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Molecular detection and predisposing factors influencing *Anaplasma marginale* prevalence in dairy cattle of Chhattisgarh, India

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ABSTRACT

Anaplasma marginale, the most common etiological agent of bovine anaplasmosis is an important cause of economic losses in livestock farming, both in the tropical and temperate regions. In the present study, 250 blood samples of cattle from organised and unorganised dairy farms in five districts of the state of Chhattisgarh, India were examined for prevalence of *A. marginale*. DNA extracted from the blood samples of these cattle was subjected to Polymerase Chain Reaction (PCR) using *A. marginale* msp1β gene. It resulted in the amplification of 265 bp PCR product in 63.6% (159/250) of animals. The prevalence of anaplasmosis was found to be significantly higher in the animals of organized dairy farms when compared with un-organized farms. Age of cattle seemed to influence the occurrence of infection with young cattle (<1 year) being more susceptible to the pathogen. Relationship between the presence of *A. marginale* in cattle and certain selected epidemiological factors was also explored. Different cattle breeds seemed to be equally susceptible to *A. marginale* infection. The study revealed a large cattle population (63.6%) in the study area as carriers of *A. marginale* infection.

Keywords: *Anaplasma marginale*, Carrier, Cattle, Chhattisgarh, Epidemiological factors, Prevalence, Polymerase chain reaction

Anaplasma marginale, a globally prevalent intracellular rickettsia found in red blood cells is an important pathogen impacting cattle health and productivity. Bovine anaplasmosis, due to A. marginale, leads to considerable monetary losses, including lower milk yield, slower weight gain, higher veterinary expenses and in severe cases, high fatality rate (M'ghirbi *et al*. 2016). Anaplasma marginale is biologically transmitted by ticks, particularly Rhipicephalus microplus, and mechanically through blood-feeding flies or contaminated instruments like needles, dehorning tools, ear tag applicators and surgical tools (Kocan *et al*. 2010). In cattle, transmission can also occur via placenta, resulting in calves that appear healthy but remain chronically infected (Kocan *et al*. 2015). The disease in cattle manifests through clinical signs such as pyrexia, reduced red blood cell count, pale mucous membranes, reduced weight gain, lethargy, jaundice, gastrointestinal problems and occasionally abortion. Animals that recover from acute anaplasmosis continue to carry a persistent infection, characterized by recurring cycles of rickettsaemia (Eriks *et al*. 1993). These animals act as carriers, capable of spreading the infection to others.

Diagnosis of carrier status is important for implementation of appropriate control measures. PCR identifies *A. marginale* in carrier cattle and provides insights into the epidemiological status of anaplasmosis in bovines from enzootic regions (Torina *et al*. 2008). Major Surface Proteins (MSPs) are the key players in the interaction between *A. marginale* and host cells, aiding in the establishment of infection. Researchers have identified six *A. marginale* major surface proteins-MSP1α, MSP1β, MSP2, MSP3, MSP4 and MSP5 isolated from bovine erythrocytes. These proteins have proven valuable for both serological and molecular diagnosis of anaplasmosis (Kocan *et al*. 2003). *A. marginale* msp1β is a specific and reliable marker for detecting infections in cattle and ticks (Carelli *et al*. 2007).

In addition to molecular detection, evaluating the risk factors associated with bovine anaplasmosis involves a multi-faceted approach. Sampling sites, age of the animal, housing system, herd management practices, use of acaricides as well as the climatic conditions play a significant role in disease dynamics (Bursakov and Kovalchuk 2019). Additionally, the antigenic and genetic variation of the pathogen, along with the genetic susceptibility of various cattle breeds to *A. marginale* infections, can affect the transmission and severity of the disease. Therefore, this study was conducted to assess the prevalence of A. marginale in cattle population of the Chhattisgarh region and to evaluate the risk factors contributing to its occurrence.

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MATERIALS AND METHODS

Place of Study: The study was conducted in five districts of Chhattisgarh, India including Durg, Rajnandgaon, Dhamtari, Kabirdham and Balod (Fig.1). The region explored in the study features a subtropical climate, marked by hot summers with monsoon rainfall, transitioning into a dry and cool winter season. The area receives an average of 1,292 mm of rainfall annually, with summer temperatures fluctuating between 30°C and 47°C, while winter temperatures range from 5°C to 25°C.

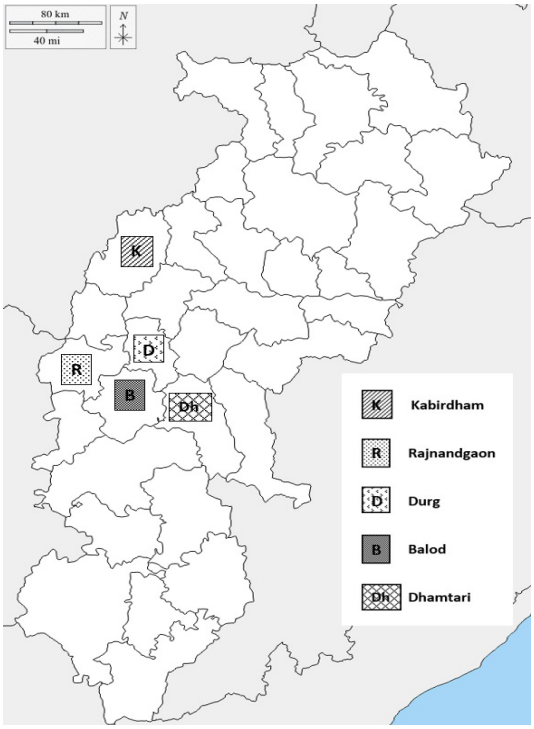

Fig. 1. A map illustrating the districts of Chhattisgarh included in the study

Collection of Blood Samples: Blood samples of 250 cattle from Durg and adjoining districts *viz*. Rajnandgaon, Dhamtari, Kabirdham and Balod were investigated during the period from January 2022 to December 2022. Cattle (cows only) were selected from the two managemental systems of un-organized dairy units (with ≤15 cattle) and organized dairy farms (with >15 cattle). A total of 25 blood samples from five organized dairy farms (5 samples from each dairy farm) and 25 samples from un-organised dairy units of each district were randomly collected. These biological samples belonged to apparently healthy cattle and those with clinical signs of fever, weight reduction, reduced milk output and low red blood cell count, suggestive of *A. marginale* infection. Blood sample of each cattle was collected from the jugular vein in a 2 mL EDTA coated vacutainer, using a sterile 18-gauge needle. The whole blood samples were brought to the Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, for investigation and were stored at -20°C for isolation of genomic DNA. Blood samples from healthy cattle with no history of anaplasmosis and with no clinical signs and confirmed negative by PCR were considered as negative controls. Questionnaire based information regarding breed, age, clinical status of the animal and also management conditions of the farm were collected to evaluate their association with the prevalence of *A. marginale*.

Detection of A. marginale infection in cattle by PCR: Genomic DNA was extracted from 100 µL of whole blood from each sample using the Hipura multi-sample DNA purification kit (Himedia, India), following the manufacturer's instructions. The isolated DNA was eluted in 50 µL of DNA elution buffer and stored at -20ºC for future use in PCR. *A. marginale* msp1β gene was used in the PCR for detection of infection in these animals.

Polymerase chain reaction was performed with msp1β specific primers (Table 1). Each PCR was performed in a 25 µL volume using a thermal cycler (Model LI96G LARK, India). The PCR master mix included 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) each of the forward and reverse primer and 3-4 µL of template DNA, with the final volume adjusted to 25 µL using nuclease-free water. The PCR cycling conditions included an initial denaturation at 94ºC for 3 min, followed by 35 cycles of denaturation at 95ºC for 45 sec, primer annealing at 50ºC for 45 sec and primer extension at 72ºC for 1 min. The amplification of the specific 265 bp PCR product was confirmed on a 1.5% agarose gel stained with ethidium bromide and the results were visualized with a gel documentation system (Bio-Rad, USA) (Syngene, UK). Twelve PCR amplicons were sequenced to confirm the identity of *A. marginale*. Additionally, the specificity of the PCR primers was assessed by testing them against the genomic DNAs of *Babesia bigemina*, *Theileria annulata*, and *Trypanosoma evansi*.

Statistical analysis: The Chi-square test was used to compare the prevalence of anaplasmosis between different sampling districts, management systems, breed, sampling age, season and health status of cattle, in accordance with Snedecor and Cochran (1994). Significance levels were set up at $p = 0.05$.

RESULTS AND DISCUSSION

Prevalence of A. marginale infection: Polymerase

Table 1. Details of primers used in the study

Gene	Primer name	Primer length	Primer Sequence (5′ → 3′)	Amplicon size	Reference
msp1β	msp1β- F	20 bp	GCTCTAGCAGGT TATGCGTC	265 bp	Bilgic *et al*. 2013
	msp1β- R	19 bp	CTGCTTGGGAGATGCACCT		

Chain Reaction amplified 265 bp of msp1β gene in 63.6% (159/250) of the cattle screened from January 2022 to December 2022 (Fig. 2). Twelve PCR amplicons were randomly sequenced, showing nucleotide sequences specific to A. marginale, with a variation of 2–4 nucleotides among the isolates. The PCR primers used in this assay did not produce any amplicons when genomic DNA of T. annulata, B. bigemina, and T. evansi was used as the template. Singh *et al*. (2012) identified 73.1% carrier cattle for *A. marginale* from Punjab by nested-PCR based on msp5 gene. George *et al*. (2017) reported an occurrence of 16.4% of *A. marginale* by PCR in cattle of Seemandhra and Telangana region using msp4 gene. Various studies have reported a wide prevalence difference (5.2% to 87.9%) of bovine anaplasmosis across different regions of the country, using different marker genes in PCR assays (Kumar *et al*. 2019, Bisen *et al*. 2021, Bhanot and Jindal 2022).

The differences in the prevalence of A. marginale observed across various regions of India are likely due to factors such as effectiveness of tick control programmes, suitability of local climate for tick development, diversity of farm management techniques, animal husbandry practices in those regions and analytical sensitivity of the PCR assay (Zafar *et al*. 2022). In this study, msp1β-based PCR detected *A. marginale* infection in 63.6% of cattle, demonstrating high detection sensitivity of this PCR assay. The study also revealed that a significant proportion of cattle in this region of Central India are carriers of *A. marginale* infection. Chhattisgarh region, characterized by its sub-tropical climate with high temperature and humidity, provides an ideal habitat for ticks specifically *R. microplus* (Sarangi *et al*. 2021) thus accounting for high occurrence of *A. marginale* infection.

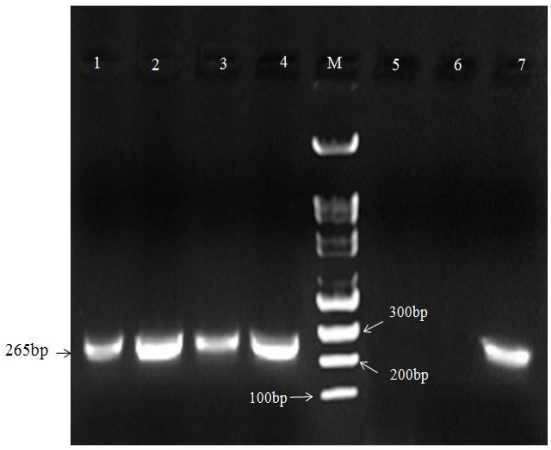

Fig. 2. *A. marginale* amplicons visualized by agarose gel electrophoresis [Lane M: 100bp DNA ladder; Lanes 1, 3, 4, 7: Samples showing *A. marginale* specific 265 bp product of msp1β gene; Lane 2: Positive control; Lane 5: Sample negative for *A. marginale*; Lane 6: Negative control

Prevalence under different cattle management systems: The management system adopted by the dairy owners influenced the occurrence of anaplasmosis in cattle. The prevalence of anaplasmosis was significantly higher ($p<0.05$) in animals from organized dairy farms compared to those from unorganized dairy units (Table 2). In the current study, it appears that high density of animals and suboptimal management in organized farms, likely increased the risk of infection spread through ticks and biting flies, compared to smaller, more dispersed operations. Kumar and Sangwan (2010) similarly reported significant variations in anaplasmosis prevalence across different cattle management systems. The differences could be attributed to farm management practices, including the use of acaricides for tick control, medication protocols, sanitation measures and other scientific methods that reduce the risk of iatrogenic transmission within the farm.

District-wise prevalence: The infection rate of A. marginale was significantly higher in cattle from Kabirdham district ($p<0.001$), while Dhamtari district reported the lowest prevalence (Table 2). As one of Chhattisgarh's leading districts in agriculture and dairy production, Dhamtari benefits from increased awareness among cattle owners and adoption of effective management practices for vector control, contributing to the reduced infection rate. In contrast, the high occurrence of anaplasmosis in Kabirdham can be attributed to limited awareness about scientific methods for tick control among cattle owners.

Breed-wise prevalence: PCR analysis detected A. marginale in 61.50% of crossbred cattle and 67.4% of indigenous cattle (Table 3), with no significant difference between the two groups ($p>0.05$). Similarly, Sarangi *et*

Table 2. Association of *A. marginale* prevalence and farm management systems

District	Samples screened			Samples Positive			Percent Positive (%)		
	UDU	OF	Total	UDU	OF	Total	UDU	OF	Average Prevalence (%)
Durg	25	25	50	16	14	30	64	56	60
Rajnandgaon	25	25	50	14	18	32	56	72	64
Kabirdham	25	25	50	20	22	42	80	88	84
Balod	25	25	50	11	21	32	44	84	64
Dhamtari	25	25	50	10	13	23	40	52	46
Total	125	125	250	71	88	159	56.8	70.4	63.6
χ^2 value	Between farm management systems and prevalence								4.99*
χ^2 value	Between districts and prevalence								28.15***

*Significant at 5% level ($p<0.05$), ***Significant at 0.1% level ($p<0.001$), UDU: Un-organized dairy units, OF: Organized farms

Table 3. Association of *A. marginale* prevalence and epidemiological parameters

		A. marginale positive	*A. marginale* negative	Degree of freedom	χ^2 value
Breed	Indigenous	60 (67.4)	29	1	0.87
	Cross-bred	99 (61.5)	62		
Health status	Healthy	122 (60.1)	81	1	5.72*
	Pyrexia	37 (78.7)	10		
Age	<1 year	31 (77.5)	09	2	6.17*
	1-5 years	71 (65.7)	37		
	>5 years	57 (55.9)	45		
Season	Summer	78 (75.7)	25	2	12.91***
	Rainy	55 (59.1)	38		
	Winter	26 (48.2)	28		

*Significant at 5% level (p<0.05), ***Significant at 0.5% level (p<0.005), (Figures in parentheses indicate percentages to the respective total)

al. (2021) reported anaplasmosis prevalence of 59.30% in indigenous cattle and 57.16% in crossbred cattle across different states in India. The present study suggests that A. marginale is prevalent in cattle of Chhattisgarh, regardless of breed, although indigenous cattle are often observed to serve as carriers of haemoprotozoan and haemorickettsial infections (Ringo *et al*. 2022).

Prevalence based on the clinical status of cattle: In the present study, prevalence of *A. marginale* infection was significantly (p<0.05) higher in cattle with history of fever as compared to apparently healthy ones (Table 3). The result is evidenced by the fact that pyrexia is usually the first recorded sign of clinical anaplasmosis (Kocan *et al*. 2010). Similar findings by Jaswal *et al*. (2014) revealed significant difference in the prevalence of anaplasmosis in animals with the history of fever and those without clinical condition. The detection of A. marginale in apparently healthy cattle suggests that such animals harbor subclinical infections, acting as silent carriers and potential sources of infection.

Season-wise and Age-wise prevalence: In this study, the prevalence of A. marginale infection was found to be significantly higher (p<0.005) during the summer and rainy seasons compared to winter, likely due to the greater abundance of ticks and blood-sucking flies during these months of the year (Table 3). A significant (p<0.05) association between age of the animal and prevalence of anaplasmosis was revealed by chi-square analysis. A distinctive aspect of A. marginale infection is that calves show more immunity compared to adult and older cattle (Jonsson *et al*. 2012). On the contrary, in the current study, the prevalence rate was higher in cattle under 1 year (77.5%), followed by those between 1-5 years (65.7%) and more than 5 years of age (55.9%). Thus, the results suggest that there is no inverse age resistance in calves to *A. marginale* and is in agreement with the reports by Sharma *et al*. (2013). This further indicates that anaplasmosis is endemic in Chhattisgarh region wherein animals are typically exposed to the infection early in life, developing immunity that allows the disease to persist in the population with minimal tick infestation and few clinical cases (Oliveira *et al*. 2003).

In conclusion, bovine anaplasmosis appears to have a significant impact on animal health and productivity in regions like Chhattisgarh, where the combination of climatic conditions and traditional management practices fosters an environment that facilitates the spread of the disease. High-precision PCR tools and serological methods need to be standardized and validated for better diagnosis and control of bovine anaplasmosis.

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