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Prevalence of *Anaplasma marginale* in Water Buffaloes (*Bubalus bubalis*) in the State of Uttar Pradesh, North India

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ABSTRACT

Background: Bovine anaplasmosis caused by *Anaplasma marginale* is highly endemic in tropical and sub-tropical regions of the world where it accounts for huge economic losses to the livestock sector. Approximately 30% of water buffalo (*Bubalus bubalis*) herds in India are located in Uttar Pradesh (U.P). However in India, most of the research studies on *Anaplasma* are focused on cattle whereas studies on this pathogen in water buffaloes have been neglected. Therefore, present investigation was undertaken for determining the prevalence of *A. marginale* infection in water buffaloes in U.P, North India.

Methods: In the current investigation during 2017-2019, the prevalence of *A. marginale* infection in 228 water buffaloes in three districts of North-West U.P was studied by microscopy and by nested-PCR amplification of major surface protein 5 (msp5) gene.

Result: Microscopic examination of blood smears of 228 water buffaloes revealed 7.89% animals positive for *A. marginale*. Conventional PCR of full length major surface protein 5 (msp5) gene detected 29.39% and nested-PCR amplification of this gene detected 64.0% of these animals as positive for *A. marginale* infection. A high incidence of the infection detected in this study area suggests that water buffaloes may serve as reservoir hosts of *A. marginale* and play a vital role in the spread of infection to other susceptible animals. However further studies are required on larger buffalo populations for determining the epidemiological status of anaplasmosis in these animals in India.

Key words: *Anaplasma marginale*, Buffaloes, msp5 gene, PCR.

INTRODUCTION

Anaplasma marginale, a tick-borne obligate intraerythrocytic rickettsia is one of the most common causative agent of bovine anaplasmosis worldwide and is endemic in several regions of Asia, Africa, Australia, North and South America (Kocan *et al*. 2010). The effects of bovine anaplasmosis on the health and productivity of cattle have been known since long but buffaloes, bison, African antelopes, mule deer can become persistently infected with *A. marginale* (Kuttler, 1984). The clinical symptoms of anaplasmosis include haemolytic anaemia, weight loss, high fever, tachycardia, jaundice, decreased milk yield, gastrointestinal signs, abortion and sudden death (Kocan *et al*. 2003, 2004).

Transmission of *A. marginale* in cattle and buffaloes occurs biologically through ticks and mechanically by blood-feeding flies and blood contaminated fomites (Scoles *et al*. 2005). Transplacental transmission of *A. marginale* occurs in cattle, resulting in healthy but persistently infected calves (Kocan *et al*. 2015). Microscopic examination of stained blood smears is the conventional method for detecting *A. marginale* infection. However, molecular techniques and serological assays have proven to be more sensitive and specific for the detection of subclinical infection and carrier status of the animal for anaplasmosis (Bisen *et al*. 2021). Major surface proteins of *A. marginale* play a vital role in the interaction and invasion of the pathogen into the host cells. Six major surface proteins (MSPs) MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5 have been identified and studied in *A. marginale* as promising molecules in the diagnosis and vaccine development (Palmer *et al*. 1985; Kocan *et al*. 2003, 2004; de la Fuente *et al*. 2010). Major surface protein 5 is a highly conserved surface protein that has been proven effective as a diagnostic antigen (de Echaide *et al*. 1998). India is a developing agricultural country and is the largest buffalo milk producer in the world. Buffaloes make an important contribution to the agricultural economy. According to 20th livestock census (Department of Animal Husbandry and Dairying, Government of India, 2019); water buffalo population is 109.85 million which contributes to about 20.5% of the total livestock population of our country. Uttar Pradesh state has the largest water buffalo population, nearing 33 million (Department of Animal Husbandry and Dairying, Government of India, 2019). Like cattle, buffaloes are also susceptible to many tick-borne parasitic infections causing huge production and economic losses to the dairy farmers (Vidotto *et al*. 1998). Bovine

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anaplasmosis caused by *A. marginale* in cattle has been reported from several parts of India but there are meagre reports on its occurrence in water buffaloes in India. Therefore, present investigation on the prevalence of *A. marginale* infection in water buffaloes in U.P, North India by microscopy and by PCR was undertaken for determining the status of this infection in this part of the country.

MATERIALS AND METHODS

Blood samples of two hundred twenty eight water buffaloes were collected from Bareilly, Moradabad and Pilibhit districts of U.P from July 2017 to June 2019. Blood samples were collected from asymptomatic buffaloes and those with signs of fever, anaemia, tick infestation and reduced milk production for screening of *Anaplasma marginale* infection. The blood samples were collected from the ear vein and jugular vein of the animals. Thin blood smears were prepared by puncturing the ear vein using 18G needle, air dried and fixed in methanol for 2 min. The thin blood smears were stained with a working dilution of 10% Giemsa stain for 40 min. The smears were washed with tap water, air dried and examined under oil immersion lens (X 100) of microscope for demonstration of *A. marginale*. Whole blood (~5 ml) was drawn from the jugular vein into EDTA coated vacutainers (BD, USA) and stored at -20°C for isolation of genomic DNA. The present investigation was performed at the Division of Parasitology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P) from July 2017 to June 2019. The percentage rickettsia was determined by counting the infected RBCs in proportion to the total RBCs in at least 15-20 microscopic fields. Percentage of rickettsia infected RBCs was determined using the following formula.

$$\% \text{ rickettsia} = \frac{\text{Total no. of infected erythrocytes}}{\text{Total no. of erythrocytes counted}} \times 100$$

Molecular detection of *A. marginale* infection in water buffaloes

Genomic DNA was isolated from 100 µl of whole blood from each sample using DNAeasy® blood and tissue kit (Qiagen, Germany) in accordance with the manufacturer's protocol. Extracted DNA was eluted in 50 µl of DNA elution buffer and stored at -20°C till further use. The concentration of genomic DNA was quantified by Nanodrop spectrophotometer. Genomic DNA of *A. marginale* isolated from blood samples showing high parasitaemia by light microscopy was used as positive control in the PCR. Genomic DNA isolated from the whole blood of water buffaloes that were maintained in tick free conditions of the experimental sheds of ICAR-Indian Veterinary Research Institute, Izatnagar and were negative in the microscopic examination of several blood smears and in two rounds of nested-PCR were used as negative controls in PCR assays.

Blood samples from 228 water buffaloes belonging to the three districts of U.P were screened for *A. marginale* infection. The msp5 gene of *A. marginale* was used for the PCR amplification and for determining the prevalence of *A. marginale* infection in these animals. Primers were designed to amplify full length msp5 gene (633 bp) of *A. marginale* as described by Visser *et al*. (1992) (Table 1) and custom synthesized based on GenBank accession number M93392. Each PCR reaction was carried out in a standard 25 µl reaction volume using thermal cycler (S1000-BIO-RAD). The master mix used for the primary PCR reaction consisted of 2.5 µl of 10X PCR Green buffer (Thermo Scientific, USA), 0.5 µl of 10 mM dNTP mix (Thermo Scientific, USA), 0.25 µl of Dream Taq DNA polymerase (Thermo Scientific, USA), 1.0 µl each (10 pmol) of the forward (msp5- FOR) and reverse (msp5-REV) primers (Eurofins, India) and 2.0 µl of template DNA. The final volume was made up to 25 µl using nuclease free water. Initial denaturation of the genomic DNA at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 45 sec, primer elongation at 72°C for 1 min and final elongation at 72°C for 10 min was the protocol used for PCR amplification of the msp5 gene.

For enhanced sensitivity of the PCR assay, nested-PCR was also performed. Forward (msp5-N FOR-1) and reverse (msp5-N REV) primers were custom synthesized (Table 1) to amplify 457 bp fragment of msp5 gene. Subsequently, second round of nested-PCR was performed on this primary PCR product using internal forward (msp5-N FOR-2) and reverse (msp5-N REV) primers to amplify a product of 343 bp (de Echaide *et al*., 1998). The PCR reaction conditions were identical for carrying out conventional and nested-PCR. Primary PCR product (1:2 to 1:10 dilution) was used as template in nested-PCR. The PCR products were checked for amplification by electrophoresis on 1.5% agarose gel and visualized in gel documentation system (Syngene, UK). A statistically significant number of PCR amplicons (n=20) (both primary and nested-PCR products) were sequenced for confirmation of the *Anaplasma* species. Sensitivity of the nested-PCR was determined by tenfold dilution of the genomic DNA retrieved from the blood samples positive for

Table 1: Primers designed for conventional and nested-PCR for msp5 gene for identification of Anaplasma marginale infection in water buffaloes.

Gene	Primer name	Primer length	Primer sequence (5′ → 3′)	Amplicon size
msp5	msp5- FOR	23 bp	ATGAGAATTTTCAAGATTGTGTC	633 bp
	msp5- REV	23 bp	CTAAGAATTAAGCATGTGACCGC	
msp5-nested	msp5- N FOR-1	20 bp	GCATAGCCTCCGCGTCTTTC	457 bp
	msp5- NREV	20 bp	TCCTCGCCTTGGCCCTCAGA	
	msp5- NFOR-2	21 bp	TACACGTGCCCTACCGAGTTA	343 bp

A. marginale infection by microscopic examination. The specificity of the nested-PCR primers was also checked with genomic DNAs of *Babesia bigemina*, *Theileria annulata* and *Trypanosoma evansi.*

RESULTS AND DISCUSSION

The microscopic investigation of Giemsa stained peripheral blood smears of 228 water buffaloes detected 18 (7.89%) animals positive for the blue/purple coloured, rounded inclusion bodies of *A. marginale* located at the margins of the erythrocytes. These results are in concurrence with the findings in South-West Gujarat where a prevalence of 7.07% of *A. marginale* infection was observed in water buffaloes by routine microscopy of Giemsa stained blood smears (Maharana *et al.,* 2015). In the present investigation, 6.58% (15/228) of animals were found to be clinically infected and showed signs of pyrexia, progressive weakness, paleness of mucous membrane, tachycardia and tick infestation. The average level of parasitaemia in clinically positive animals was found as 1570.5± 876.8 × 10^6 infected RBCs per ml of blood which coincided with the observations of Gale *et al.* (1996). Rickettsiaemia level exceeding 10^9 infected erythrocytes per ml of blood is indicative of acute infection, generally characterized by clinical manifestations. Recovery from acute anaplasmosis results in persistent infection and in such cases the level of rickettsiaemia is generally below the threshold levels of microscopic detection (10^6 infected erythrocytes per ml of blood). Moreover, *A. marginale* organisms remain undetected during conventional microscopic investigation and microscopy is, therefore, not a reliable tool for detecting presymptomatic or carrier animals (Carelli *et al.,* 2007; Aubry and Geale, 2011).

The PCR of the full length msp5 gene resulted in an amplicon of 633 bp in 29.39% (67/228) of animals (Fig 1). However, nested-PCR was highly sensitive in the detection of rickettsia in buffaloes using primers designed by de Echaide *et al*. (1998). In the nested-PCR assay, *A. marginale* infection was revealed as the primary-PCR product of 457 bp in 35.5% (81/228) of buffaloes (Fig 2) while secondary-PCR generated amplicon of 343 bp in 64.0% (146/228) of these animals (Fig 3). The analytical sensitivity of the PCR assay for detection of genomic DNA of *A. marginale* using

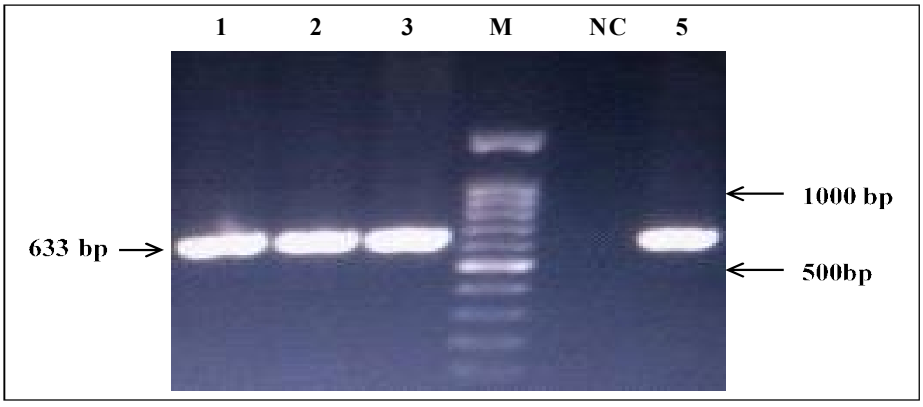

Fig 1: PCR amplification of full length msp5 gene of *A. marginale* in water buffaloes.
Lane M: 100 bp DNA ladder.
Lanes 1, 2, 3, 5: PCR positive samples (633 bp).
Lane NC: No DNA template control.

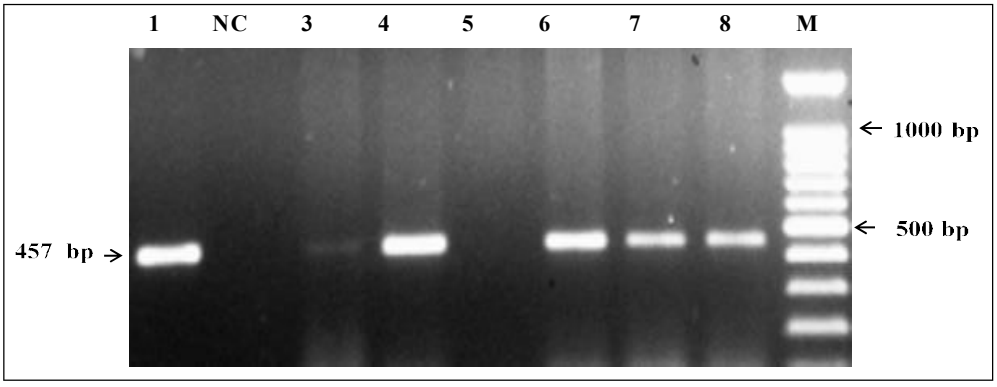

Fig 2: Nested-PCR (primary) amplification of *A. marginale* msp5 gene.
Lane M: 100 bp DNA ladder.
Lanes 1, 3, 4, 6, 7 and 8: msp5 PCR primary amplicon (457 bp).
Lane 5: PCR negative sample.
Lane NC: No DNA template control.

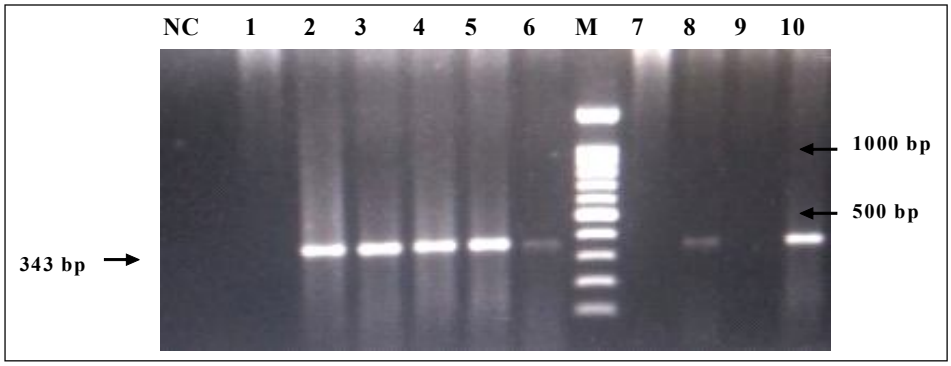

Fig 3: Nested-PCR (secondary) amplification of *A. marginale* msp5 gene.
Lane M: 100 bp DNA ladder.
Lanes 2, 3, 4, 5, 6, 8, 10: msp5 PCR secondary amplicon (343 bp).
Lane 1, 7, 9: PCR negative sample.
Lane NC: No DNA template control.

present set of primers was 200 picograms. A significant number of PCR amplicons (*n*=20) were randomly sequenced which revealed nucleotide sequence specific to *A. marginale*. 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. Based on the msp5 gene PCR assay, the prevalence of *A. marginale* was reported to be 57.5% in buffaloes in Sri Lanka (Zhtldyz *et al*., 2019). In Colombia, a prevalence of 13.1% was observed in buffaloes using semi-nested PCR targeting the msp5 gene of *A. marginale* (James-Duenez *et al*., 2018). Molecular detection of *A. marginale* infection in buffaloes by PCR based on msp4 gene observed a prevalence of 8.0% in North-East Thailand (Saetiew *et al*. 2015). A study on buffaloes in Pakistan by Ashraf *et al*. (2013) using 16S RNA gene reported a prevalence of *A. marginale* in 7.0% of animals. The nested-PCR based on msp5 gene has been reported as the most sensitive assay (de Echaide *et al*. 1998; Scoles *et al*. 2005; Singh *et al*., 2012; Mason *et al*., 2017; Bisen *et al*., 2021) for detecting *A. marginale, A. centrale* and *A. ovis*. Very few reports on the prevalence of *A. marginale* infection in water buffaloes are available from India. A three year old buffalo manifesting clinical signs of anaplasmosis and its complete recovery after treatment was reported by Vatsya *et al*. (2013). A prevalence of anaplasmosis in 18.18% (4/22) buffaloes was reported by msp1β gene based PCR (Sharma *et al*. 2013). Based on the amplification of 576 bp msp5 DNA, the detection rate of *A. marginale* was found to be 18.33% (11/60) in buffaloes in coastal South Gujarat (Kumar *et al*., 2019). A study on *A. marginale* using msp5 based nested-PCR observed a prevalence of 73.1% in carrier cattle of North-west India (Singh *et al*., 2012). Similar trends were observed in a study conducted in carrier cattle of North India which revealed a prevalence of 87.9% by nested-PCR based on msp5 gene of *A. marginale* (Bisen *et al*., 2021). In the present study, a large number of buffaloes found positive for anaplasmosis by microscopy and PCR were asymptomatic but only 6.58% of these buffaloes presented clinical signs of anaplasmosis. The clinical picture of *A. marginale* infection was revealed in only 1.90% of buffaloes in a study in coastal South Gujarat by Kumar *et al*. (2019). Buffaloes are reported to be less susceptible to *A. marginale* infection than cattle as they are naturally resistant to tick infestation and can reduce the intensity of infection by preventing the multiplication of the pathogen in the cells (Amira *et al*., 2020). Detection of persistently infected buffaloes and cattle is important to control the movement of infected animals into disease-free regions and also to formulate and implement strategic control measures against the spread of infection.

CONCLUSION

The present study carried out in water buffaloes in U.P, North India detected a large percentage of these animals as positive (64.0%) for *A. marginale* by nested-PCR. However, a large buffalo population needs to be screened for anaplasmosis in different geographical regions of the country for a complete epidemiological study of the infection.

Conflict of interests

The authors declare that they have no conflict of interest.

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