The Pharma Innovation Journal 2023; SP-12(7): 315-318

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(7): 315-318

www.thepharmajournal.com
Received: 16-04-2023
Accepted: 22-06-2023

T Arulkumar
Assistant Professor, Department of Clinics, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

KM Palanivel
Professor and Head, Department of Veterinary Preventive Medicine, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu

R Rishikesavan
Assistant Professor, Department of Veterinary Preventive Medicine, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

G Selvaraju
Professor and Head, Department of Veterinary Epidemiology and Public Health, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

S Sivaraman
Assistant Professor, Department of Clinics, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

Corresponding Author:
T Arulkumar
Assistant Professor, Department of Clinics, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

Molecular detection and characterization of A*naplasma marginale* in dairy animals by targeting different genes

T Arulkumar, KM Palanivel, R Rishikesavan, G Selvaraju and S Sivaraman

Abstract
This study describes the detection and characterization of *A. marginale* infection in dairy cows by targeting different genes. The PCR assay was done using two sets of primers targeting Amar 16S and MSP5 of *A. marginale*. 245 blood samples were collected from cows in and around Namakkal region, suspicious of *A. marginale* infection, and were subjected to direct blood smear examination to analyze the status of infection and meanwhile genomic DNA extracted from whole blood samples was subjected to PCR assay. Blood smear examination revealed presence of *A. marginale* in 18.7% of cases. The PCR analysis targeting Amar16S and MSP5 genes revealed presence of *A. marginale* (18.35%) and (20.8%) respectively, both targeting genes were present (16.5%). Gene sequencing and phylogenic tree study were performed. Compared to traditional diagnostic method, the PCR assay was more sensitive. So it could be used as a tool to detect subclinical and carrier state animals in Large-scale epidemiological survey for identification of *A marginale* infection in dairy cattle.

Keywords: PCR, *Anaplasma marginale*, genomic

Introduction
Anaplasmosis is caused by the Obligate intracellular organism *A. marginale*, which was first described by (Theiler, 1910) [10] as the causative agent of gall sickness in cattle. *A. marginale* infection is most economically important vector-borne diseases in India, losses directly attributed to mortality, morbidity, production loss, cost of treatment and diagnosis and vector control (Maharana *et al.*, 2016) [7].

The infection is characterized by dullness, emaciation, anemia, fever, lethargy, pale mucous membranes, icterus, decreased milk production and often death in animals older than two years (Kundave *et al* 2018) [6]. Laboratory diagnosis of haemoparasites in cattle is largely based on the microscopic examination of peripheral blood smears. It depends on various factors including the level of parasitaemia, quality of blood smear, technical expertise and the staining method used (Radostitis *et al*., 2000) [8]. Diagnosis of infected carrier animals is always difficult as the level of pathogen remains far below the detection limit of microscopy, under low parasitaemia (Hove *et al.*, 2018) [5]. Hence, carrier individuals play a critical role in disease epidemiology by serving reservoirs for naive vectors and introducing infection to healthy animals. Current serological assays such as the immunofluorescent antibody test (IFAT) and Latex agglutination test (LAT) used for the diagnosis of *A. marginale* suffer from the inherent. Limitation of antibody cross-reactivity among species and failure to differentiate between past and present infections. (Almeria *et al*., 2001) [1]. The effective management of hemoparasitic diseases. Requires rapid, reliable, and highly sensitive diagnostic tests, which can also serve to monitor the effectiveness of Therapeutic and prophylactic measures. Targeting gene Amar 16S (Kundave *et al*., 2018B) [6] and MSP 5 gene (Anita *et al*., 2020) [2] used for identification of target genomes in *A. marginale*. Nucleic acid-based. Detection of parasites in clinical samples can be considered as an evidence. Of active and ongoing infection. Timely and accurate diagnosis of the causative agent before implementation of appropriate treatment and related control measures is very important (Reetha *et al*. 2012) [9]. The present study was aimed at the simultaneous detection of different targeting genes of *A. marginale* infection.

Materials and Methods

Samples collection and blood smear screening

Sample collection: Blood samples and peripheral blood smear were collected from Dairy animals (3–6 years; 245) brought to Veterinary Clinical Complex, Veterinary College and Research Institute, Namakkal with history and clinical signs of dull and depression, fever, severe anemia, weight loss, jaundice, loss of appetite, muscular tremors, pale mucous membrane. (Fig 1 and Fig 2), labored breathing and loss of milk yield etc., The blood samples and blood smears from infected were labeled properly. Blood smears were fixed with absolute methanol, air-dried and stained with Giemsa stain (1:20) as per standard protocol. Stained smears were examined microscopically to assess the infection. EDTA Blood samples were stored. at -20 °C for molecular diagnosis and further examination.

Fig 1: Pale conjunctiva mucus membrane

Fig 2: Pale vaginal mucus membrane

DNA isolation from blood samples

Genomic DNA was extracted from 200µl of. each blood sample using commercial kit (DNeasy blood kit, Qiagen, Germany) according the manufacturer's protocol. The concentration and purity of isolated DNA was measured using spectrophotometer and quality was checked in agarose gel electrophoresis (1.5% gel). Aliquots of extracted DNA were labeled properly and stored at –20 °C until further use.

Designing of different gene primers for PCR

Two sets of primers targeting the Amar16S of *A. marginale* and MSP5 gene of *A. arginale* were designed (Table I) on the basis of sequence. Information in the public domain (Gen Bank) with the help of Primer. Select (DNASTAR-Laser gene) software. All the primers were. Checked. For their properties like Tm value, length, presence of self, and cross dimer using. Oligonucleotide analyzer software 1.0.2 and were also checked for their specificity in the BLAST tool of NCBI. Before being custom synthesized.

Table 1: List of primers used in PCR

S. No	Oligomer name and sequences (5'-3')	Amplicons Size
1	Amar16S-F: GGC GGT GAT CTG TAG CTG GTC TGA Amar16S-R: GCC CAA TAA TTC CGA ACA ACG CTT	270 bp Kundave *et al*., 2018 [6]
2	AMF 5′-ACAGGCGAAGAAGCAGACAT-3′ AMR 5′-ATAAATGGGAACACGGTGGA-3′	382 bp Anitha *et al*., 2020

PCR assay

Target genes Amar 16S

The synthesized PCR primer Amar16S F/R was initially used to optimize PCR assay in order to amplify the template DNA of *A. marginale*. PCR technique was. Performed in a final volume of 25 µl containing multiplex master mix (Qiagen, Germany) (2X) 12.5 µl, gene-specific primer pair (F/R, 10 pmol/µl) 0.5 µl each, known positive template DNA 2.0 µl and nuclease-free water (NFW) to make the final volume. PCR conditions were optimized as initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation (95 °C for 45 s), primer annealing (55 °C for 45 s), and extension (72 °C for 45 s) along with a final extension of 72 °C for 10 min. PCR Reaction was performed in an automatic thermal cycler (Bio-Rad, USA). A total 10 µl PCR product sample was electrophoresed in 2.0% agarose gel containing 10 µl/ml *Ethidium bromide*. in Tris-Acetate- EDTA (TAE) buffer at 85 V for 45mins and visualized under UV light.

Target gene MSP5

Identification of the MSP5 gene, the PCR was performed using *A. marginale* primer MSP5 F/R with a reaction volume of 25 µL. The reaction mixtures contained 2X PCR master mix (QIAGEN, Germany) 12.5 µL, gene-specific primer pair (F/R, 10 pmol/µL) 0.5 µL each, known positive template DNA 2 µL and nuclease-free water to make the final volume up to 25 µL. The reactions mixtures were subjected to an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 53 °C for 30 s, and 72 °C for 45 s, with a final extension step at 72 °C for 10 min. For each PCR, a negative control was run together. PCR amplicons (10 µL) were analyzed in 2% agarose gel containing 10 µg/ml *Ethidium bromide* in Tris-Acetate- EDTA (TAE) buffer at 80 V for 48 mins and visualized under UV light.

Sequencing of target genes

PCR amplified products with respect to target gene Amar 16S and MSP5 of *A. marginale* were gel purified, subsequently PCR product was sent to Genurem Biosciences Ltd., for sequencing.

Results

Microscopic examination

Microscopic examination of Giemsa stained blood smears revealed intensely stained dark blue-purple coloured 1-3 numbers of inclusions in the periphery of RBC's were seen in 46 (18.7 percent) animals. (Fig. 3 and Fig 4).

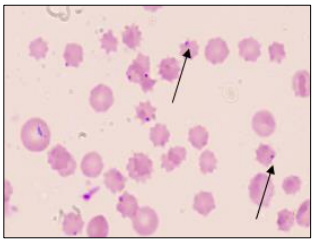

Fig 3: Blood smear *A. marginale*

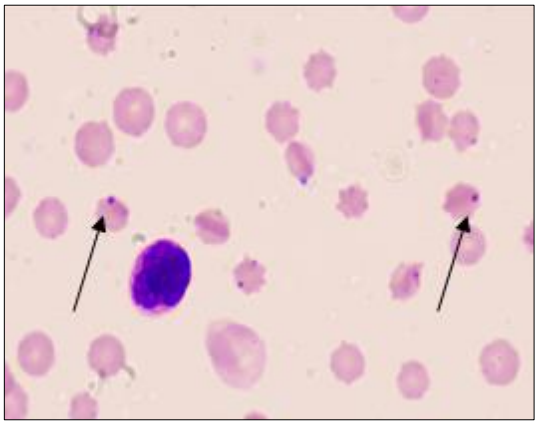

Fig 4: Blood smear *A. marginale*

Molecular assay

The PCR amplification of *A. marginale* targeting gene Amar 16S and MSP 5 were Observer in 270bp and 382 bp respectively. Nonspecific amplification was not observed in conducted test. PCR revealed positive case in 96 animals, among these Amar 16S gene (fig 5) was identified in 45 animals (18.35%) and MSP 5 gene (fig 6) was identified in 51 animals (20.8%) both genes were identified in 41 animals (16.5%). Overall positive case were recorded in 96 numbers (39.18%).

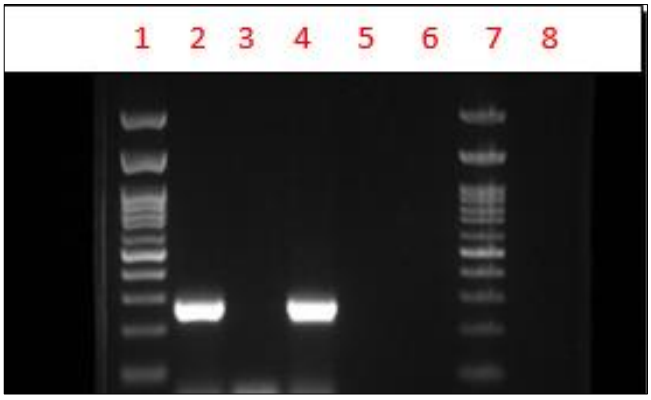

Fig 5: *A. marginale* Amar 16 S gene 270 bp lane 2 and 4, ladder lane 1 and 7

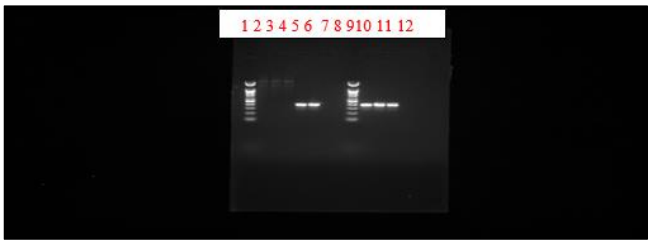

Fig 6: *A. marginale* MSP 5 gene 382 bp lane 5,6 ,10,11, and 12, ladder lane 1 and 6.

Discussion

In the present study PCR assay with various primers of *A. marginale* was employed to detect *A. marginale* infection in dairy cows. To ensure species specific amplification, oligonucleotide primers were designed from unique gene sequence selected from different target gene Amar 16S and MSP 5 of *A. marginale*. The PCR assay has been employed for sensitive detection of *A. marginale* and also considered as best alternative to microscopy. Blood smear examination revealed low prevalence of infection, whereas PCR confirmed the sub-clinical and carrier infection (Kundave *et al*., 2018) [6]. Targeting a +Single gene of *A. marginale* Amar 16S gene showed positive 18.35% MSP 5 shown positive in 20.81% of both genes identified in 16.52% of cases. The optimized different target genes PCR was able to detect parasite DNA from field cases at higher level. In this study overall prevalence of *A. marginale* infection in cattle was found to be higher in PCR assay than microscopic study. An important feature of Haemo protozoan infection in cattle is that survivors act as carriers (Anitha *et al*., 2020 B). PCR based assays targeting major surface protein (MSP) genes (especially of MSP1α, MSP4, and MSP5) of *A. marginale* have high degree of sensitivity and specificity. Therefore, MSP5 has been used as an ideal marker for specific diagnosis of *A. marginale* infection (de la Fuente 2005) [3]. The test was found highly specific as well as in none of the cases nonspecific amplification was recorded and sequencing of PCR products also confirmed the specific amplification. In the present study, the overall prevalence of *A. marginale* infection in cattle was found to be higher by PCR assay (39.18%) than the microscopic examination (18.7%). Different target genes in particular infection were found high sensitive and able to detect all parasitic DNA.

Genetic heterogeneity among the population of *A. marginale* was previously studied from two South Indian states *viz*., Seem Andhra and Telangana (George *et al*. 2017) [4]. Based on phylogeny using MSP5, was reported that most of the strains (21/24) from these South Indian states showed close proximity with strains from Mexico and Brazil.

In the present study, revealed that minimal heterogeneity exists within 16S rRNA and MSP5 genes among the field isolates from Kerala. The results of phylogeny using MSP4 in the present study are similar to those of South Indian isolate. Hence, it could be inferred that isolates of *A. marginale* of South India are genetically close.

Conclusion

The present study confirmed the existence of *A. marginale* infection of dairy cattle infecting in Namakkal Tamil Nadu. *A. marginale* isolates of Namakkal were genetically conserved based on phylogenetic analysis of MSP5 and Amar 16S genes. Targeting two genes will be best tool to Identified subclinical and carrier state animals. The PCR assay represents a rapid, robust, highly sensitive and specific diagnostic tool than traditional methods. It offers a more practical alternative for accurate and simultaneous detection of different target genes of *A. marginale* in clinical samples as well as in large scale epidemiological studies. Prompt diagnosis and targeted selective control measures will help in diminishing the losses incurred to the poor livestock owners.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgement

The authors express their sincere sense of gratitude to the Vice Chancellor, TANUVAS, and Chennai for providing research facilities and financial support.

References

1. Almeria S, Castella J, Ferrer D, Ortuno A, Estrada-Pena A, Gutierrez JF. Ovine piroplasms in Minorca (Balearic Islands, Spain): A comparison of PCR-based and light microscopy detection. Veterinary Parasitology.

2001;99:249-59.
2. Anita G, Biswa RM, Indrajit G. Pentaplex PCR assay for rapid differential detection of *Babesia bigemina*, *Theileria annulata*, *Anaplasma marginale* and *Trypanosoma evansi* in cattle. Biologicals. 2020;63:81-88.
3. De la Fuente J, Lew A, Lutz H, Meli ML, Hofmann-Lehmann R, Shkap V, *et al*. Genetic diversity of Anaplasma species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. Anim. Health. Res. Rev. 2005;6:75-89.
4. George N, Bhandari V, Sharma P. Phylogenetic relationship and genotypic variability in *Anaplasma marginale* strains causing anaplasmosis in India. Inf. Genet. Evol. 2017;48:71-75.
5. Hove P, Khumalo ZTH, Chaisi ME, Oosthuizen MC, Brayton KA, Collins NE. Detection and Characterisation of *Anaplasma marginale* and *A. centrale* in South Africa. Vet Sci. 2018;35(1):26.
6. Kundave VR, Ram H, Banerjee PS, Garg R, Mahendran K, Ravikumar GVPPS, *et al*. Development of multiplex PCR assay for concurrent detection of tick borne haemoparasitic infections in bovines. Acta Parasitol. 2018;63(4):759-65.
7. Maharana BR, Tewari AK, Saravanan BC, Sudhakar NR. Important haemoprotozoan diseases of livestock: challenges in current diagnostics and therapeutics: An update. Vet World. 2016;9(5):487-95.
8. Radostitis OZ, Gay CC, Blood DC, Hinchliff KW. Veterinary Medicine., A Text Book of the Diseases of Cattle, Sheep, Goats and Horses, W.B. Saunders Co., London, New York, Philadelephia. 2000;9:1261.
9. Reetha TL, Thomas KS, Babu M. Occurrence of haemoprotozoan infection in bovines. Int. J App Bioresearch. 2012;13:1-2.
10. Theiler, A. Gall-sickness of South Africa. (Anaplasmosis of Cattle.). J Comp. Pathol. Therap. 1910;23:98-115.