STANDARDISATION OF REAL TIME PCR METHODOLOGY FOR DIAGNOSIS OF *ANAPLASMA MARGINALE* AFFECTING BOVINE POPULATION TARGETING *MSP1 BETA* GENE

Sunitha Karunakaran[1*], Hareesh Sasidharan[2], Gopika Thattat Gopinathan[3]

[1]District Laboratory Officer, Clinical Laboratory, Animal Disease Control Project

[2]Veterinary Surgeon, Veterinary Dispensary, Ayyanthole, Thrissur

[3] Research Assistant, Clinical Laboratory, Animal Disease Control Project

Paravattani, Thrissur East P.O-680005

Department of Animal Husbandry, Kerala

*Corresponding author: drsunivet@yahoo.co.in

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ABSTRACT

The present study was aimed to standardise Taqman based Real Time PCR (qPCR) protocol as an aid in accurate and confirmatory diagnosis of bovine anaplasmosis due to *Anaplasma marginale* targeting *Msp1 Beta* gene.

Key Words:-Real Time PCR, Tick Transmitted Pathogen, *Anaplasma marginale*, Bovine population

INTRODUCTION

A very high prevalence of tick transmitted diseases especially anaplasmosis is observed among bovine population in Kerala now days. Tentative diagnosis of bovine anaplasmosis is based on clinical signs and peripheral blood smear examination. Conventional diagnostic methods based on direct microscopy will not be sufficient to identify whether single or multiple pathogen is responsible for bovine anaplasmosis and to identify *Anaplasma species* prevalent among bovine population in Kerala state. The present study was aimed to standardise Taqman based Real Time PCR (qPCR) protocol as an aid in accurate and confirmatory diagnosis of bovine anaplasmosis due to *Anaplasma marginale* targeting *Msp1 Beta* gene.

MATERIALS AND METHODS

Previously reported, forward and reverse primers and probe sequences specific for *A. marginale* msp1β gene (Table.1) designed and custom synthesised from Tibmolbiol, (Roche, India PVT, LTD) were used in the study for standardisation of Real Time

PCR experiments. Entire sequence of the target genes (*Msp*1*Beta*) custom synthesised and cloned into pUGM Plasmid (SciGenom Labs, PVT LTD, Kakkanad, Cochin) was used as positive control for primer standardisation and optimization of PCR conditions. Format of Real Time PCR followed with total reaction volume of 20 µl, using Fast Start Essential DNA Probes Master 2X concentration-10µl (Roche Diagnostic's, Version 6), 1 µl each of forward and reverse primers (10µM), 1 µl of probe (10µM), 2 µl of water and 5µl of template DNA.

Primer efficiency testing was conducted by plotting a standard curve. Custom synthesized positive clone in pUGM plasmid (with initial concentration 10ng/µl) was used for primer efficiency testing and standardization of cycling conditions. The positive clone was diluted in serial 1:10 dilutions in PCR grade water (Roche) so as to contain one genomic equivalent (GE) per 5 µl in last dilution. Six serial dilutions in duplicate were prepared for the experiment and all the dilutions were tested in RT PCR assay to find out the limit of detection and efficiency of the primers designed and to generate a standard curve. Cycling conditions optimized for primer efficiency testing included an initial activation (pre incubation) at 95° C for 10 min followed by 50 cycles of 15s denaturation at 95° C followed by a 1 min annealing -extension step at 60° C.

RESULTS AND DISCUSSION

Primer efficiency testing was conducted by plotting a standard curve, custom synthesized positive clone in pUGM plasmid, with initial concentration 10ng/µl for *Anaplasma marginale* *Msp* 1 *Beta* was used as template for the plotting of standard curve. Six tubes in duplicate with serial 1:10 dilutions prepared for the experiment showed, change of approximately 3.3 cycles between 10 fold dilutions of the template. The slope of the standard curve generated after primer efficiency testing showed an efficiency of 1.93 and R value 1.0 for *A. marginale* (Fig.1).

TaqMan-based real-time PCR assay developed for the diagnosis of *A. marginale* infection of cattle by Carelli *et.al*. (2007) was proven to be highly specific, cross-reactions were not observed with other *Anaplasma* species of ruminants, including the closely related *Anaplasma centrale*, or other haemoparasites of ruminants *Anaplasma bovis*, *Anaplasma ovis*, *Anaplasma phagocytophilum*, *Babesia bovis*, *Babesia bigemina*, *Theileria annulata* and *Theileria buffeli*. The same primer pairs and probe was used in the present study and could successfully amplify target gene Msp1β from positive template used. Primers and probe pair used, format for Real Time PCR followed and PCR conditions optimized could successfully amplify target gene *Msp* 1 *Beta*

and this methodology can be used for testing of clinical samples.

SUMMARY

The present study shows that Taqman based qualitative Real Time PCR (qPCR) protocol can be successfully standardized and used in laboratories for accurate and confirmatory diagnosis of tick transmitted pathogens like *Anaplasma marginale* up to species level and this protocol can later be used for testing of clinical samples under filed conditions.

Table.1. Details of primers and probes used

Oligo Name	Primer Sequence (5'-3')	Product Size	Target Genome	References
Anaplasma marginale A.mar_F	ttggcaaggcagcagctt	95 bp	Msp1 β gene Accession Number M59845	1
A.mar_R	ttccgcgagcatgtgcat			
A.mar_P	FAM-tcggtctaacatctccaggctttcat-BBQ			

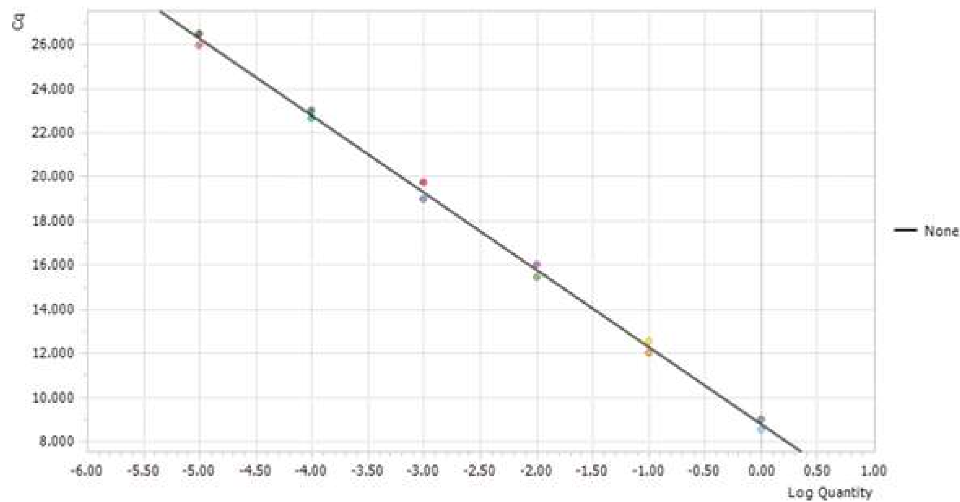

Fig.1. Slope of standard curve for primer efficiency testing- Primers used *A.mar_F, A.mar_R*, along with Taqman probe *A.mar_P*

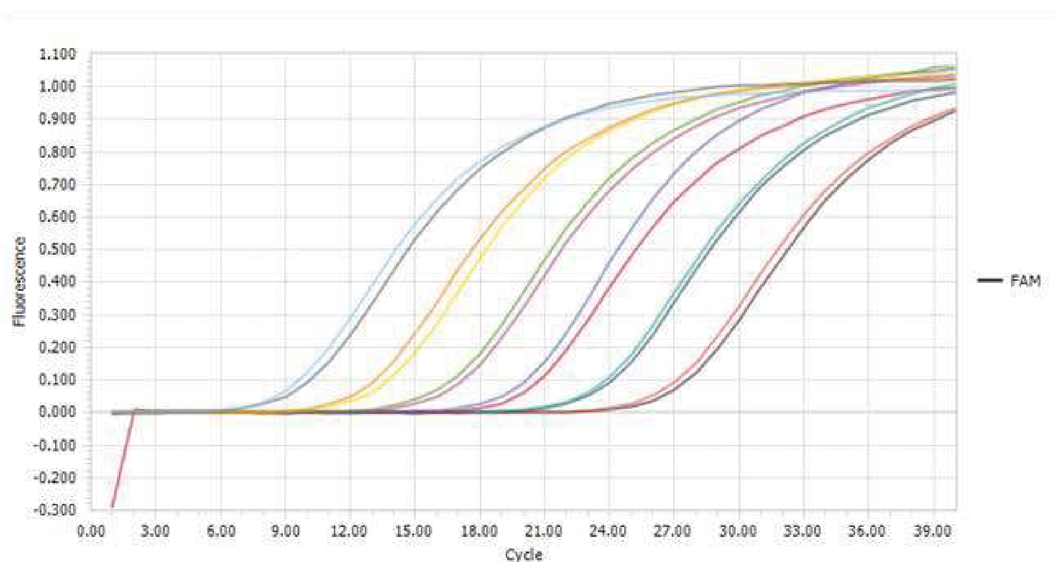

Fig.2. Amplification curve of pzositive control serial dilution

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REFERENCES

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L., Buonavoglia, C. 2007. Detection and quantification of Anaplasma marginale DNA in blood samples of cattle by real-time PCR. *Vet Micobiol.* **124**: 107-114.

Hoar, B. R., Nieto, N. C., Rhodes, D. M., Foley, J.E. 2008. Evaluation of sequential coinfection with Anaplasma phagocytophilum and Anaplasma marginale in cattle. *Am. J. Vet. Res.* **69**:1171–1178.

Reinbold, J. B., Coetzee, J. F., Sirigireddy, K. R., Ganta, R. R. 2010. Detection of Anaplasma marginale and A. phagocytophilum in Bovine Peripheral Blood Samples by Duplex Real-Time Reverse Transcriptase PCR Assay. *J. Clin. Microbiol.* **7**:2424-2432.