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Evaluation of real time PCR for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in faecal samples of cattle

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Abstract

The efficiency and suitability of a MAP F57 based SYBR Green qPCR assay for the detection of Mycobacterium avium subsp. *paratuberculosis (MAP) using a set of known MAP positive (12) and MAP negative (23) DNA samples that were previously identified by conventional IS 900 PCR were assessed. These DNA samples were isolated in our previous study from faecal samples collected from cattle in the livestock farms under government sector with a previous history of Johne's disease. The MAP F57 qPCR was able to identify all the positive samples accurately and rapidly with Cq values ranging from 20-29. The efficiency of qPCR using recombinant plasmid for standard curve was 0.991 and limit of detection was 10 MAP organisms per microlitre of DNA sample.*

Keywords: *Johne's disease, MAP, qPCR, F57*

Running title: Real time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle faeces

Johne's disease (JD) is characterised by chronic enteritis and wasting in ruminants. The causative agent is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease is associated with prolonged course and animals may remain infected sub-clinically for several years with low and intermittent shedding of MAP in faeces (Whittington and Sergeant, 2001). Transmission occurs majorly through faeces which contaminates pastures and farm environment. Animals with clinical disease also shed large number of MAP (10^6 MAP cells per gram) in the faeces (Eamens *et al*., 2007). The subclinical form of Johne's disease is difficult to be diagnosed by culture and such animals spread infection silently without manifesting any clinical signs. As the organism is very slow growing and fastidious, PCR based methods for detection have been widely in use and are recommended by OIE (*OIE terrestrial manual*, 2021).

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Most of the diagnostic PCR tests for MAP detection are based on amplification of the MAP specific insertion sequence IS*900,* which is present in multiple copies in MAP genome and hence offers greater sensitivity (Vansnick *et al*., 2004). The MAP F57 gene is unique to MAP and absent in other mycobacterial species. Hence PCR assay targeting this gene offers greater specificity (Poupart *et al*., 1993; Herthnek and Bolske, 2006). Moreover, F57 being a single copy gene is mostly targeted in quantitative real time PCR assays (qPCR) that aim to assess the MAP burden in the clinical samples (Tasara and Stephen, 2005; Irenge *et al*., 2009). Real time PCR methods offer greater sensitivity than bacterial culture (Schonenbrucher *et al*., 2008) and can detect very low numbers less than 10 CFU (Mahony and Hill, 2004; Nelli *et al.,* 2008) of MAP.

In this study, a qPCR assay based on SYBR Green chemistry targeting a 195 bp fragment of MAP F57 gene, developed in our previous study (Chaitanya *et al*., 2019) has been evaluated for its diagnostic efficacy using known MAP positive and negative DNA samples. These DNA samples were isolated in our previous study (Priyanka, 2019) from faecal samples collected from cattle in the government livestock farms with a history of Johne's disease incidence. The DNA extraction kit used was QIAamp DNA stool mini kit (Qiagen). They were identified by IS*900* PCR previously as per the method of Vansnick *et al*. (2004).

A set of twelve MAP IS*900* PCR positive DNA samples and 23 PCR negative samples were chosen for evaluating F57 qPCR. The qPCR assays were performed using an Eppendorf Real Plex Master cycler as described by Chaitanya *et al.* (2019). A standard calibration curve with a series of MAP quantification standards with 10^9 to 10^0 copies of recombinant plasmid carrying F57 target sequence (195 bp) was included in each qPCR run. The DNA (20pg/µL concentration) from known MAP isolate from a previous study (Chaitanya *et al*., 2015) in Tamil Nadu was used as a positive control. All the samples, standards and positive control were run in duplicate. No template control is included to check the specificity of amplification process.

Standard curve of cycle threshold (Cq) values increasing with increase in dilution of MAP F57 plasmid insert was obtained. The Cq values plotted against the dilution factor were found to not deviate much from the mean. The Cq values of standards ranged from 4.2 to 33.1. The least copy number of the plasmid that was able to be detected was 10^1. Slope of the standard curve was –3.297 and the assay efficiency in terms of 'R^2' value was 0.991. From the standard curve, the copy number of F57 plasmid that correspond to each Cq value and the approximate number of MAP organisms in each microlitre of DNA sample could be calculated. This qPCR technique was able to detect as low as 10 MAP per microlitre of the DNA.

In MAP F57 qPCR all the 12 DNA samples that were positive in IS*900* PCR were positive with Cq values ranging from 20 to 29. One of the 23 IS*900* PCR negative samples was considered to be inconclusive or doubtful in qPCR with Cq value 30 and this might be because of high sensitivity of amplification detection in real time PCR when compared to the gel electrophoresis in conventional PCR. Such animals should be tested again after a couple of months with a fresh faecal sample. The Cq values above 35 were considered as negative because all other 22 samples that were IS*900* PCR negative did not show any initiation of amplification curve before 35^{th} cycle. The melt curve analysis confirmed that all the amplicons have acceptable Tm value of 86.8±0.2 indicating the specificity of the assay.

It is evident that this qPCR assay is rapid than conventional PCR. It is suitable for direct detection of MAP in the clinical samples. Developed countries like Australia have adopted real time PCR testing of faecal samples for diagnosis and surveillance of Johne's disease (Plain *et al.,* 2014). However, the assay needs further refinement by inclusion of internal control target for checking the DNA extraction losses and for monitoring PCR amplification conditions. It is well known that DNA extraction procedure may cause reduction in copy number and lead to under estimation of MAP load in the clinical samples. The complex and lipid rich cell wall of MAP results in poor

DNA recovery and PCR inhibitors in faeces affect amplification of the target. Assay should be also evaluated using faecal samples spiked with known number of MAP to find out limit of detection in its actual sense.

Summary

The MAP F57 qPCR assay detected all the 12 IS*900* PCR positive DNA samples as positive with Cq values ranging from 20 to 29 that corresponds to 10^4 MAP copies to 100 copies per microlitre of DNA. The Cq values above 35 were considered as negative. The qPCR assay is rapid and sensitive than conventional PCR detect as low as 10 MAP per microlitre of the DNA.

Conflict of interest

The authors declare that they have no conflict of interest.

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