Explor Anim Med Res.
Vol. 15, Issue 2, 2025
DOI: 10.52635/eamr/15.2.235-240

ISSN 2277-470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

INCIDENCE AND PHYLOGENETIC STUDY OF *ANAPLASMA MARGINALE* IN CATTLE OF CHHATTISGARH PLAINS

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Received 29 April 2025, revised 18 July 2025

ABSTRACT: *Anaplasma marginale*, the most common aetiological agent of bovine anaplasmosis, is a highly pathogenic, intra-erythrocytic rickettsia. It is one of the causative agents of economic losses to livestock farming, both in tropical and in temperate regions. The present study was aimed at studying the incidence and molecular characterization of *A. marginale* in cattle of Chhattisgarh plains by Polymerase Chain Reaction (PCR). Microscopic investigation of Giemsa stained peripheral blood smears of 150 apparently healthy and clinically ill cattle revealed *A. marginale* organisms in 11.33% (17/150) animals. For molecular incidence studies, major surface protein-1β (msp1β) of *A. marginale* was selected as the target gene for amplification by PCR. The DNA samples of these cattle when subjected to conventional PCR resulted in amplicon of 265 bp in 59.33% (89/150) animals. The PCR results of the incidence of *A. marginale* were significantly higher ($p<0.001$) as compared to microscopy. Phylogenetic study of the two isolates collected locally from Chhattisgarh showed that these two isolates clustered in two clades. Considering that the cattle population of 9.983 million in Chhattisgarh is targeted for productivity improvement but faces the risk of anaplasmosis, it is essential to analyze incidence trends and develop appropriate control measures to manage anaplasmosis in livestock effectively.

Keywords: *Anaplasma marginale*, cattle, Chhattisgarh, Polymerase Chain Reaction, msp1β.

INTRODUCTION

Livestock production is an integral part of the agricultural production system in India and plays an important role in the national economy as well as the socio-economic development of millions of rural households. In India, tick-borne diseases (TBDs) caused by haemoparasites *viz*. *Babesia*, *Theileria* and *Anaplasma* are the major impediments to livestock production that causes significant economic losses corresponding to 8.7 million USD [1]. *A. marginale*, frequently identified as the primary pathogen responsible for causing bovine anaplasmosis is observed as dense, rounded, intra-erythrocytic, gram-negative bodies situated on or near the margin of the erythrocytes. *A. marginale* may be biologically transmitted by ticks, primarily by *Rhipicephalus microplus* [2], mechanically by blood sucking flies, contaminated fomites [3], and also transplacentally to new born calves [4]. It is a potent rickettsial pathogen that can lead to both acute and chronic infections in cattle. The disease caused by *A. marginale* is characterized by fever, anaemia, pale mucous membranes, weight loss, decreased milk production, lethargy, icterus, gastrointestinal signs, abortion and often death in animals [5]. The interaction between *A. marginale* and its host relies significantly on the Major Surface Proteins (MSP) which plays a vital role in the invasion of host cells by the rickettsia. Typically this multigene protein family experiences antigenic shifts, leading to distinctive antigens identified in specific

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geographical regions. Thus, *A. marginale* employs antigenic variation as a strategy to evade the host's immune response enabling the pathogen to establish persistent infections [6]. Cattle may develop persistent infection after recovery from acute anaplasmosis, characterized by recurring bouts of rickettsaemia with levels ranging from around $10^{2.5}$ – 10^{7} infected erythrocytes per milliliter of blood [7]. Cattle with persistent infections may act as long-standing reservoirs facilitating ongoing transmission within herds. Identification of persistently infected cattle is crucial for managing the movement of infected cattle in and out of regions free from the disease.

The clinically suspected cases of bovine anaplasmosis can be conventionally diagnosed by microscopic detection of *A. marginale* in Giemsa-stained blood smears. But microscopy is not reliable for the determination of carrier or pre-symptomatic animals as it has the detection limit of about 0.03 percent and moreover, distinguishing the pathogen from similar organisms including artifacts, necessitates keen expertise and experience. Hence, sensitive and specific molecular tool *viz.* PCR is being used to detect *A. marginale* in carrier cattle and tick vector [8, 9] that has proved useful in mapping the disease.

In Chhattisgarh, data on the incidence of *A. marginale* and the circulating genotypes are lacking. Thus, the primary objective of the present study was to gather data on the incidence of *A. marginale* infections in cattle within the Chhattisgarh plain region using both conventional and molecular diagnostic techniques. Furthermore, the study aimed to explore the phylogenetic relationships of the isolates infecting cattle in this region of the country.

MATERIAL AND METHODS

Collection of blood samples

Blood samples of one hundred and fifty cattle from five districts of Chhattisgarh *viz.* Durg, Rajnandgaon, Dhamtari, Kabirdham and Balod were randomly screened for their infection status with *A. marginale* during the period from November 2021 to September 2022. To accomplish this, blood samples, thirty each from five districts, were randomly collected in EDTA coated vacutainer tubes from apparently healthy cattle and those with clinical signs of fever, weight loss, decreased milk production and anaemia. Two thin blood smears were prepared immediately after each blood collection and air dried. The blood samples were stored at -20°C in the Department of Veterinary Parasitology, College of Veterinary Science & Animal Husbandry, Anjora, Durg (Chattisgarh) for investigation. Blood samples that tested positive for *A. marginale* infection under light microscopy were utilized as positive controls. Conversely, blood samples were collected from cattle with no prior anaplasmosis history, showing no clinical symptoms and confirmed negative through examination of Giemsa stained blood smears and PCR serving as negative control.

Microscopic examination

For the microscopic detection of *A. marginale*, thin blood smears were fixed with absolute methanol for 1-2 min and air dried. Giemsa stain was diluted to 1: 9 in distilled water and blood smears were covered with the stain solution. The smears were kept undisturbed for ~40 min, washed in running tap water and air dried. These blood smears were examined under oil immersion lens (100X). At least 15-20 microscopic fields of stained blood smears were observed under oil immersion lens. The Incidence of *A. marginale* will be calculated as:

$$\text{Incidence (\%)} = \frac{\text{Total no. of animals positive for } \textit{A. marginale}}{\text{Total no. of animals examined}} \times 100$$

Molecular detection

Genomic DNA was isolated from 100 µl of whole blood from each sample using Hipura, multi-sample DNA purification kit, (Himedia, India) in accordance with the manufacturer's protocol. Extracted DNA was eluted in 50 µl of DNA elution buffer and stored at -20ºC for further use. The template DNA thus isolated from whole blood of cattle was used in PCR for detection of *A. marginale* infection. The target gene of *A. marginale* selected for PCR amplification was msp1β. The forward primer (msp1β-FOR) 5´-GCTCTAGCAGGTTATGCGTC-3´ and reverse primer (msp1β-REV) 5´- CTGCTTGGGAGATGCACCT -3´were used in the PCR [10]. Each PCR reaction was carried out in 25 µl volume in thermal cycler (Model LI96G LARK, India). In the PCR assay, the master mix consisted of 2.5 µl of 10X PCR buffer, 0.5 µl of 10 mM dNTP mix, 1.0 U of Taq DNA polymerase, 0.5 µl (20 pmol/µl) of forward primer, 0.5 µl (20 pmol/µl) of reverse primer and 3-4 µl of template DNA. The volume was made up to 25 µl with nuclease-free water. The cycling conditions of initial denaturation at 94ºC for 3 min, primer annealing at 50ºC for 45 sec and primer extension at 72ºC for 1 min, 35 cycles of denaturation at 95ºC for 45 sec and final extension at 72ºC for 10 min were followed. The amplification of specific PCR

product of 265 bp was checked by electrophoresis on 1.5% agarose gel stained with ethidium bromide in horizontal electrophoresis apparatus and visualized in gel documentation system (Bio-rad, USA) (Syngene, UK).

Determination of analytical sensitivity and specificity

The analytical sensitivity of the msp1β based PCR assay was determined by tenfold dilution of the genomic DNA retrieved from the blood samples positive for *A. marginale* infection by microscopic examination. The specificity of the PCR primers was also checked with genomic DNA of *Babesia bigemina*, *Theileria annulata* and *Trypanosoma evansi.* The PCR amplicons of 02 samples were sequenced for confirmation of the *A. marginale* species.

Determination of diagnostic sensitivity and specificity

The diagnostic sensitivity and specificity of microscopic investigation of Giemsa stained blood smears were calculated with following formulae [11].

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100$$

Where, TP indicates true positive i.e. samples positive with msp1β based PCR and microscopy both; FN indicates false negative defined as samples positive in PCR but negative in microscopy.

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100$$

Where, TN indicates true negative i.e. samples negative with both msp1β based PCR and microscopy; FP indicates false positive i.e. samples negative in PCR but positive with microscopy.

Sequencing and phylogenetic analysis

For molecular characterization, PCR products of two isolates *i.e.* Chhattisgarh-India isolate 1 and Chhattisgarh-India isolate 2 were sequenced that belonged to Rajnandgaon and Kabirdham districts of Chhattisgarh, respectively. PCR amplified product of each selected isolate was gel purified using Minelute gel extraction kit (Qiagen, Germany). The eluted products were sequenced at Eurofins Genomics, Bangalore, Karnataka. Nucleotide sequences (n = 2) generated in the study were truncated at both ends, so as to obtain sequences that started and ended at the homologous nucleotide positions. The sequences were compared with the available sequences in GenBank using BLAST program of NCBI. The sequences were aligned with clustal W programme of MEGA 11 software. Phylogenetic trees were constructed using neighbor joining (NJ) method.

Statistical analysis

The association of incidence of *A. marginale* infection as observed by the two diagnostic methods *viz*. PCR and microscopy in the plain region of Chhattisgarh was determined by Chi-square test [12].

RESULTS AND DISCUSSION

Investigation of Giemsa-stained thin blood smears of 150 apparently healthy and clinically ill cattle revealed inclusion bodies of *A. marginale* in 11.33% (17/150) animals (Fig. 1). The diagnostic sensitivity of microscopy was 18.48%, whereas its diagnostic specificity was 100%. In the present study, 10.00% (15/150) animals were found to be clinically infected. In clinical cases, conventional parasitological techniques like light microscopic examination of Giemsa or Romanowsky stained thin blood smears is a widely accepted, cost effective technique and always remains as gold standard for the diagnosis of bovine anaplasmosis

Table 1. Relationship between *A. marginale* infected and uninfected cattle (PCR confirmed) and microscopy results for 150 samples from naturally infected herds

Microscopy	PCR		Total number of samples
	Infected	Uninfected	
Positive	17	0	17
Negative	75	58	133
Total	92	58	150

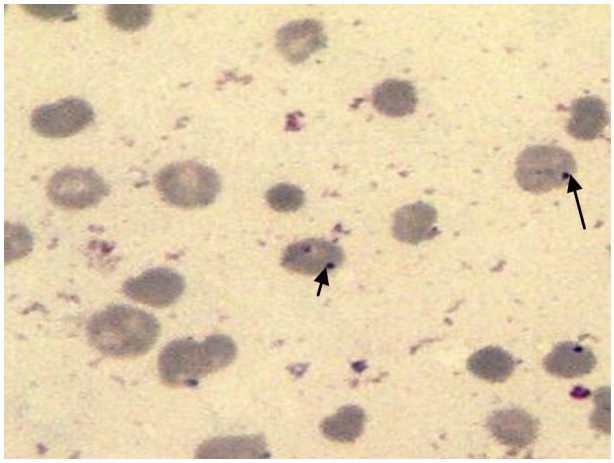

Fig. 1. Photomicrograph of blood smear showing inclusion body of *A. marginale* in erythrocytes

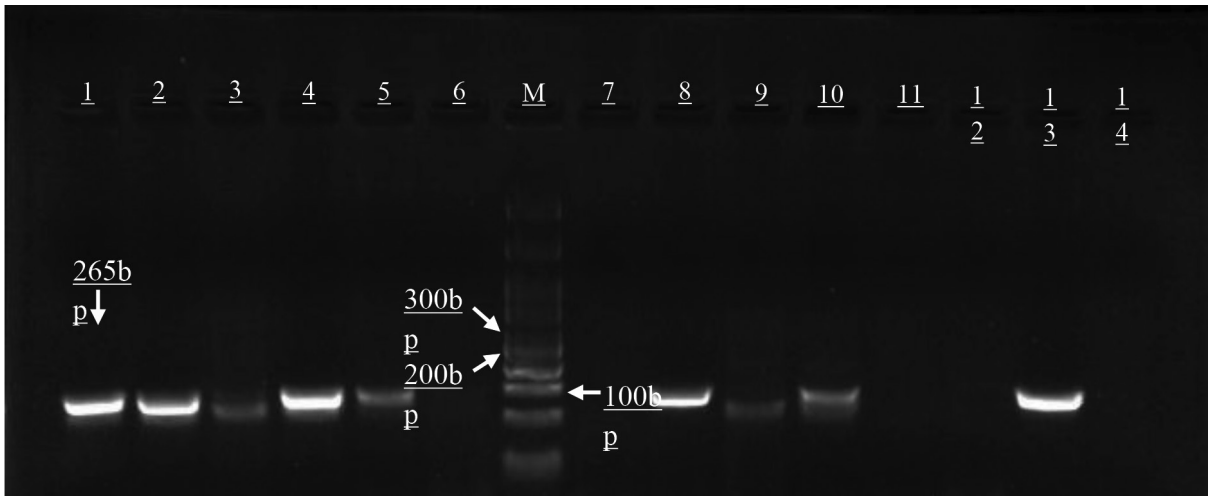

Fig. 2. PCR amplification of msp1β gene of *A. marginale.* [Lane M: 100 bp plus DNA Ladder; Lane 7: Negative control; Lane 8: Positive control; Lane 1, 2, 3, 4, 5, 9, 10, 13 : Positive for *A. marginale*].

[13]. However, in carrier or in pre-symptomatic animals, the level of rickettsaemia is typically too low (<10^6 infected erythrocytes per ml of blood) to be identified by microscopy [14]. Our results corroborates with the findings from West Bengal, wherein 11.93% blood samples of cattle revealed inclusion bodies of *A. marginale* [15]. Examination of Giemsa stained thin blood smears of cattle in the states of Uttar Pradesh and Uttarakhand reported 12.0% animals positive for *A. marginale* infection [16].

Bovine anaplasmosis is known for causing long term infection in animals, even after they have recovered from initial acute infection. As a result, diagnosing *A. marginale* is crucial for identifying carrier animals. Of the 150 total samples subjected to conventional PCR, 61.33% (92/150) were found positive for *A. marginale* infection as revealed by the amplification of 265 bp product (Fig. 2). The result showed the incidence of *A. marginale* by PCR significantly higher (P<0.001) as compared to microscopy, and is depicted in Table 1. The analytical sensitivity of the PCR assay for detection of genomic DNA of *A. marginale* using present set of primers was 220 picograms. The PCR primers used in the present assay did not generate any amplicon when the genomic DNA of *T. annulata, B. bigemina* and *T. evansi* were used as template.

Various authors have recorded variable prevalence of bovine anaplasmosis in different regions of the country by targeting different genes in PCR assays. In Egypt, prevalence of *A. marginale* in bovine was found to be 68.3% by msp1α gene based qPCR assay [6]. By performing nested-PCR targeting msp5 gene 87.9% cattle were detected positive for *A. marginale* infection in the states of Uttar Pradesh and Uttarakhand [16]. In cattle of Jammu region, *A. marginale* infection was diagnosed in 16.5% (46/278) animals by amplification of 16S rRNA gene [1]. Because of the varying detection thresholds of PCR assays, there is a need for diagnostic assays with high analytical sensitivity and specificity to accurately assess the true prevalence of *A. marginale* infection in domestic ruminants. The msp1β gene of *A. marginale* is a sensitive and specific marker for detecting infection in both ticks [17] and cattle [10]. In different regions of the world, many researchers targeted msp1β gene for prevalence of bovine anaplasmosis and obtained the results as 89.7% in Madagascar [18], 28.13% in Iraq [19], 27.0% in Brazil [20] and 37.24% in Pakistan [21]. In India, few workers have performed msp1β gene based molecular detection of *A. marginale* organisms [22, 23]. In the current investigation, the major surface protein 1β gene (msp1β) based PCR assay deciphered the true epidemiological picture of bovine anaplasmosis in the plains of Chhattisgarh state and demonstrated high sensitivity in detecting latent *A. marginale* infections in cattle. The study also showed that a large population of cattle is carrier of *A. marginale* infection in this part of Central India.

The sequence similarity searches in BLAST revealed that Chhattisgarh-India isolate 1 (PV919925.1), from Rajnandgaon district showed 100% similarity with genomic sequences from Myanmar (LC764755.1, LC764754.1 and LC764753.1) (Fig. 3). Chhattisgarh-India isolate 2 (PV919926.1) from Kabirdham district showed marked diversity with the sequence of Chhattisgarh-India isolate 1. It showed 98.70% similarities with isolates from Brazil (CP023731.1) and IVRI, Izatnagar, Bareilly (OR333501.1). In phylogenetic analysis, the two isolates from Chhattisgarh clustered

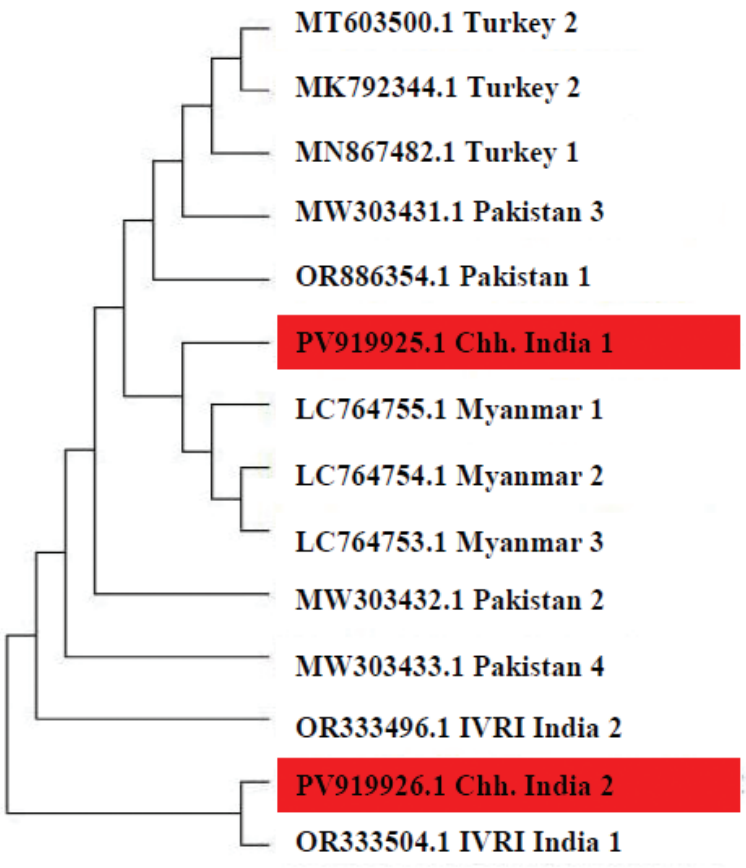

Fig. 3. Phylogenetic relationship of *A. marginale* isolates based on msp1β gene. [All accession numbers corresponds to different *Anaplasma marginale* isolates followed by their country of origin. The sequences generated in the present study are PV919925.1 Chhattisgarh India 1 and PV919926.1 Chhattisgarh India 2 as depicted in red boxes].

in two clades. Chhattisgarh-India isolate 1, formed a group with the isolates of Myanmar, Pakistan and Turkey whereas Chhattisgarh-India isolate 2 made a compilation with the isolates from IVRI, Izatnagar, India and Pakistan.

CONCLUSION

The findings of the present study indicate that *A. marginale* is observed in cattle in Chhattisgarh plain region. *A. marginale* was identified by amplification of msp1β gene. The phylogenetic analysis indicated that the parasite isolates prevailing in the Chhattisgarh region are showing variations. However, a comprehensive study on different *Anaplasma* species prevalent in our country along with their genetic diversity needs to be carried out.

ACKNOWLEDGEMENT

The authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Director, Research Services and Vice Chancellor, Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Durg, Chhattisgarh for providing necessary facilities to carry out this work.

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Cite this article as: Sao SK, Bisen S, Baghel KR, Pal S, Sonkusale PM, Gumasta P. Incidence and phylogenetic study of *Anaplasma marginale* in cattle of Chhattisgarh plains. Explor Anim Med Res. 2025; 15(2), DOI: 10.52635/eamr/15.2.235-240.