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# Molecular diagnosis of anaplasmosis caused by *Anaplasma marginale* in cattle

**B Subramanian, P Vijayalakshmi, S Venkatesa Perumal and D Selvi**

**Abstract**
Polymerase chain reaction (PCR) assay is considered the "gold standard" for detection of persistently infected cattle with *Anaplasma marginale* infection. Major surface protein 4 gene is a stable marker for the genetic classification of *A. marginale* strains. The present study was conducted for the molecular diagnosis of anaplasmosis in cattle infected with *Anaplasma marginale* in the Puducherry state. Each blood sample was subjected for Deoxyribonucleic Acid (DNA) extraction as per HiPurA™ SPP Blood DNA Isolation Kit (Himedia®). On Basic Local Alignment Search Tool analysis, the sequence showed 100% identity with the sequence of *A. marginale* and GenBank accession number: MG720555. Out of 73 anaplasmosis suspected samples, 15 were found to be positive for *A. marginale* by PCR. PCR was found highly sensitive and best diagnostic tool in the diagnosis of anaplasmosis, when compared to blood smear examination.

**Keywords:** Polymerase chain reaction, major surface protein 4, deoxyribonucleic acid, *Anaplasma marginale*

## Introduction
Anaplasmosis is mainly a disease of adult cattle, while younger animals may remain susceptible but exhibited little detectable signs. Adult animals are considered as carrier resultant from their prior contacts in life to *Anaplasma marginale* infection (Soulsby, 1982 and Singh *et al*. 2003) [1, 2]. Office of the International Epizootics (2003) [3] stated that anaplasmosis is currently classified in List B of the Terrestrial Animal Health Code due to its socio-economic importance and significance in terms of limitations in the international trade of animals and animal products. *A. marginale* is the communal pathogen of cattle and is responsible for substantial financial loss in livestock production in emerging countries (Dreher *et al*., 2005) [4].

Disease is characterized by progressive hemolytic anemia associated with pyrexia, jaundice, decreased milk production, abortion, hyperexcitability and in some cases sudden death (Aubry and Geale, 2011) [5]. Giemsa stained blood smear examination can be used as a appropriate method to detect Anaplasma in the animals clinically suspected for acute diseases, but it is not appropriate for the purpose of pre-symptomatic and carrier animals. The level of parasitemia is often too low for detection by microscopy (Carelli *et al*., 2007) [6]. Microscopic examination which is used to confirm acute anaplasmosis, can only detect levels of $>10^6$ infected erythrocytes per ml (Gale *et al.*, 1996) [7].

PCR assay is considered the "gold standard" for detection of persistently infected cattle with *A. marginale* infection (Torioni *et al.*, 2005) [8]. The diagnostic sensitivity of polymerase chain reaction (PCR) - based methods has been predictable as 0.0001% in infected erythrocytes (Torioni De Echaide *et al*., 1998) [9]. Molecular methods, with a high degree of sensitivity and specificity, have been developed to identify Deoxyribonucleic Acid (DNA) of *A. marginale* (Carelli *et al*., 2007) [6] and also been used in identifying the occurrence of low-level infection in carrier cattle and tick vectors (Kocan *et al*., 2010) [10].

Major surface protein 4 gene is a stable marker for the genetic classification of *A. marginale* strains (Fuente *et al.*, 2004) [11]. Major surface proteins (Msps) genes are good diagnostic marker and also used in phylogenetic studies, since their known role in host pathogen relations and they may more rapidly progress than other nuclear gene because of selective burdens (Almazan *et al.*, 2008) [12]. *A. marginale* infection in examined cattle was validated by msp4 gene sequencing, indicating a low geographic segregation. Only two different *A. marginale* msp4 sequences were isolated from cattle in different Tunisian areas (Belkahia *et al*., 2015)

The present study was conducted for the molecular diagnosis of anaplasmosis in cattle infected with *Anaplasma marginale* in the Puducherry state.

## Materials and Methods

A total of 586 Cattle brought to Large Animal Medicine Unit, Teaching Veterinary Clinical Campus, Rajiv Gandhi Institute of Veterinary Education and Research and Ambulatory clinic for treatment of various medical ailments. Seventy three cases with clinical signs of pale to icteric mucous membrane and tick infestation were subjected for blood smear examination and PCR. Blood smear from the suspected cattle were collected from the ear vein and thin blood smears were made and air dried immediately. The blood smear was stained using Giemsa stain. Two milliliter of blood was collected from jugular vein in a dry vial containing 3.6 mg of EDTA. The vials were stored at -20ºC and the sample was subjected to PCR analysis.

An initial volume of 300 µl blood from each sample was subjected for DNA extraction as per HiPurA™ SPP Blood DNA Isolation Kit (Himedia®). The extracted DNA samples were stored at -20 ºC for further proceedings. A pair of primers was custom synthesized (Eurofins) for PCR amplification of Major Surface Protein (MSP) 4 of *A. marginale* as described by Joazeiro *et al.* (2015) [14]. The details of the primers are given below in the table.

The details of the primers are given below in the table.

| Gene | Primer | Primer sequence | Expected product size |
|---|---|---|---|
| MSP4 | MSP F – Forward | 5'-CCCATGAGTCACGAAGTGG-3' | 753 bp |
| | MSP R – Reverse | 5'-GCTGAACAGGAATCTTGCTCC-3' | |

PCR was carried out as per Joazeiro *et al.* (2015) [14] for amplification of MSP4 gene (753 bp) for the confirmation of *A. marginale* infection in suspected cattles. PCR was set up in 25 µl reaction mixtures which consists of 5 µl of template DNA, 1 µl of H forward and reverse primer (5 picomol / µl) each, 12.5 µl of 2X Red dye Master Mix (Amplicon) and 5.5 µl of Nuclease free water.

PCR was performed with the following condition in Eppendorf thermal cycler with 35 cycles of initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR products were analyzed in 2 % Agarose gel electrophoresis. The gel was visualized under UV trans-illuminator and images were documented in a gel documentation system (Gel Doc It. Images System, UVP). The amplified PCR product was purified and was subjected for custom sequencing at Eurofins Genomics India Private Limited, Bengaluru. The sequence was subjected for Basic Local Alignment Search Tool (BLAST) analysis to check the specificity of the product Altschul *et al.* (1990) [15] (www.ncbi.nlm.nih.gov).

## Results

Of the 73 cattle suspected for anaplasmosis, 3 cases (4.11%) showed round intra-erythrocytic bodies near the margin of the erythrocyte in the peripheral blood smear. DNA was isolated from the blood as per procedure of DNA isolation kit (HiPurA™ SPP Blood DNA Isolation Kit - Himedia®). The yield of the DNA ranges from 20 – 50 ng/µl. The purity of DNA assessed using the ratio of absorbance at 260 to 280nm ranges from 0.9 to 2.0.

Agarose gel electrophoresis of PCR product revealed the amplicon of 753 bp of *A. marginale* specific (Fig.1). As there was no positive control, the PCR product was custom sequenced. On BLAST analysis, the sequence showed 100% identity with the sequence of *A. marginale* and (GenBank accession number: MG720555)

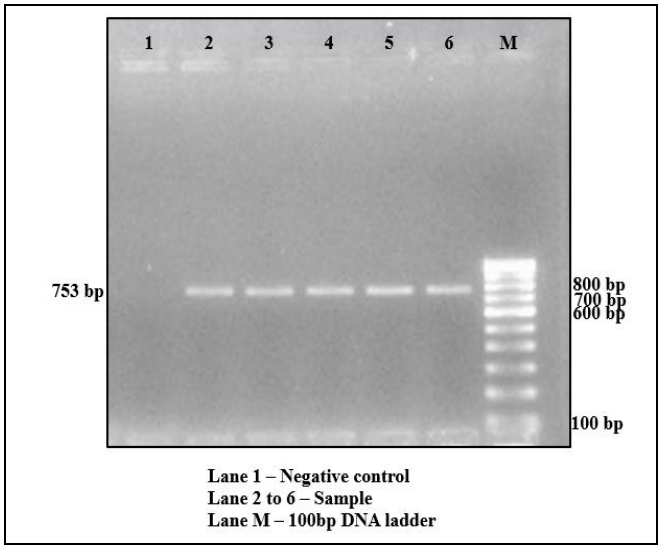


**Fig 1:** Agarose gel electrophoresis of PCR product of *Anaplasma marginale*

Out of 73 anaplasmosis suspected samples, 15 were found to be positive for *A. marginale* by PCR. Table 1 shows the comparison between the PCR assay and blood smear examination for the identification of anaplasmosis.

**Table 1:** Comparison of the results of PCR and blood smear examination

| Technique | Blood smear positive | Blood smear negative | Total |
|---|---|---|---|
| PCR positive | 3 | 12 | 15 |
| PCR negative | 0 | 58 | 58 |
| Total | 3 | 70 | 73 |

## Discussion

In the present study, out of 73 anaplasmosis suspected samples, 15 were found to be positive for *A. marginale*, while only three turned positive by blood smear examination. The peripheral blood smears were stained using Giemsa stain, as suggested by Kelly (1984) [16] for the demonstration of intra-erythrocytic bodies in the RBC. In the present study, peripheral blood smears were examined for the demonstration of round intra-erythrocytic bodies near the margin of the erythrocyte as per OIE (2008) [17].

Hamid *et al.* (2014) [18] stated that traditional Giemsa staining method is not applicable for identification and diagnosis of

persistently infected cattle with no signs and apparently healthy in contact with diseased animals. Diagnosis of anaplasmosis in cattle is difficult as differentiation between *Anaplasma* organisms, structures like Heinz bodies, Howell-Jolly bodies or staining artifacts are often seen in Giemsa stained blood smears. During the persistent infection, infected erythrocytes are not always detectable in stained blood smears as also reported by Birdane *et al.* (2006) [19]. Hence, blood smear cannot be considered as a reliable diagnostic aid for *Anaplasma* detection.

This shows that PCR was found to be more profound for the detection of *A. marginale* which harmonizes with the findings of Torioni De Echaide *et al.* (1998), Carelli *et al*. (2007) and Kocan *et al*. (2010) [9, 6, 10] who reported that PCR was more consistent, precise and delicate method for detection of anaplasmosis. Keiser *et al.* (1990) [20] from Kerala stated that higher incidence of anaplasmosis in clinically normal crossbreds of South India specified subclinical infections or carrier status of these vector borne diseases which could be identified by PCR. Singh *et al*. (2012) [21] reported in Punjab, 45.2 % of cases identified by PCR while 12.5 % identified by blood smear examination

## Conclusion

PCR was found highly sensitive and best diagnostic tool in the diagnosis of anaplasmosis, when compared to blood smear examination.

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