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The investigation of the truncated mbtA gene within the mycobactin cluster of *Mycobacterium avium* subspecies *paratuberculosis* as a novel diagnostic marker for real-time PCR

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ABSTRACT

The inability of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) to produce endogenous mycobactin in-vitro is most likely due to the presence of a truncated mbtA gene within the mycobactin cluster of MAP. The main goal of this study was to investigate this unique mbtA truncation as a potential novel PCR diagnostic marker for MAP.

Novel primers were designed that were located within the truncated region and the contiguous MAP2179 gene. Primers were evaluated against non-MAP isolates and no amplicons were generated. The detection limit of this mbtA-MAP2179 target was evaluated using a range of MAP DNA concentrations, MAP inoculated faecal material and 20 MAP isolates. The performance of mbtA-MAP2179 was compared to the established f57 target. The detection limits recorded for MAP K-10 DNA and from MAP K-10 inoculated faecal samples were 0.34 pg and 10^4 CFU/g respectively for both f57 and mbtA-MAP2179. A detection limit of 10^3 CFU/g was recorded for both targets, but not achieved consistently. The detection limit of MAP from inoculated faecal material was successful at 10^3 CFU/g for mbtA-MAP2179 when FAM probe real-time PCR was used. A MAP cell concentration of 10^2 CFU/g was detected successfully, but again not consistently achieved. All 20 mycobacterial isolates were successfully identified as MAP by f57 and mbtA-MAP2179. Interestingly, the mbtA-MAP2179 real-time PCR assay resulted in the formation of a unique melting curve profile that contained two melting curve peaks rather than one single peak. This melting curve phenomenon was attributed towards the asymmetrical GC% distribution within the mbtA-MAP2179 amplicon.

This study investigated the implementation of the mbtA-MAP2179 target as a novel diagnostic marker and the detection limits obtained with mbtA-MAP2179 were comparable to the established f57 target, making the mbtA-MAP2179 an adequate confirmatory target. Moreover, the mbtA-MAP2179 target could be implemented in multiplex real-time PCR assays and with its unique melting curve profile adds increased specificity to MAP diagnostic tests.

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1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a slow-growing, Gram-positive, acid-fast mycobacterial pathogen that infects dairy cattle, sheep, goats and other non-ruminant animals. These MAP infections lead to intestinal inflammation that causes severe diarrhea, loss of weight, inclined milk production and eventual death of infected dairy cattle. These clinical symptoms are associated with Johne's disease, which has a serious impact on the dairy economy and animal health worldwide (Li et al., 2016).

To detect MAP in dairy herds from faecal material, the choice to use the solid culture media method is still regarded as the gold standard detection method in MAP diagnostics. However, this MAP screening method is troublesome. Impairments such as decontamination difficulties of the samples, labor intensiveness and the extensive time taken before positive MAP results can be observed are problems associated with the solid culture method (Soumya et al., 2009; Whittington et al., 1998). Many quantitative PCR (qPCR) molecular-based methods have been developed that offer a rapid and sensitive approach for the detection of MAP from a faecal environment (Imirzalioglu et al., 2011; Sidoti et al., 2011).

The multi-copy insertion sequence 900 (IS900) has been widely implemented in molecular MAP diagnostics (Li et al., 2005; Semret et al., 2006a). Other multi-copy insertion sequences, such as ISMAP02, IS1311 and ISMav2, were identified within the MAP K-10 genome and

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several were used in studies as a molecular target for MAP detection (Li et al., 2005; Stabel and Bannantine, 2005; Sting et al., 2014). The discovery of single-copy elements such as the f57, locus 251 and hspX revealed that these sequences were specific and unique only to MAP, making these elements attractive molecular targets in MAP diagnostics despite their single-copy nature (Tasara and Stephan, 2005; Clark et al., 2006; Rajeev et al., 2005). The single-copy f57 element has been utilised as a molecular target in MAP diagnostics from various bovine matrices (Keller et al., 2014; Donaghy et al., 2011).

Two unique phenotypical differences between MAP and other mycobacteria is that MAP is one of the slowest growing known mycobacteria and secondly, MAP has the inability to produce mycobactin in an in-vitro environment and is therefore dependent on mycobactin supplementation. Mycobactin is an iron binding siderophore, which is important for growth and survival of mycobacteria. These mycobactins are important for the binding and the transportation of ferric iron from the environment into the mycobacterial cell (Li et al., 2005; Wang et al., 2015; Timms et al., 2015). These cells require iron, which is vital for processes such as oxidative stress defense, nucleic acid synthesis and the transportation of electrons (Janagama et al., 2009). The mycobactin operon (mbtA-J) was identified in *Mycobacterium tuberculosis* and consists of 10 genes, which are important for the mycobactin synthesis within *M. tuberculosis* (Quadri et al., 1998). The complete sequencing of the MAP K-10 genome showed that homologs of the mycobactin operon in *M. tuberculosis* also occurred within the MAP K-10 genome. This revealed that a truncation was identified within the mbtA gene of the mycobactin cluster that is unique only to MAP compared to *M. tuberculosis* and *Mycobacterium avium* (Li et al., 2005).

The mbtA gene is the initiator in the mycobactin synthesis and it has been postulated that this truncation in the mbtA gene most likely prevents MAP from synthesising endogenous mycobactin in-vitro resulting in slow mycobacterial growth (Li et al., 2005; Wang et al., 2015). This truncation should be further investigated and evaluated as a potential novel diagnostic marker for MAP detection. This truncation within the mbtA gene is unique only to MAP and could therefore, enhance the specificity of MAP qPCR detection tests. This mbtA gene is truncated at the C-terminus by a 19 kb large sequence polymorphism ($LSP^{p}12$) and the first gene within this $LSP^{p}12$ is a hypothetical gene designated MAP2179 (Li et al., 2005; Semret et al., 2004; Semret et al., 2006b). This MAP2179 gene is situated downstream of mbtA and primer sets could be designed, where the forward primer is located on the mbtA gene truncation and the reverse primer is located on the MAP2179 gene, thus creating a unique, albeit a single copy, specific amplicon for MAP.

This study investigated the potential of the single-copy mbtA-MAP2179 target as a novel molecular diagnostic marker for the detection of MAP. The mbtA-MAP2179 target was evaluated and compared with the established f57 target using qPCR assays performed on MAP K-10 DNA concentrations, MAP isolate cultures and from bovine faecal samples that were artificially inoculated with different MAP K-10 cell concentrations. A hydrolysis probe targeting the mbtA-MAP2179 was also evaluated for additional specificity to the qPCR assay for MAP detection. This new mbtA-MAP2179 diagnostic target would beneficially increase the repertoire of specific and unique MAP detection targets that can be implemented in qPCR tests or implemented in multiplex qPCR assays with other multi-copy or single-copy targets and therefore, further increase the specificity of MAP diagnostic tests.

2. Materials and methods

2.1. Bacterial strains, cultivation conditions and DNA preparations

The mycobacterial strains used were *Mycobacterium smegmatis* mc^2 155, *Mycobacterium hominissuis*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium tuberculosis* H37Rv and *Mycobacterium avium* subspecies *avium* ATCC 25291. These mycobacterial strains (from Cork Institute of Technology culture collection, CIT) were all cultured stationary at 37 °C, up to four weeks in 40 ml Middlebrook 7H9 broth (Sigma-Aldrich) and supplemented with 10% OADC enrichment (Becton Dickinson, BBL). *M. smegmatis* mc^2 155 was cultured in 10 ml Brain Heart Infusion broth (Sigma-Aldrich) at 37 °C for two to three days. MAP K-10 was cultured stationary at 37 °C for 12 to 16 weeks in 40 ml MB 7H9 broth supplemented with 10% OADC enrichment and Mycobactin J (2 mg/l, Serosep Limited). The Central Veterinary Research Laboratory (CVRL, Cellbridge, Kildare, Republic of Ireland) supplied twenty Middlebrook 7H10 agar slants containing 10% OADC enrichment and Mycobactin J supplementations (Becton Dickinson, BBL) to CIT that contained MAP colonies cultured from faecal material. Individual colonies were selected and cultured as described for the MAP K-10 strain. Mycobacterial DNA was extracted using the TetraCore MAP purification system (TetraCore Inc., Rockville MD, USA) as per manufacturer's protocol. The MagNA lyser system (Roche Diagnostics, Germany) was used at 6500 rpm for 2 min instead of the beatbeater mentioned in the manufacturer's manual.

Escherichia coli TOP10 (Invitrogen) was cultured at 37 °C, shaking at 150 rpm, in 10 ml of Luria-Bertani broth (Sigma-Aldrich) for 16 h and DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany). DNA preparations from the following Gram-positive and Gram-negative bacterial strains were donated by colleagues at CIT and these included *Staphylococcus epidermidis* ATCC12228, *Streptococcus agalactiae* (CIT clinical isolate), *Campylobacter ureolyticus* CIT007, *Campylobacter jejuni* DSM4688, *Lactobacillus brevis* ATCC8287 and *Pectobacterium atrosepticum* DSM18077. These DNA concentrations were normalised to 35–40 ng/μl and Table 1 shows a complete list of all bacterial strains used during this study.

2.2. Primer design and qPCR amplification conditions of mbtA-MAP2179

The sequences of the mbtA gene (MAP K-10 GenBank: AE016958.1, Gene tag: MAP_2178, protein ID: AAS04495.1) and the MAP2179 gene (Gene tag: MAP_2179, protein ID: AAS04496.1) were retrieved from the NCBI database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) and the entire sequence was 1711 bp long. A 46 bp sequence was situated between the truncation of the mbtA

Table 1
The complete list of bacterial strains used during this study. Primer specificity of the single-copy mbtA-MAP2179 target was determined on these bacterial strains.

Bacterial strains	Source[a]	mbtA-MAP2179
Mycobacterium avium subspecies *paratuberculosis* K-10	CIT	+
Mycobacterium smegmatis mc^2 155	CIT	–
Mycobacterium hominissuis	CIT	–
Mycobacterium intracellulare	CIT	–
Mycobacterium kansasii	CIT	–
Mycobacterium tuberculosis H37Rv	CIT	–
Mycobacterium avium subspecies *avium* ATCC 25291	ATCC	–
Staphylococcus epidermidis ATCC12228	ATCC	–
Streptococcus agalactiae	CIT (clinical)	–
Lactobacillus brevis ATCC8287	ATCC	–
Campylobacter ureolyticus CIT007	CIT (clinical)	–
Campylobacter jejuni DSM4688	DMSZ	–
Escherichia coli TOP10	Invitrogen	–
Pectobacterium atrosepticum DSM18077	DMSZ	–

[a] Abbreviations of the bacterial strain sources used. CIT; Cork Institute of Technology culture collection (Cork, Republic of Ireland). ATCC; American Type Culture Collection (USA). DMSZ; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell cultures, Germany).

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