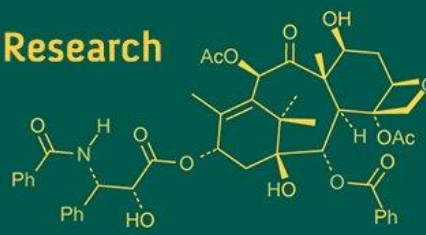
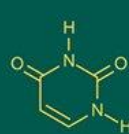


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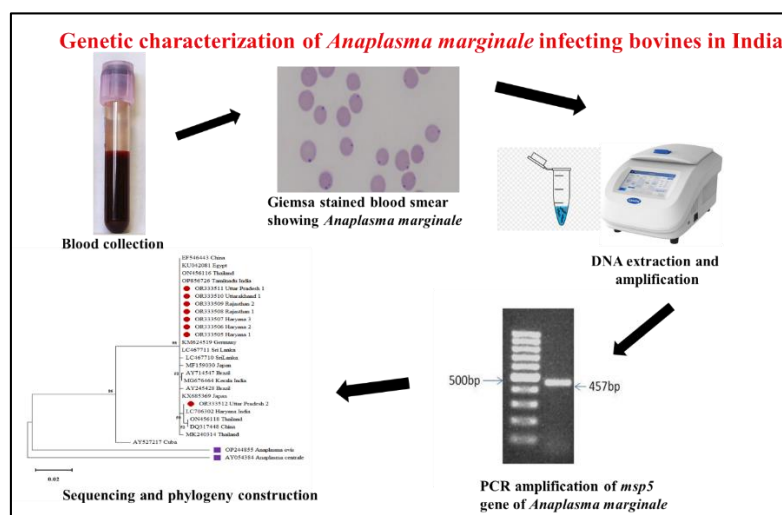
Genetic characterization of *Anaplasma marginale* infecting bovines in India

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Abstract

Genetic characterization of *Anaplasma marginale* infecting bovines in India was attempted targeting the *msp5* gene of the parasite. Blood samples of bovines (n = 721), suspected for haemoprotozoan infections, from 4 different states viz. Uttar Pradesh, Uttarakhand, Haryana and Rajasthan of the country were microscopically examined for *A. marginale* infection. Microscopy recorded 8.46% positivity of the infection. Out of 61 *Anaplasma* positive blood samples, a total of 8 isolates representing 4 different states (Uttar Pradesh, Uttarakhand Haryana and Rajasthan) were utilized for *msp5* gene based genetic characterization. Selected isolates were PCR amplified (457bp), gel purified and then amplicon of each isolate was submitted for sequencing. The sequence data received with respect to the newly generated isolates were analysed, edited and submitted in the GenBank database to obtain the accession numbers (OR333503 to OR333512). BLAST search analysis revealed very high level of sequence similarity of these isolates with published sequences of *A. marginale* originating from different countries such as China, Egypt, India and Thailand. In the phylogenetic tree constructed using MEGA11 software, newly generated isolates (OR333503 to OR333512) showed clustering (high bootstrap support) with isolates originating from China, Egypt, Germany, India, Thailand and Sri Lanka; suggesting negligible genetic variability across the country.



Graphical abstract

Keywords: *Anaplasma marginale*, bovines, genetic characterization, *msp5* gene, India

1. Introduction

Bovine anaplasmosis also known as gall sickness, is a tick born rickettsial disease caused by an obligate intra-erythrocytic pathogen belonging to the genus *Anaplasma*, family Anaplasmataceae, order Rickettsiales (Aktas and Ozubek, 2017) [1]. The genus comprises seven recognized species namely *A. centrale*, *A. marginale*, *A. bovis*, *A. caudatum*, *A. ovis*, *A. platys* and *A. phagocytophilum* (Hove et al., 2018) [8]. Among these, *A. marginale* is the

most pathogenic and principle aetiological agent for bovine anaplasmosis worldwide, causing substantial economic losses to livestock industry in temperate, tropical and sub-tropical regions (Kocan *et al.*, 2003) ^[12]. In India, anaplasmosis is ranked among the top 10 rickettsial diseases of ruminants (PDADMAS, 2005) ^[20]. The disease primarily affects adult cattle and characterised by progressive haemolytic anaemia. The organism appears as small, spherical, red stained bodies within the erythrocytes (at the periphery of cell) on Giemsa-stained blood smears (Soulsby, 1982) ^[23].

Transmission of *A. marginale* occurs biologically through several tick genera involving *Rhipicephalus*, *Hyalomma*, *Dermacentor*, *Ixodes* and *Argas*, with *Rhipicephalus* (*Boophilus*) *microplus* recognized as principle vector in India (Kolte *et al.*, 2017) ^[13]. Mechanical transmission may also occur via biting flies, mosquitoes and contaminated instruments such as needles, syringes, dehorning and castration equipment's (Marchette and Stiller, 2018) ^[17]. Trans-placental transmission has also been described in cattle (Kocan *et al.*, 2015) ^[11]. Amaro-Estrada *et al.* (2020) ^[3] demonstrated transovarial transmission of *A. marginale* by identifying the pathogen in unfed larvae hatched from fully engorged *R. microplus*, using PCR targeting both *msp5* and *msp1a* genes. The clinical signs of *A. marginale* infected animals are fever, severe anaemia, jaundice, pale mucous membranes, depression, weight loss, dyspnea, muscular tremors, abortion and, in severe cases, eventual death. Cattle are generally more susceptible than buffaloes, and outbreaks often results in significant mortality and reduced productivity (Minjauw and McLeod 2003; Kahn, 2005; Awad *et al.*, 2011) ^[18, 4]. Diagnosis of *A. marginale* infection in bovines can be done by different approaches. Clinical form of disease is routinely diagnosed by microscopic examination of Giemsa-stained peripheral blood smears. However, microscopic detection of parasite is more difficult in chronic infection. Molecular techniques such as polymerase chain reaction (PCR) provide higher sensitivity and specificity by detecting pathogen DNA and are especially useful for identifying persistently infected carrier animals (Ramos *et al.*, 2019; Aktas and Ozubek, 2021; Teja *et al.*, 2023) ^[22, 1, 24].

In general, severity of haemoparasitic infection is determined by parasite and host related several factors including the parasite strain or genotype, virulence, quantum of infection besides host susceptibility and age. Further, in order to assess the virulence of a parasitic strain, comparative genetic analysis is playing very important role. However, very limited information is available on genetic characterization of *A. marginale* from India. Keeping in view, the present study was undertaken to characterize the locally circulating *A. marginale* infecting bovine population in India targeting the *msp5* gene.

2. Materials and Methods

2.1 Blood sample collection and microscopic examination

Blood samples (approx. 3ml) were collected from a total of 721 bovines of four North Indian States (Uttar Pradesh-339, Uttarakhand-101, Haryana-131 and Rajasthan-150) and transported to the laboratory in cold chain. In the laboratory, thin blood smear was prepared from all the samples and microscopy was performed following staining with Giemsa stain. A total of 8 representative *Anaplasma* sp. positive

samples were utilized for genomic DNA isolation.

2.2 Isolation of genomic DNA

Out of 61 microscopically positive blood samples for *A. marginale*, a total of 8 isolates (UP-2, UK-1, HR-3 and RJ-2) were used for genomic DNA isolation using Qiagen Blood DNA isolation kit. The recovered DNA was tested for its concentration and purity using Nanodrop spectrophotometer.

2.3 PCR amplification of major surface protein 5 (*msp5*) gene

Targeted gene was PCR amplified using gene specific primer pair (*msp5*-For: GCATAGCCTCCGCGTCTTTC and *msp5*-Rev: TCCTCGCCTTGGCCCTCAGA). PCR reaction was performed in 25 µl total volume containing 30 ng of template DNA, 2.5 µl of 10× PCR Green buffer (Puregene), 0.5 µl of 10mM dNTP, 0.5 µl of each forward and reverse primer (20 pmol/µl), 0.2 µl Dream Taq DNA polymerase and nuclease free water to make the volume 25 µl. PCR amplification (primary) was performed using a S1000 thermal cycler Bio-Rad, USA) under optimized conditions as follows: initial denaturation at 94 °C for 5 min, followed by 35 amplification cycles (94 °C for 45s, 58 °C for 45s and 72 °C for 90s) and a final extension step at 72 °C for 10 min. Known *A. marginale* DNA was used as positive control, while nuclease free water (NFW) was used as negative control. The PCR products were electrophoresed in 1.5% agarose gel (Tris-borate-EDTA), stained with ethidium bromide and visualized under UV light. After confirming gene specific amplification in primary PCR, a bulk PCR reaction (25 µl volume in triplicate) was run for each one of the selected isolate. Amplified product was analysed in agarose gel electrophoresis as above and then the specific band was excised from the gel. Thereafter, PCR product was purified using the gel extraction kit (Qiagen) and concentration as well as purity of the each elute was analysed using the Nanodrop spectrophotometer. Finally, purified fragment of a total of 8 isolates was then submitted for custom DNA sequencing.

2.4 Sequence analysis

The raw data obtained with respect to different isolates of *msp5* gene was analysed and submitted to the DNA database of NCBI. Subsequently, accession numbers were obtained for all newly generated sequences. Newly generated sequences of *msp5* gene were subjected for homology search analysis using the BLAST programme of the NCBI database. Further, representative sequences were also downloaded from the NCBI database for its utilization in the phylogenetic analysis.

2.5 Phylogenetic analysis

A total of 8 newly generated gene sequences of *msp5* gene (457 bp) were used to establish the phylogenetic tree of *A. marginale* circulating in North India. A total of 19 published sequences were also retrieved from nucleotide database using the nucleotide BLAST program of the NCBI. In order to align all the sequences, most sequences were truncated at both ends using the MegAlign program of DNASTAR software. Lastly, phylogenetic tree was constructed using the Maximum Likelihood method using the Tamura-Nei parameter as best substitution model.

3. Results

3.1 Microscopic examination

Examination of Giemsa-stained blood smear recorded 8.46% overall positivity (61 samples detected positive) for *A. marginale* infection. Infection was recorded relatively high in Rajasthan than the other states included in the study. Level of parasitemia was mild to moderate indicating persistence of chronic infection in animals (Fig.1).

3.2 Genetic characterization *A. marginale* using *msp5* gene

Optimization of PCR assay revealed specific amplification of *msp5* gene of

A. marginale (Fig. 2). Raw sequence data obtained for all 8 isolates were analysed and submitted in GenBank database and subsequently accession numbers were obtained (OR333505 to OR333512). BLAST search analysis of these sequences recorded 100% sequence similarity with published isolates from different countries including Sri Lanka (LC467711) and India (LC706302). Sequence alignment study involving a total of 26 isolates (8 newly generated & 19 published) recorded very high level of sequence similarity amongst the isolates. The phylogenetic tree constructed based on the Maximum Likelihood method and Kimura-2 parameter model using the MEGA 11 software (Fig. 3) revealed clustering of all isolates in a single clade along with the majority of the published sequences (98 bootstrap value) included for establishing the phylogenetic tree (Table 1). Further, the sequences of *A. ovis* (OP244855) and *A. centrale* (AY054384) included in the study as out group member were placed at the bottom of the tree (Fig.3).

4. Discussion

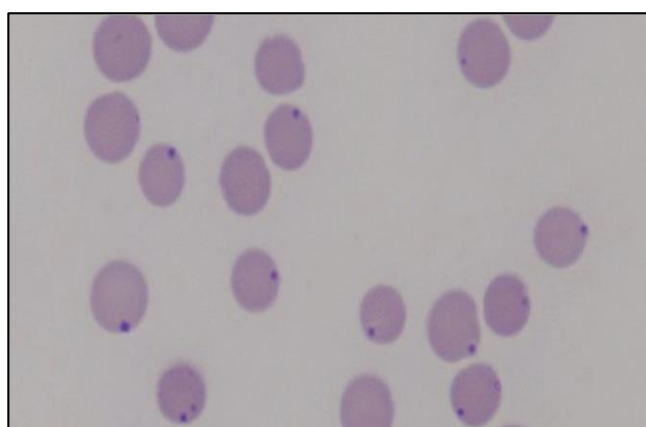
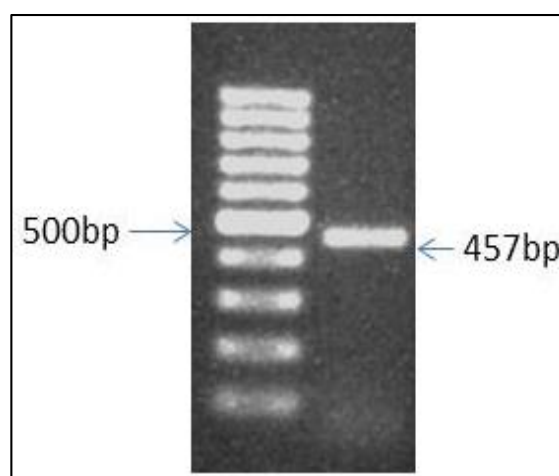
Tick borne haemoparasitic diseases are the major health concern in bovine population especially in tropical and sub-tropical part of the world including India. The most common tick borne diseases infecting bovines are; bovine tropical theileriosis, bovine babesiosis and anaplasmosis. Animals may be infected with single or mixed species infection depending on infestation by multiple species of ticks on the animal body. Bovine Anaplasmosis caused by *Anaplasma marginale* is transmitted by many genera of ticks including *Rhipicephalus*, *Hyalomma*, *Dermacentor* and *Ixodes*, besides the mechanical means through the bites of flies and mosquitoes (Marchette and Stiller, 2018) [17]. Disease can also be transmitted accidentally via syringes, needles, ear tagging, dehorning saws, castration, and other contaminated surgical equipment (Demessie and Derso, 2015) [6]. Bovine anaplasmosis has very serious impact on the health and production of animals in India. Further, variable prevalence rate of *A. marginale* infection has already been recorded from different parts of the country. The global and national prevalence of *Anaplasma* infection was estimated through a meta-analysis by Paramanandham *et al.* (2019) [19]. The study reported a higher prevalence of anaplasmosis in South America, whereas India showed a comparatively lower prevalence than the global average. In India, the overall prevalence was 11%, with the highest occurrence (61%) in the central zone and the lowest (6%) in the western and southern zones. The clinical signs produced in anaplasmosis are general in nature and that includes high fever, anaemia, icterus, inappetence, dyspnoea, tachycardia, lymphadenopathy, weakness, loss of body weight and

depression. Further, morbidity rate may be very high, if animals are already under stress condition (extreme weather, transportation, pregnancy, lactation). Clinical infection can be easily diagnosed by the microscopic examination methods, but it is very difficult if infection is in carrier stage (post recovery, chronic infection). Such carrier animals are playing major role in the maintenance of infection in a given geographical area and hence determination of real prevalence of individual parasitic agent is very much essential (Minjauw and McLeod 2003; Kahn, 2005) [18, 9].

In the present investigation, microscopic examination of the blood smear revealed 8.46% overall prevalence of *A. marginale*. Genetic characterization of *A. marginale* was performed to ascertain that whether single genotype of this organism is in circulation or other variants are also circulating in the country. For this purpose, one of the reported genetic marker *i.e.* major surface protein 5 (*msp5*) gene was targeted. A total of 8 new sequences for different isolates were generated and used for establishing the phylogenetic tree along with 19 retrieved sequences. In the study, sequence variability was recorded almost negligible within the newly generated sequences. This least sequence variability resulted in the clustering of most of the isolates included (newly generated and retrieved) in a single clade, excepting one isolate from Cuba (AY527217). In a study, Kumar *et al.* (2015) [15] generated 5 sequences from Mathura region and found complete nucleotide homology in the sequences. As, this gene encodes an immunodominant protein and present across the genus including *A. marginale*, *A. centrale*, *A. ovis* and *A. phagocytophilum* (de la Fuente *et al.*, 2005) [5]. Highly conserved nature of this gene (worldwide) made the choice for its use as an important diagnostic marker and developed cELISA for the recognition of anti-*msp5* antibodies in bovine using purified recombinant (rMSP5) fusion protein (Knowles *et al.*, 1996) [10]. Other workers have also performed phylogenetic analysis of *A. marginale* using different genetic markers from India (George *et al.*, 2017; Kumar *et al.*, 2019; Pradeep *et al.*, 2019) [7, 14, 21]. George *et al.* (2017) [7] performed a study to investigate presence of *A. marginale* infection in cattle from Telangana and Seemandhra states using *msp4* gene based PCR assay. Kumar *et al.* (2019) [14] assessed the presence of *A. marginale* in bovines and vector tick (*Rhipicephalus microplus*) by microscopy and *msp5* gene based PCR assay. Ticks were found present on the body of total 20 cattle (out of 39 *A. marginale* positive) and rickettsial infection was detected in 18 animals (90%) in infected animals. Pradeep *et al.* (2019) [21] revealed phylogenetic analysis of *msp4* gene of *Anaplasma* spp. in bovines in Kerala, India. In the study, all the field isolates of *A. marginale* from Kerala clustered in a single clade with others isolated from around the world. The phylogenetic analysis of *msp4* gene sequences confirmed the presence of *A. marginale* in the 10 isolates. Eight field isolates (Trissur-4,-5,-6,-7,-8, Wayanad-18,-20 and TVM-2) of Kerala clustered together along with isolates from around the world, including isolates from North America, South America and Asia, and revealed only minor levels of genetic diversity with intra-clade divergence of 0-0.9% within the *msp4* gene. Lira-Amaya *et al.* (2022) [16] analysed the nucleotide sequences of gene of *A. marginale* from Mexico and reported sequence identities ranging from 96 to 99% among the isolates from Mexican water buffaloes.

Table 1: Sequences of *msp* 5 gene used for phylogenetic analysis (* denote present study)

S. No.	Isolate name (origin place)	Host	Accession number	Accession showing maximum coverage & homology (%)
1	Haryana 1	Cattle	OR333505*	LC467711 (100% C, 100% H)
2	Haryana 2	Cattle	OR333506*	LC467711 (100% C, 100% H)
3	Haryana 3	Cattle	OR333507*	LC467711 (100% C, 100% H)
4	Rajasthan 1	Cattle	OR333508*	LC467711 (100% C, 100% H)
5	Rajasthan 2	Cattle	OR333509*	LC467711 (100% C, 100% H)
6	Uttarakhand 1	Cattle	OR333510*	LC467711 (100% C, 100% H)
7	Uttar Pradesh 1	Cattle	OR333511*	LC467711 (100% C, 100% H)
8	Uttar Pradesh 2	Cattle	OR333512*	LC706302 (100% C, 100% H)
9	Tamil Nadu, India	Cattle	OP856726	-
10	Haryana, India	Cattle	LC706302	-
11	Thailand	Cattle	ON456116	-
12	China	Yak	EF546443	-
13	Egypt	Cattle	KU042081	-
14	Sri Lanka	Buffalo	LC467705	-
15	Sri Lanka	Buffalo	LC467711	-
16	Japan	Cattle	KX685369	-
17	Germany	Cell line	KM624519	-
18	Sri Lanka	Buffalo	LC467710	-
19	Japan	Tick	MF159030	-
20	Brazil	-	AY714547	-
21	Kerala, India	Cattle	MG676464	-
22	China	Buffalo	DQ317448	-
23	Thailand	Cattle	ON456118	-
24	Thailand	Cattle	MK240314	-
25	Cuba	-	AY527217	-
26	Egypt	Goat	OP244855	<i>Anaplasma ovis</i> (as out group)
27	Israel	Vaccine strain	AY054384	<i>Anaplasma centrale</i> (as out group)

**Fig 1:** Giemsa stained blood smear showing *Anaplasma marginale* infection**Fig 2:** PCR amplification of *msp*5 gene of *Anaplasma marginale*

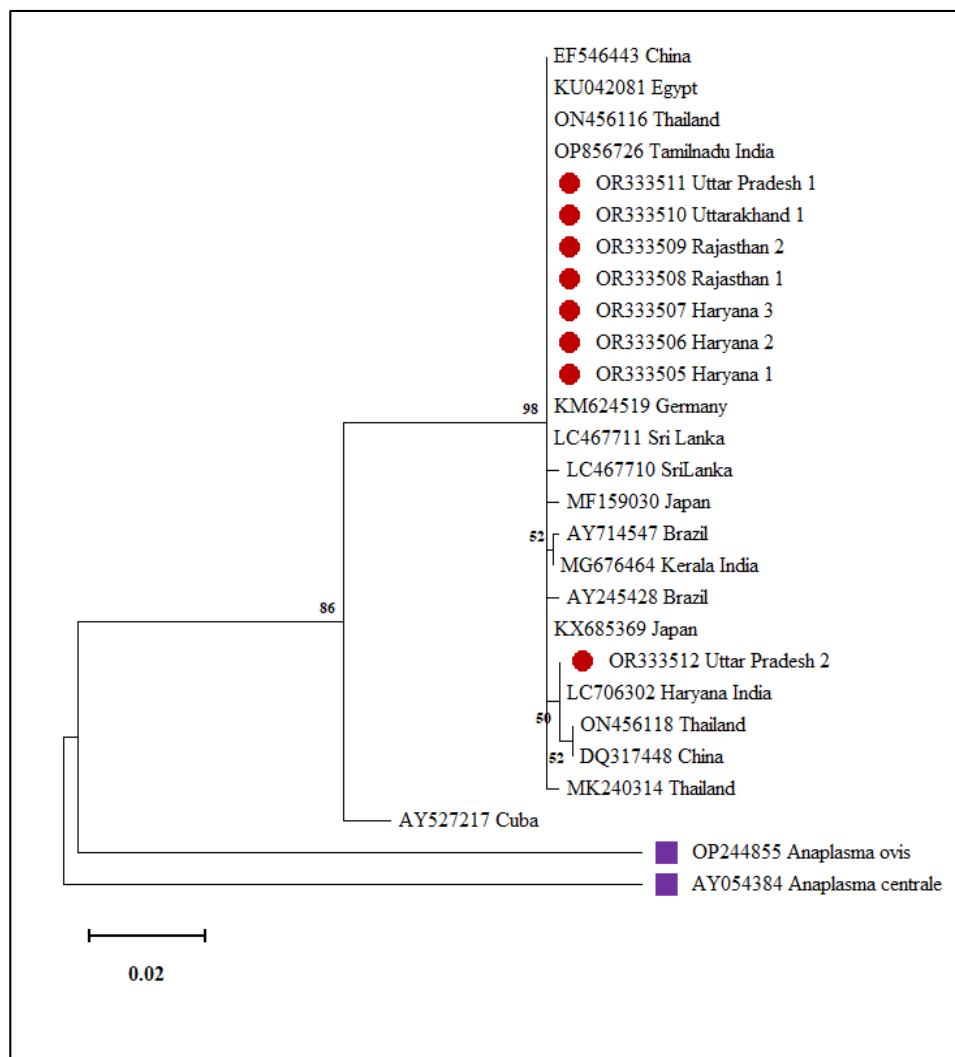


Fig 3: Phylogenetic tree based on *msp5* gene of *Anaplasma marginale*

5. Conclusion

The study recorded an overall prevalence of 8.46% of *A. marginale* infection based on blood smear examination, with a higher occurrence in Rajasthan. The mild to moderate parasitemia observed in the study suggests the presence of chronic carrier animals. PCR targeting the *msp5* gene confirmed the infection, and sequence analysis showed 100% similarity with previously reported isolates from India and Sri Lanka. Phylogenetic analysis demonstrated that all isolates clustered in a single group, indicating high genetic similarity. These results confirm the circulation of genetically conserved *A. marginale* strains and support the use of molecular methods for accurate detection and surveillance of bovine anaplasmosis.

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