Discrimination between some Mycoplasma spp. and Acholeplasma laidlawii in bovine milk using high resolution melting curve analysis

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Abstract

Objectives: This study aimed to provide a rapid, accurate and cost-effective diagnostic real time polymerase chain reaction-high resolution melting curve assay (PCR-HRM) to identify and distinguish between four different mycoplasmas and Acholeplasma laidlawii isolated at cow-level from a single commercial dairy farm in South Australia. One set of genus-level universal primers was designed targeting the 16S ribosomal RNA gene.

Results: Real time PCR-HRM analysis was able to identify and distinguish between five different mollicutes, namely A. laidlawii, M. arginini, M. bovirhinis, M. bovis and uncultured Mycoplasma. Results were confirmed through sequencing. Our developed assay provides rapid and accurate screening for Mycoplasma mastitis detection.

Keywords: Mycoplasma, Mastitis, Cattle, Milk, Acholeplasma

Introduction

Mycoplasma mastitis is of emerging significance worldwide, posing significant economic impacts on the dairy industry. Early detection of Mycoplasma mastitis is important to disease control strategies [1]. Several Mycoplasma spp. are mainly responsible for mastitis, including M. bovis, M. bovoculi, M. alkalessence, M. alvi, M. bovigenetallium, M. bovirhinis, Mycoplasma species bovine group 7, M. californicum, M. dispar, M. canis, M. verecundum, M. canadense and M. mycoides subsp. mycoides [2]. Acholeplasma spp. may be isolated from milk either as a contaminant [3] or as a co-invader with other mycoplasmas [4, 5]. Conventional microbial culture of mollicutes can be laborious and time-consuming with a variety of species-specific growth requirements [6]. Misdiagnosis of Mycoplasma using serological detection is common due to the lag period required for antibody formation. Therefore, a rapid and accurate diagnostic assay is required for screening of mycoplasma in dairy herds. High resolution melting curve analysis (HRM) has been recently developed and widely used for phenotyping at strain or species-level of various organisms including mycoplasmas [7, 8]. However, field isolates of mastitis related mycoplasmas and other milk environmental mollicutes have not been assessed previously using this method.

The aim of this study was to provide a suitable diagnostic real time polymerase chain reaction-high resolution melting curve analysis (PCR-HRM) to identify and distinguish between five different mollicutes isolated at cow-level from a single commercial dairy farms in South Australia.

Main text

Methods

Samples were selected based on conventional PCR findings of a previous study conducted on single commercial dairy farm in South Australia. This farm had a history of repeated mastitis treatment failure with high somatic cell
count (SCC) and poor response to antimicrobials [4]. Six isolates for each of the following species were selected in this study: A. laidlawii, M. arginini, M. bovirhinis, M. bovis and uncultured mollicutes. One set of genus-level universal primers targeting the 16S rRNA gene was designed for real time-PCR. Forward primer Mol-F: GGC GAAYGGGTAGTAACAC and reverse primer Mol-R: CATHGYCTTGGTRGCYNTTA. The real time PCR mixture was prepared using HRM kit AccuMelt HRM SuperMix (Quantabio, Australia). DNA amplification was conducted in a 96 microplate (Illumina, San Diego, CA, USA). Each well contained 10 µL reaction solution of 5 µL HRM SuperMix, 1 µL DNA template (approximately 20 ng), 1 µL each primer (0.2 nmol) and 2 µL nuclease free water (Qiagen, Germany). The reaction was conducted using an Illumina Thermal Cycler with preheating activation for 2 min followed by 40 PCR cycles of three steps: denaturation at 95 °C for 15 s, annealing at 60 °C for 45 s, then extension at 72 °C for 15 s. HRM was performed at 55–95 °C at the rate of 0.1 °C. Results were analysed via EcoStudy software (version 5.0, Illumina). PCR products were subject to electrophoresis in 1.5% agarose gels and visualised by staining with Gel Red. PCR products from the 16S rRNA gene were submitted to the Australian Genome Research Facility Ltd (AGRF, Adelaide, South Australia) for Sanger sequencing. Each fragment was sequenced in forward and reverse directions. To reconstitute the sequence, forward and reverse sequences were edited and assembled using BioEdit package v.7.0.4.1. Edited sequences were blasted against existing sequences in GeneBank using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences from relevant Mycoplasma strains were used as reference strains for nucleotide alignments using ClustalW program version 2.

Results
Five different mollicutes, A. laidlawii, M. arginini, M. bovirhinis, M. bovis and uncultured mollicutes, produced normalised and derivative melt curves (Fig. 1). A. laidlawii (Accession No. LC201977.1) generated one melting peak at 81.2 °C, M. arginini (Accession No. LC158832.1) generated two melting peaks at 88.5 and 84.7 °C. M. bovirhinis (Accession No. AP018135.1) generated three melting peaks at 85.7, 77.6 and 88.2 °C. M. bovis (Accession No. KX462439.1) generated two melting peaks at 77.6 and 85.2 °C. Uncultured Mycoplasma spp. (Accession No. LT679634.1) generated one melting peak at 83.9 °C.

Discussion
While conventional culture, the traditional method for mollicute detection, imposes technical challenges in distinguishing between milk pathogenic and saprophytic mollicutes, our study indicated that real time PCR-HRM assay provides a sensitive, rapid and cost-effective screening method to identify and discriminate between

![Normalized Melting Curve](image1)
![Difference Melting Curve](image2)

Fig. 1 Normalised and different high resolution melting curves generated by EcoStudy software of five different mollicutes isolated from bovine milk: purple—Acholeplasma laidlawii, green—Mycoplasma arginini, blue—M. bovirhinis, yellow—M. bovis, red—uncultured Mycoplasma spp.
some pathogenic and environmental mollicutes isolated from milk DNA. Effects of some of these mollicutes on SCC and milk composition have been previously studied [4]. We considered SCC as the crucial factor that discriminates between contagious and environmental mollicutes. M. bovis was widely reported as a main mastitis causing Mycoplasma [9, 10]. Inconsistent results have been reported regarding involvement of M. arginini, M. bovirhinis and A. laidlawii in bovine mastitis, particularly with co-infection Mycoplasma mastitis [5, 11, 12]. However, several studies indicate these mollicutes are not significant pathogens [3, 13].

Melting profile, introduced in 2002, is widely used for genotyping a wide range of microorganisms [14–17]. HRM-based assay describes correlation between temperature and DNA extent of denaturation [18]. The variety of melting temperatures for different species is attributed to DNA length, sequencing and GC content [14]. In summary, as an alternative to sequencing, our developed real time PCR-HRM assay offered a rapid, low-cost and simple discriminative method to distinguish between some mastitis causing pathogenic mycoplasmas and other saprophytic mollicutes in bovine milk. This method was useful for screening of Mycoplasma mastitis and can be extended to identify more mollicutes species.

Limitations
One of the limitations of HRM-based analysis in Mycoplasma mastitis detection is the inability to detect co-infection cases due to amplimer concentration differences and the requirement of separation each individual amplicon Tm [18]. Primers used in this study were designed to target more spp. of major Mycoplasma-causing mastitis in dairy herds. However, in this study, we used only field isolates of five different mollicutes.

Abbreviations
HRM: high resolution melt; PCR: polymerase chain reaction; SCC: somatic cell count.

Authors’ contributions
AAA, FH and KP participated in the study design and coordination. AAA, KP and AH contributed to sample collection. AAA and FH contributed in sample processing and real time PCR in the PC2 laboratory. RU designed the primers for HRM analysis All authors were involved in drafting the manuscript, corrected. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent to publish
Not applicable.

Ethics approval and consent to participate
Not applicable. Samples were collected by field veterinary support as a part of the mastitis investigation as per farmer request and no Animal Ethics application was required (Australian code for the care and use of animals for scientific purposes, 8th edition, 2013).

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