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Anaplasma marginale: Detection, Characterization and Phylogenetic Analysis of Major Surface Protein 5 from Buffalo Isolates in South Coastal Region of Andhra Pradesh, India

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A B S T R A C T

The intracellular pathogen *Anaplasma marginale* is one of the main causing agents of bovine anaplasmosis, a mild to severe haemolytic illness that causes significant economic damage in both dairy and meat sectors. Although there have been several reports of anaplasmosis outbreaks in Andhra Pradesh, the molecular studies on *A. marginale* in the state are minimal. Therefore, we aim to detect and characterize the rickettsia organism. From 2022- 2023, we collected a total of 103 blood samples from buffalos showing clinical signs of bovine anaplasmosis across four districts in south coastal region of Andhra Pradesh, India. Upon blood smear examination bovine anaplasmosis was detected in 15.53% of cases, while the msp5 gene-based polymerase chain reaction assay detected *A. marginale* in 32.0% of cases. The coding region of msp5 gene from four field isolates was sequenced. The phylogenetic analysis revealed that, three of the present study isolates were in one clade and clustered with isolates from Kerala, Uttar Pradesh and Thailand while one isolate clustered separately with isolates from Tamil Nadu, Tirupati and Sohag. The PCR assay was shown to be more sensitive than standard diagnostic techniques. Therefore, it might be employed as a tool in large-scale epidemiological surveys to detect animals in the carrier and subclinical states. The current data may also be useful in vaccine development studies.

Introduction

Bovine anaplasmosis is a tick-borne intraerythrocytic rickettsial disease and it is considered as one of the top 10 economically important livestock diseases affecting ruminants in India according to the PD-ADMAS annual report, 2011-12. The *Anaplasma marginale* is an obligatory intracellular gram-negative bacterium that belongs to the *Rickettsiales* order under the *Anaplasmatacea* family (Zafar *et al.*, 2022). It exhibits diverse modes of transmission, including transmission through tick bites and other blood-feeding arthropods. Additionally, the bacterium can be transmitted through the use of contaminated equipment for procedures like ear tagging, tattooing and needle injection. The prevalence of bovine anaplasmosis is notably high in tropical and subtropical regions, where environmental conditions create a favourable habitat for blood-sucking

vectors (Kocan *et al.,* 2010 and Arnuphapprasert *et al.,* 2023). Clinical signs include anaemia, icterus without haemoglobinemia and haemoglobinuria as a result from the massive phagocytosis of infected erythrocytes by the bovine reticuloendothelial system (Aubry and Geale, 2011). Other symptoms include dullness, emaciation, fever, lethargy, pale mucous membranes, dyspnoea, cough, hyperexcitability, reduced milk supplyand mortality in animals over two years old (Kundave *et al.,* 2018). The laboratory diagnosis of haemoparasites in buffaloes primarily relies on microscopic examination of peripheral blood smears.

The accuracy of this diagnostic approach is influenced by several factors, such as the extent of parasitemia (presence of parasites in the blood), the quality of the blood smear, technical expertise and the specific staining method applied (Radostits *et al.,* 1994). Hence, to diagnose the infected carrier animals PCR assay may be used.

Bovine anaplasmosis has significant impact on farmers such as economic losses, decreased productivity and increased veterinary costs. To mitigate the impact of illness, we need to implement comprehensive herd health management practices including vaccination strategies, tick control measures, regular veterinary care and proper diagnosis of the disease. So, present study was taken up for detection, characterization and phylogenetic analysis of *A. marginale* from buffaloes in south coastal region of Andhra Pradesh, India.

Materials and Methods

Ethical approval

The investigation necessitated the collection of blood samples from buffaloes showing symptoms of bovine anaplasmosis. Prior to sample collection, informed consent was obtained from responsible authorities. A formal permission was sought and obtained from NTRCVSc, Gannavaram to conduct this research, ensuring that it aligns with established ethical standards and guidelines.

Study area

The study was carried out in four of the south coastal region districts (Prakasam, Bapatla, Krishna, West Godavari) of Andhra Pradesh from November, 2022 to December, 2023 (Fig. 1).

Sample collection and blood smear examination

During the study, about 3 ml of blood and thin blood smears were collected in aseptic conditions using labelled sterile EDTA vacutainers and sterile glass slides respectively from each of the 103 buffaloes with clinical signs of anaemia, icterus, pale mucous membranes (Fig. 2: A-D), dullness, emaciation and fever. The blood smears stained with Leishman's stain as per standard protocol (Benjamin, 1986) and they were examined under oil immersion objective microscope for the detection of infection. The blood samples were stored at 4°C for further examination.

Molecular detection

DNA extraction from blood samples

The genomic DNA was extracted from each blood sample using spin column-based DNA extraction Kit (HiPurA® Blood Genomic DNA Miniprep Purification kit) as per the manufacturer's guidelines. The concentration and purity of the extracted DNA was measured using Nanodrop Spectrophotometer 200C (Thermo Scientific, USA) and subsequently stored at -80°C until further use.

Amplification of major surface protein 5 gene (msp5)

The DNA samples were tested for detection of *A. marginale* using species specific oligonucleotide primers of msp5 gene (Table 1). The PCR reaction mixture, comprising 12.5 µl of GoTaq® Green Master Mix (2X), 1.25 µl each of forward and reverse primers (20pmol/µl) and 150 ng of DNA was prepared with a final volume of 25 µl using nuclease-free water.

The components of the GoTaq® Green Master Mix included GoTaq® DNA Polymerase, 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM each of dATP, dGTP, dCTP, dTTP and 3 mM MgCl2 (Promega, USA). The PCR assay was conducted in Proflex PCR systems (Applied Biosystems) following the standardized cycling conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1min, 65 °C for 2min, and 72 °C for 1min, with a final extension step at 72 °C for 10 min. For each PCR reaction, a negative control was run together. The PCR amplified products were analyzed by gel electrophoresis in a 1.5% agarose gel and

observed under UV illumination using the BIO-RAD gel documentation system.

Nucleotide sequencing and Phylogenetic analysis

The PCR products of four blood samples positive for msp5 gene were sequenced at Barcode Biosciences Private Limited, Bangalore, Karnataka, India. The obtained sequences were edited for errors using Codon Code aligner, Version 10.0, (Sequence Assembly and Alignment software). Homology searches were conducted using the NCBI program BLAST. The phylogenetic analysis was performed in MEGA 11 utilizing ClustalW and a maximum likelihood tree was constructed. The significance of deduced phylogenetic tree was verified by bootstrap analysis of 1000 replicates (Tamura *et al.,* 2021). The nucleotide sequences of the four isolates have been deposited in GenBank.

Results and Discussion

Microscopic examination of blood smears stained with Leishman stain

The microscopic examination of Leishman-stained blood smears under oil immersion revealed presence of *Anaplasma marginale* organism in 15.53% buffaloes (16/103) as dense, homogenously stained blue-purple inclusions located towards the margin of the infected erythrocyte (Fig.3 A-C).

Detection of *A. marginale* by PCR

All the DNA samples were tested by msp5 gene primer pair. Out of 103 samples 33 samples (32.0%) were positive for *A. marginale* and produced the amplification product of 457bp (Fig. 4).

Nucleotide sequencing and Phylogenetic analysis

The partial coding region of msp5 gene from four isolates, namely APM1-WG, APM2-KSN, APM3-BPL and APM4-PKM was sequenced to determine the source and level of genetic affinity among *A. marginale* strains found in various nations and those originating in India and submitted to the GenBank with the accession numbers OR800334, PP236017, PP236018 and PP216656 respectively. The blast analysis showed 100% similarity with isolates from Thailand, Kerala, Sri Lanka, Uttar Pradesh and Sohag.

A Phylogenetic tree (Fig. 5) was constructed, which revealed that present study isolates from West Godavari, Krishna and Bapatla were in one clade and clustered with isolates from Uttar Pradesh, Kerala and Thailand. The isolate from Prakasam region was in one clade and clustered with isolates from Tirupati, Sohag, Tamil Nadu and Sri Lanka.

The development of livestock industry has been hampered by the high occurrence of tick-borne diseases. In India bovine anaplasmosis is considered as one of the most economically significant livestock diseases affecting ruminants.

The economic loss combined due to babesiosis and anaplasmosis in India has been estimated to be $ 57 million (Nair *et al.,* 2013). In the current investigation, blood samples were collected from a total of 103 buffaloes which showed clinical signs of anaemia, icterus, pale mucous membranes, dullness, emaciation, fever and lethargy. These clinical findings we observed were in accordance with Das *et al.,* (2022) and Kumar *et al.,* (2023).

In the current study, bovine anaplasmosis was detected through Leishman's-stained thin blood smear examination in 16 buffaloes (16/103, 15.53%) and the findings were in concurrence with Wahba *et al.,* (2017); Ntesang *et al.,* (2022) and Jalali *et al.,* (2023) who recorded the occurrence as 14.89, 15.83, and 15.53 percent respectively. The organisms appeared as dense, homogenously stained bluish-purple inclusions located towards the margin of the infected erythrocyte in a stained blood smear under oil immersion in the present study and the findings were in accordance with Namratha and Ramesh (2020); Bisen *et al.,* (2021) and Coetzee (2022).

In accordance with Nair *et al.,* (2013) and da Silva *et al.,* (2014) amplification of msp5 gene (457bp) was used for the detection of *A. marginale* by PCR assay. A total of 103 DNA samples were analyzed by PCR assay, out of which 33 samples were found positive for *A. marginale* contributing an occurrence of 32.00% (33/103).

The present findings were in line with Ntesang *et al.,* (2022) and Jalali *et al.,* (2023) who detected *A. marginale* infection in 32.48 (127/391) and 31.10 (32/103) percent respectively. The PCR assay demonstrated higher sensitivity in comparison to the microscopic examination of blood smears.

Table.1 Primers used for detection of *Anaplasma marginale*

Organism	Target gene	Primer sequence	Amplicon size	Reference
A.marginale	msp5	**F:** 5′- GCATAGCCTCCGCGTCTT TC-3′ **R:**5′-TCCTCGCCTTGGCCCTCAGA-3′	457 bp	Nair *et al.*, 2013

Figure.1 Map of Andhra Pradesh showing sample collection areas

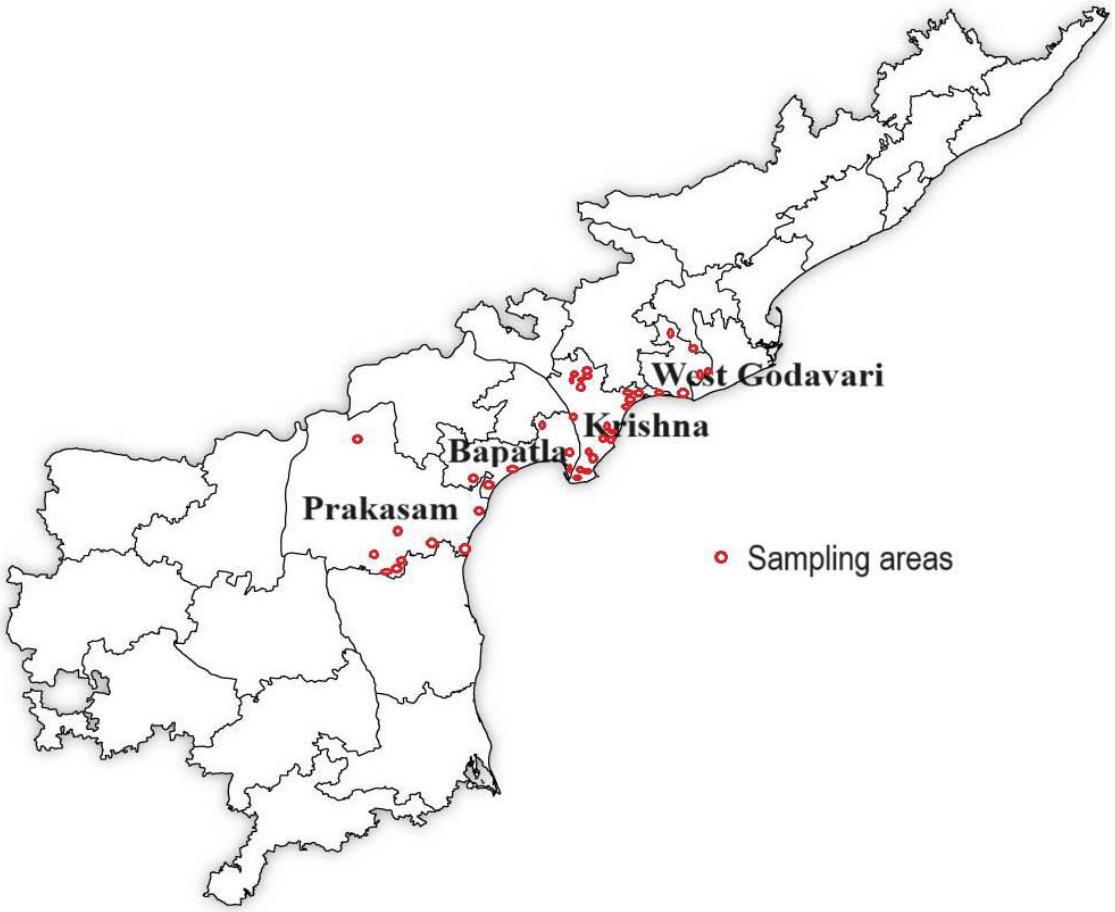
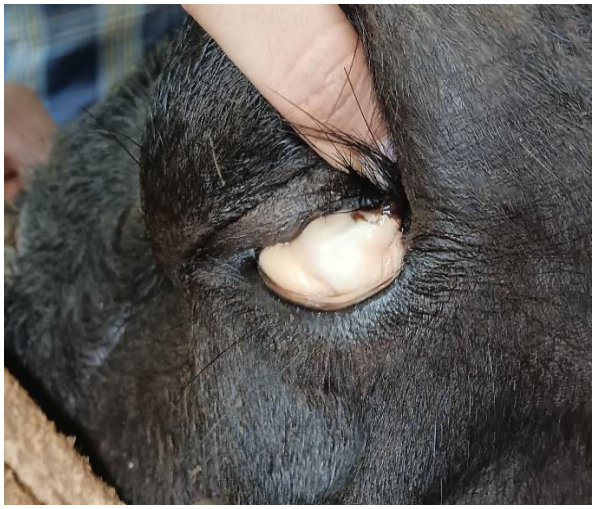
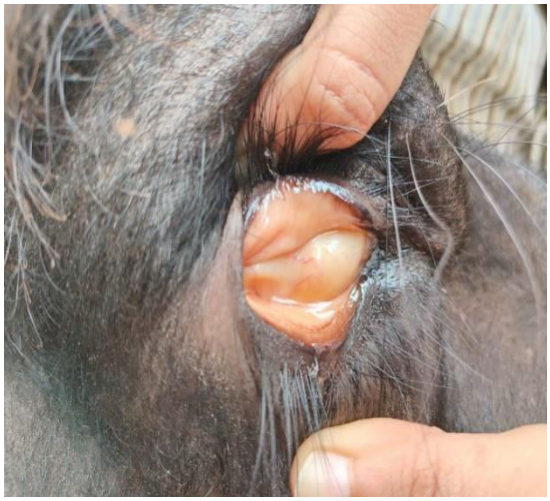


Figure.2 (A-D): Clinical signs in buffaloes affected with anaplasmosis.

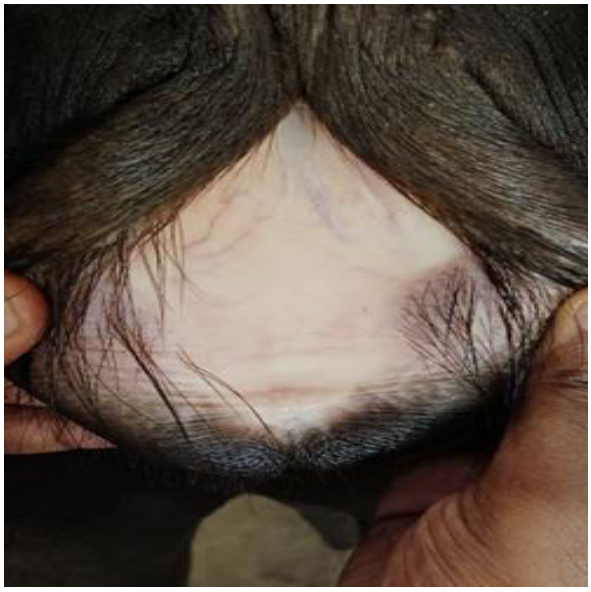
A: Pale conjunctival mucous membrane



B: Icteric Conjunctival mucous membrane



C: Pale vaginal mucous membrane



D: Icteric vaginal mucous membrane

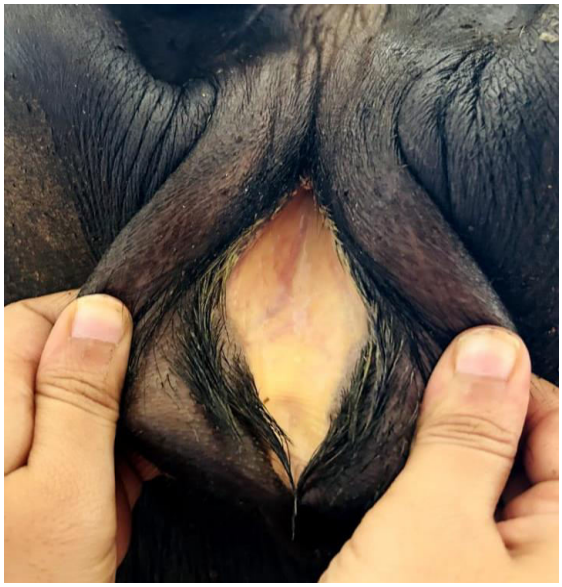
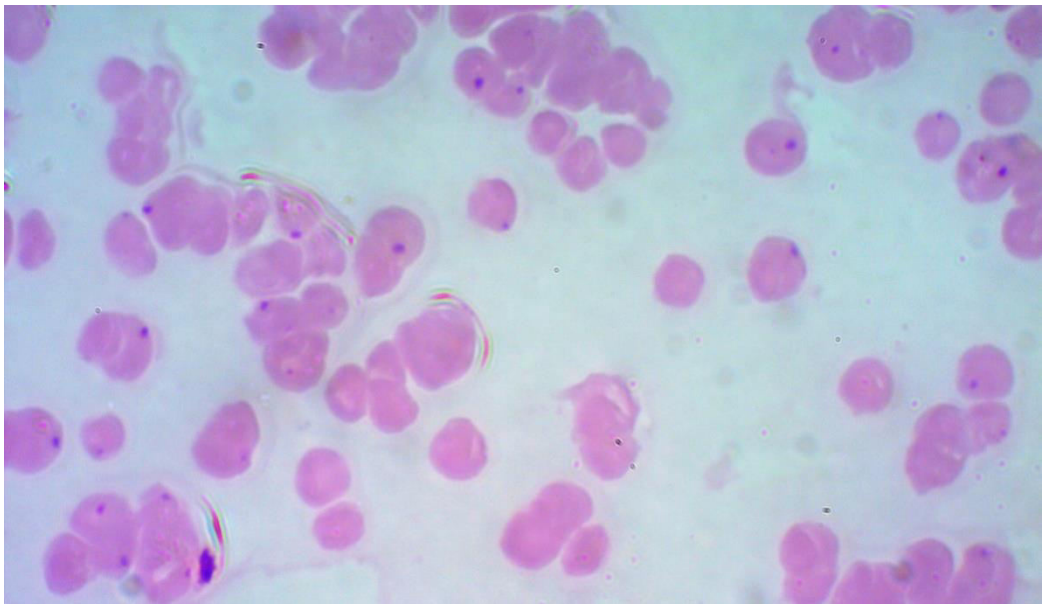
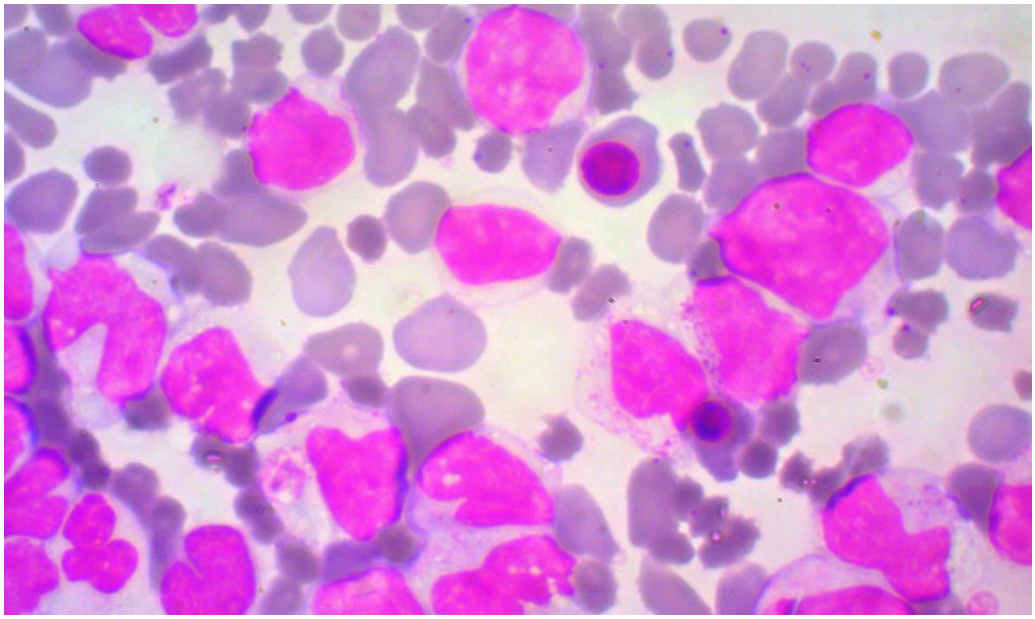


Figure.3 (A-C): Microscopic findings in a Leishman's-stained blood smear of *A. marginale* infected buffaloes

A) *A. marginale* infected erythrocytes observed under oil immersion microscope (100X)



B) Regenerative anaemia in anaplasmosis infected buffalo exhibiting nucleated RBC's and anisocytosis



C) Blood smear of a buffalo noticed with inclusion bodies in the cytoplasm of monocyte

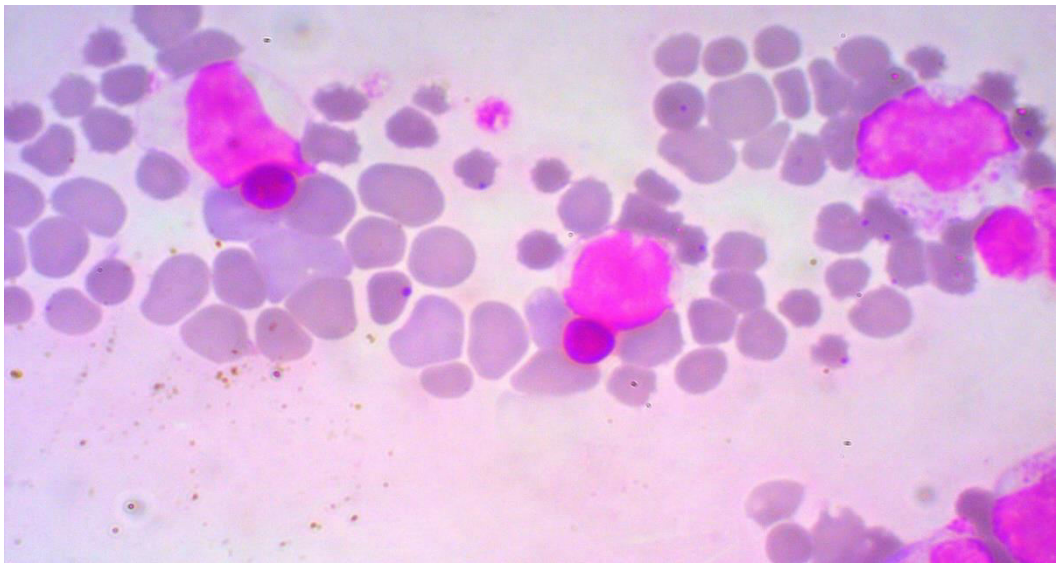
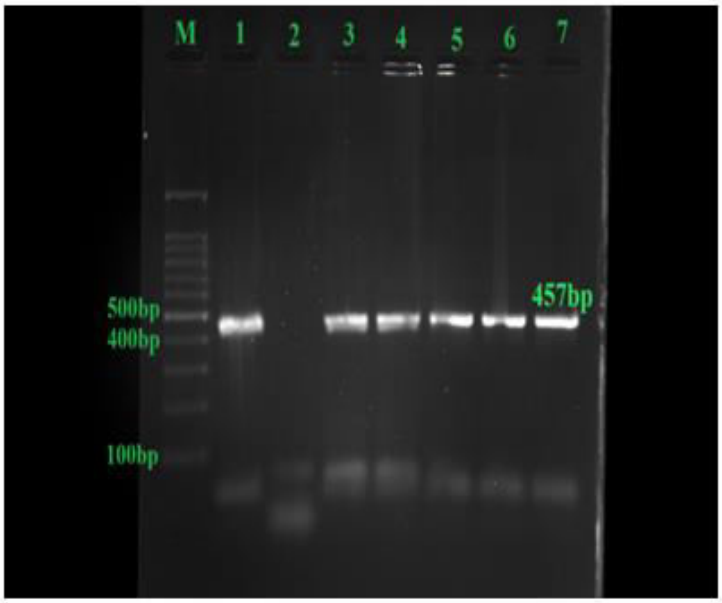


Figure.4 Amplification of msp5 gene (457bp)



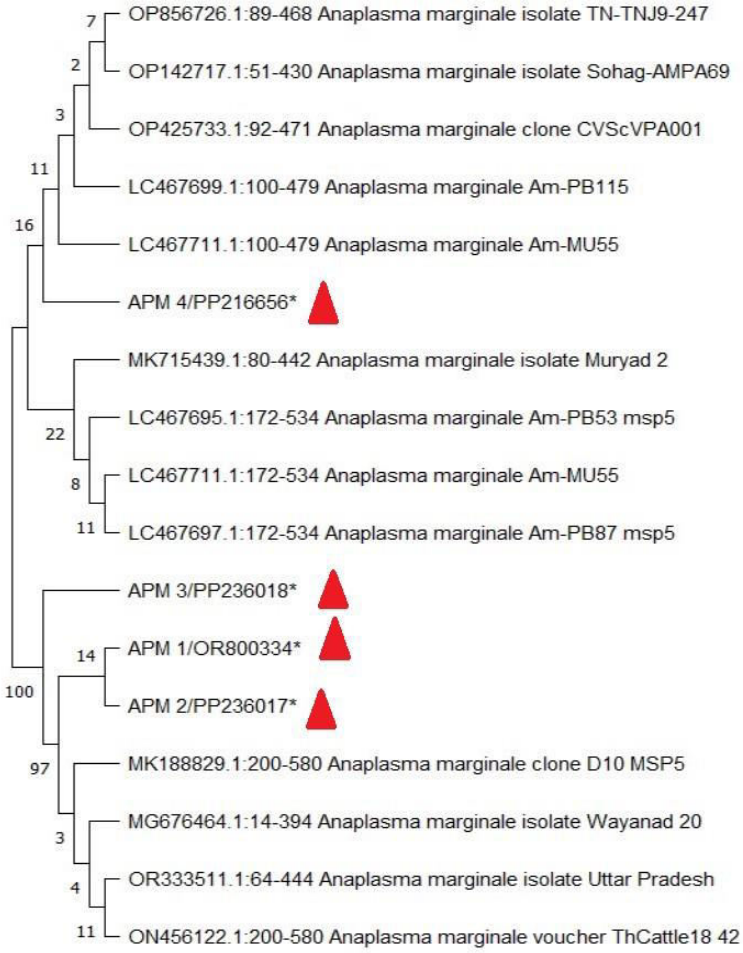
Lane M: DNA ladder (100bp)

Lane 1: Positive control (457bp)

Lane 2: Negative control

Lane 3-7: Positive samples of *A. marginale*

Figure.5 Phylogenetic tree based on major surface protein 5 gene sequences by Maximum Likelihood method.



(**Note**: The sequences obtained through this investigation were identified by (▲) labels. Other *A. marginale* sequences used in this analysis were obtained from GenBank.)

The PCR was proved to be more advantageous owing to its high sensitivity and efficacy in detecting both active infections and in cases of low parasitaemia. Similar findings were reported by Ganguly *et al.,* (2018); Subramanian *et al.,* (2019) and Das *et al.,* (2022).

The phylogenetic tree revealed that current study south coastal Andhra region isolates were in cluster with isolates from other states of India, Thailand and Sri Lanka. This indicated that *A. marginale* strains circulating in the present study region were mostly came from other surrounding states of Andhra.

This was in agreement with Kumar *et al.,* (2020). The major surface protein 5 gene, which is a conserved gene was shown to be more useful for phylogenetic analysis among various geographical isolates of *A. marginale* strains. The same also suggested by Nguyen *et al.,* (2020). The current study confirmed the presence of *A. marginale* infection in south coastal region of Andhra Pradesh, India. The PCR assay is more reliable, sensitive and specific diagnostic tool than traditional techniques.

The nucleotide sequencing of field isolates will be helpful in selection of vaccine candidates. However, in this study sequencing was done based on limited information and the whole genome sequence analysis should be undertaken for better understanding of genetic variation and molecular epidemiology of *A. marginale* strains.

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Author Contribution

G. Saikumar: Investigation, formal analysis, writing—original draft. N. Likitha: Validation, methodology, writing—reviewing. G. Sharmila:—Formal analysis, writing—review and editing. V. Navya Surekha: Investigation, writing—reviewing. Y. V. S. Rajesh: Resources, investigation writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Conflict of Interest: The authors declare no competing interests.

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