacutainers for haematology and 4 ml was added into vacutainer with clot activator for biochemical analysis. Further, the coagulated blood samples were subjected to centrifugation at 2000 rpm for 5 min and the separated serum was collected for estimation of biochemical parameters.

Polymerase Chain Reaction

The DNA extraction was done from anticoagulant added blood by modified high salt method (Montgomery and Sise, 1990) with minor modifications. Blood samples were screened for the presence of *A. marginale* by PCR using species specific primers (Joazeiro *et al.,* 2015). The primer sequences are given in Table 1. The genomic DNA extracted from cattle blood sample which was found positive for *A. marginale* microscopically was used as the positive control. No template control was maintained with nuclease free water in all amplifications. The reaction mixture was reconstituted into 10 µL total volume with 5 µL EmeraldAmp® GT PCR Mastermix 2X (Takara Bio, USA), 0.5 µL each of forward and reverse primers (10pM/µL), 2 µL DNA template and 2 µL nuclease free water. The amplification of genes was carried out with initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. A total of 139 samples were screened using newly standardised PCR protocol.

After completion of the PCR reaction, the amplified DNA products were subjected to agarose gel electrophoresis in a submarine electrophoretic apparatus (Bio-Rad Laboratories, USA) using a 1.5 per cent agarose gel using Tris Aetate EDTA buffer at a voltage of 70 V for 1 hr. The gel was documented in the gel documentation system (Bio-Rad Laboratories, USA). Further, sequencing was done at Genespec Private Limited, Kakkanad and were aligned using EMBOSS (www.bioinformatics.nl>cgi-bin>merger). Similarity with other published sequences were analysed by NCBI BLAST (www.blast.ncbi.nlm.nih.gov>blast) and submitted to GenBank using bankit (gb-admin@ncbi.nlm.nih.gov).

A phylogenetic tree and distance matrix of *A. marginale* and *A. bovis* was constructed from corresponding gene sequences of msp4 gene and 16S rRNA along with other sequences reported in the GenBank database using the software MEGA 11.0 (Tamura *et al.,* 2021). The nucleotide sequences were aligned by clustalW. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) using the Kimura 2-parameter method (Kimura, 1980).

Haemato-biochemical analysis

Haematological parameters analysed include total erythrocyte count (TEC), haemoglobin (Hb), volume of packed red cells (VPRC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total leukocyte count (TLC) using automatic haematological analyser (Orphee Mythic Vet 18). The serum albumin, blood urea nitrogen (BUN), alanine transaminase (ALT) alkaline phosphatase (ALP) and total bilirubin were estimated by semi-automatic analyser (Alpha technologies, Chennai).

Statistical analysis

Statistical analysis was carried out using IBM-

Table 1. Primer sequences targeting msp4 gene specific for *A. marginale*

Gene	Primer	Expected product size
msp4F	5' – CCCATGAGTCACGAAGTGG – 3'	753 bp
msp4R	5'- GCTGAACAGGAATCTTGCTCC - 3'	

SPSS software version 24. One way analysis of variance was used to compare between the groups.

Results and discussion

Microscopic examination of 139 Field stained blood smears from cattle, revealed round dense intracytoplasmic inclusion bodies in the periphery of the erythrocytes suggestive of *A.marginale* (Fig.1) in 22 animals. The overall occurrence of *A. marginale* in the cattle under study was found to be 15.82 per cent by blood smear examination. Arulkumar *et al*. (2023) observed *A. marginale* infection 18.7 per cent animals in symptomatic cattle of Namakkal by blood smear examination. On contrary, Kumar and Sangwan (2010) recorded a higher prevalence of *Anaplasma* organisms in 46.9 per cent of cattle in Haryana.

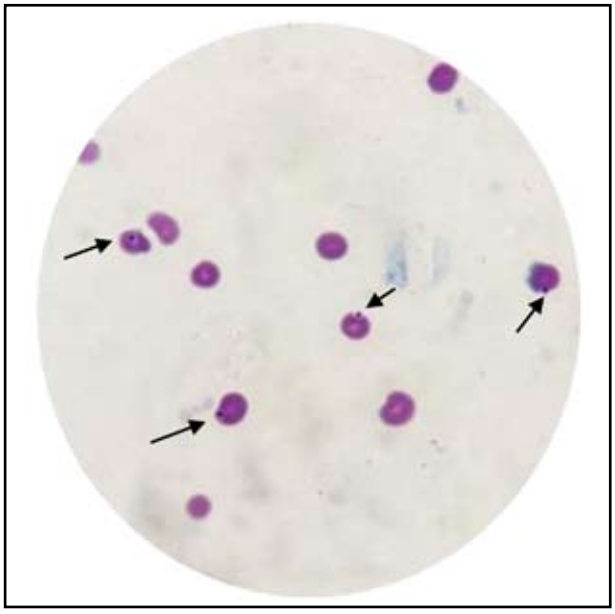

Fig. 1 Intraerythrocytic inclusions of *A. marginale*

Polymerase chain reaction targeting *msp4* of *A. marginale* resulted in amplification of 753 bp product in 86 out of 139 samples by showing an overall occurrence of 61.87 per cent of *A. marginale* infections in cattle under study (Fig.2). The *msp4* of *A. marginale* has been widely used for detection of *A. marginale.* Rjeibi *et al.* (2018) reported a prevalence of 39.4 per cent of *A.marginale* in cattle of Algeria. The prevalence of *A.marginale* in cattle detected by PCR were also reported from different parts of south India as in 16.4 per cent of cattle in Seemandhra and Telangana (George *et al.,* 2017) and in 20.54 per cent of cattle in Puducherry (Subramanian *et al*., 2020) . The increased occurrence of anaplasmosis in cattle observed in PCR may be attributed to the presence of subclinical infections or carrier status in recovered animals, as opined by Nair *et al*. (2013). Hamid *et al*. (2014) emphasised the importance of PCR in assessing the carrier status of cattle with *Anaplasma* infections, highlighting its superiority over conventional microscopic examination as observed in the

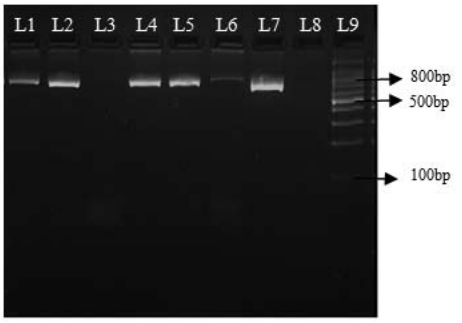

Fig. 2. Gel picture showing amplified products of *msp4* of *A. marginale*

Lanes: L1 to L6- samples
L7- positive control
L8- no template control
L9- 100 bp Ladder

current study. Jaswal *et al*. (2014) detected *A. marginale* in 39.16 per cent of cattle by PCR compared to 15.83 per cent by conventional blood smear examination indicating the high sensitivity of PCR supporting the results of the present study.

Amplified PCR product was sequenced and submitted to Genbank (Accession No: PQ309681). The sequence revealed 100 per cent similarity with other published sequences in Genbank.

Haematological findings

The haematological values of *A. marginale* infected group and control group (healthy animals) are given in Table 3. *Anaplasma marginale* infected group had a significantly decreased TEC, Hb and VPRC when compared to control group. The invasion of *Anaplasma* organisms into red blood cells, followed by their multiplication, leads to the rupture of these cells and the subsequent spread of the organisms to other erythrocytes (Sivajothi and Reddy, 2022; Biswas *et al.,* 2023). This process triggers erythrophagocytosis due to parasitic damage to the erythrocytes and an increased activation of complement products, resulting in the removal of destroyed cells by the bovine reticuloendothelial system (Sharma *et al.,* 2013). The primary clinical sign of anaplasmosis in cattle is extravascular haemolytic anaemia (Bhanot and Jindal, 2022), although both intravascular and extravascular haemolysis are typically observed (Riond *et al.,* 2008).

A notable reduction in TLC and granulocyte count was observed in the study, consistent with the findings of Hussein *et al.* (2007). They suggested that the decrease in TLC could be due to the persistent detrimental effects of toxic metabolites produced by *Anaplasma* on haemopoietic organs, particularly the bone marrow, and their interference with the process of leucogenesis.

The significant increase in MCH observed in case

of infected animals when compared to control group was similar to the findings of Al-Gharban and Dhahir (2015). Abbas *et al.* (2020) opined that the increase in MCH could be ascribed to the release of haemoglobin into plasma due to erythrolysis (Abbas *et al.,* 2020).

In this study, although the MCV values were elevated compared to the control group, the changes were not statistically significant. Typically, an increase in MCV indicates regenerative anaemia, characterised by the release of a large number of immature red blood cells (RBCs) from the bone marrow (Riond *et al.,* 2008). This response might be due to the rapid destruction of RBCs through phagocytosis. As the immature RBCs are larger than mature ones might lead to an increased MCV. Subramanian *et al.* (2020) also noted that regenerative anaemia could be a positive indicator in bovine anaplasmosis, which aligns with our current observations.

No significant difference was noticed in lymphocyte count, monocyte count, MCHC and platelet count.

Biochemical findings

The biochemical values of *A. marginale* infected group and control group (healthy animals) are given in Table 4. A statistically significant increase in bilirubin levels was noted in animals infected with *A. marginale* compared to healthy controls. This hyperbilirubinemia may result from the extensive destruction of parasitised erythrocytes by the bovine reticuloendothelial system (Debbarma *et al*., 2020) and indirect hepatocellular damage (Sharma *et al*., 2013).

Phylogenetic analysis

Evolutionary analyses were conducted in MEGA11 and the evolutionary history was inferred using the Neighbor-Joining method with bootstrap value of 1000. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 27 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The final data set contained a total of 878 positions.

The isolate *Anaplasma marginale* Mannuthy was positioned separately but within the larger *A. marginale* clade. It showed close genetic relationships with isolates from geographically diverse regions, including Turkey and Malaysia. This placement suggests that *A. marginale* Mannuthy is part of a broader group of isolates

Table 3. Haematological parameters in *A. marginale* infected group and control group

Haematological parameter	*A. marginale* infected group (n=21)	Control Group (n=10)	F value
Total leucocyte count (10^3/µL)	6.64 ± 0.88^b	10.46 ± 0.21^a	3.069*
Lymphocyte count (10^3/µL)	4.59 ± 0.71	6.23 ± 0.33	2.995^{NS}
Monocyte count (10^3/µL)	0.65 ± 0.11	0.61 ± 0.03	1.154^{NS}
Granulocyte count (10^3/µL)	1.40 ± 0.22^b	3.61 ± 0.38^a	10.827**
Total erythrocyte count (10^6/µL)	3.99 ± 0.28^b	8.32 ± 0.13^a	43.418**
Haemoglobin (g/dL)	6.94 ± 0.38^b	10.51 ± 0.53^a	11.964**
VPRC (%)	19.88 ± 1.04^b	33.73 ± 1.60^a	15.491**
MCV (µm^3)	53.70 ± 3.90	45.73 ± 1.44	1.618^{NS}
MCH (pg)	17.97 ± 0.55^a	14.40 ± 0.53^b	4.617**
MCHC (g/dL)	34.90 ± 1.02	32.34 ± 0.48	4.006^{NS}
Platelet count (10^3 per µL)	312.76 ± 39.81	290.80 ± 14.29	1.339^{NS}

** Significant at 5 per cent level; **Significant at 1 per cent level; NS: non-significant*

Table 4. Biochemical parameters in *A. marginale* infected group and control group

Biochemical parameters	*A. marginale* infected group (n=21)	Control Group (n=10)	F value
Blood urea nitrogen (mg/dL)	15.95 ± 0.95	16.08 ± 1.21	0.187^{NS}
Alkaline phosphatase (IU/L)	63.00 ± 13.61	26.94 ± 1.51	1.676^{NS}
Alanine transaminase (IU/L)	22.43 ± 2.79	20.77 ± 1.16	0.120^{NS}
Serum albumin (g/dL)	3.34 ± 0.20	3.83 ± 0.10	1.505^{NS}
Total bilirubin (mg/dL)	0.94 ± 0.14^a	0.45 ± 0.06^b	3.938*

** Significant at 5 per cent level; NS: non-significant*

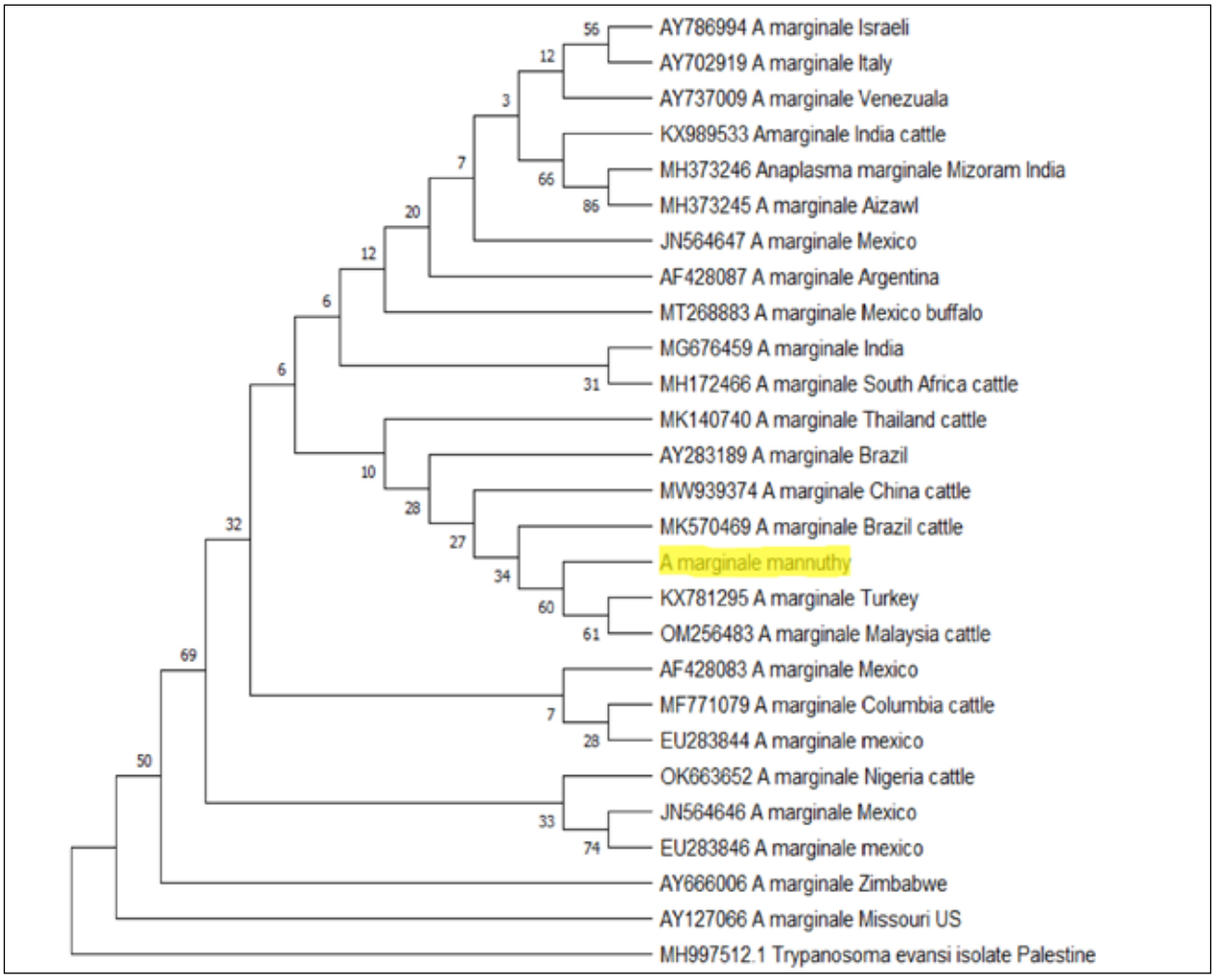

Fig. 3. Phylogenetic tree constructed using *msp4* sequence of *A. marginale*

sharing a relatively recent common ancestor, indicating genetic similarities across different regions. However, *A. marginale* Mannuthy exhibited moderate divergence from its closest neighbors, such as the isolates from Turkey and Malaysia, indicating a level of genetic conservation among these geographically separated isolates. The presence of *A. marginale* Mannuthy within a well-supported clade, with relatively high bootstrap values at the relevant nodes, underscores the reliability of its placement and suggests strong evolutionary ties with other isolates in the same group. Similarly, Pradeep *et al.* (2019) reported the clustering of *Anaplasma* isolates from Kerala with other isolates from north America, south America and Asia. Therefore, it could be inferred that *A. marginale* isolates from south India exhibit close genetic similarity.

Conclusion

An overall occurrence of 15.82 per cent of anaplasmosis could be identified in central Kerala by blood smear examination. A high prevalence of 61.87 per cent observed in PCR might be attributed to the prevalence of subclinical and chronic infections. Haematobiochemical analysis revealed anaemia with hyperbilirubinaemia associated with parasite induced erythrocyte damage. Phylogenetic analysis revealed the close genetic similarity of *A. marginale* strains in south India. Consequently, this study provides the molecular characterisation of *A. marginale* and evaluates its effects on the haematological and biochemical profiles of infected cattle, which will help in developing suitable treatment protocols and control measures.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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