



High-resolution melting curve analysis: a novel method for identification of *Mycoplasma* species isolated from clinical cases of bovine and porcine respiratory disease

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Abstract

Mycoplasma species cause wide ranges of infectious diseases in human and animals. The aim of the present study was to evaluate a real-time polymerase chain reaction (RT-PCR) followed by a high-resolution melting curve assay (HRM) for rapid differentiation of *Mycoplasma* species isolated from clinical cases of bovine and porcine respiratory disease. Lung samples from suspected cases to respiratory infections from cows and pigs were cultured on specific media, and the extracted DNA were tested by conventional polymerase chain reaction (PCR) assays for *Mycoplasma*. A set of universal primers specific for the 16S ribosomal RNA gene was designed and used for RT-PCR and HRM. The HRM analysis was able to differentiate between five different species of *Mycoplasmas*, namely, *M. hyopneumoniae*, *M. bovis*, *M. hyorhinitis*, *M. hyosynoviae* and other uncultured *Mycoplasma*. All results were confirmed based on 16S rRNA gene sequencing. This rapid and reliable assay was as a simple alternative to PCR and sequencing, differentiating bovine and porcine mycoplasmas in species level.

Keywords *Mycoplasma* · Identification · Bovine · Porcine · Respiratory disease

Introduction

Mycoplasmas are very small cell-wall deficient bacteria belong to the Mycoplasmataceae family in the Mollicutes class, which also contains ureaplasmas, achleplasmas, spiroplasmas and the newly classified haemoplasmas (Nicholas 2004; Caswell and Archambault 2008). Many species of *Mycoplasma* are clinically important in veterinary medicine, because they cause a range of diseases in animals including respiratory infection, mastitis, conjunctivitis, arthritis and abortion (McAuliffe et al.

2004). Mycoplasmas are responsible for huge economic losses in production animals worldwide particularly in the dairy industry as a cause of mastitis, and in intensive livestock industries primarily as respiratory pathogens. For example in Europe, the losses attributed to respiratory disease in cattle caused by *Mycoplasma bovis* are estimated to be €576 million per year. In the USA, this organism causes losses of approximately 32 million USD per year due to the loss of the weight gain and the reduced carcass value (Surýnek et al. 2016). It seems that the losses due to mastitis caused by *M. bovis* are higher than those due to respiratory disease in which the loss of milk production due to mastitis is estimated to be \$108 million per year in the USA (McAuliffe et al. 2004).

To identify veterinary important mycoplasmas, isolation on specific selective media is routinely used; however, *Mycoplasma* spp. are generally fastidious and slow growing; consequently, their isolation in pure culture is very difficult and time consuming (Jeffery et al. 2007). Conventional methods based on serological tests, such as the indirect fluorescent antibody test (IFAT), can be used for *Mycoplasma* species identification; however, they are time-consuming and need professional interpretation (McAuliffe et al. 2003; Ghorashi et al. 2010).

Recently, emphasis has been given to rapid identification of *Mycoplasma* species. For *Mycoplasma* speciation, 16S

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ribosomal RNA (rRNA) gene sequence analysis has been applied but this test is also time consuming and expensive. Most recently, high-resolution melting (HRM) curve analysis of real-time PCR products has been used for *Mycoplasma* speciation and strain typing (Jeffery et al. 2007; Ghorashi et al. 2010; Rebelo et al. 2011).

The aim of this study was to evaluate a reliable and rapid test to detect *Mycoplasma* species by applying real-time polymerase chain reaction (PCR) with high-resolution melting curve analysis (RTPCR-HRM) using 1 set of universal primers targeting the 16S rRNA gene.

Materials and methods

Collection and preparation of samples

Lung samples from clinical cases of bovine and porcine respiratory disease were aseptically collected from different feedlots and abattoirs involved in intensive piggy herd health monitoring over a 4-month period (from June to September 2017). Samples were kept on ice and were sent immediately to the infectious diseases laboratory at school of animal and veterinary sciences, The University of Adelaide. Upon arrival, lung samples were subjected to conventional bacterial culture and susceptibility testing. All samples were then frozen at $-20\text{ }^{\circ}\text{C}$ for further analysis.

Isolation and identification of *Mycoplasma*

The samples were cultured in Mycoplasma broth base (Oxoid™) enriched with Mycoplasma selective supplement G (Oxoid) and incubated at $37\text{ }^{\circ}\text{C}$ for 5 days. Five hundred microliters of a well-grown culture were transferred into a fresh Mycoplasma broth and agar using a sterile syringe through a $0.45\text{-}\mu\text{m}$ filter and spread plated on the agar. After 5–10 days of incubation at $37\text{ }^{\circ}\text{C}$ in a 5% CO_2 atmosphere, typical Mycoplasma colony morphology was evaluated and subcultured to a fresh agar plate for storage at $4\text{ }^{\circ}\text{C}$.

DNA extraction

A fresh supplemented Mycoplasma broth was inoculated with a single Mycoplasma colony and incubated 5 days at $37\text{ }^{\circ}\text{C}$ for DNA extraction. One milliliter of the culture was used for DNA extraction using QIAamp DNA extraction Kit (Qiagen, Germany), according to manufacturer's instructions. Genomic DNA concentration was measured using Nanodrop 1000c (ThermoFisher Scientific Inc., Waltham, MA, USA) and stored at $-20\text{ }^{\circ}\text{C}$ for further use.

Polymerase chain reaction (PCR)

A conventional genus-specific PCR was performed on all isolates using the primers that their sequences are displayed in Table 1 with the resulting 280-bp amplicon. Amplification of genomic DNA to detect *Mycoplasma* was carried out in $25\text{ }\mu\text{L}$ volumes consisting of $0.5\text{-}\mu\text{L}$ Taq DNA polymerase, $5\text{ }\mu\text{L}$ of $\times 5$ reaction buffer (Bioline, UK), $1\text{ }\mu\text{L}$ forward primer, $1\text{ }\mu\text{L}$ reverse primer, $1\text{ }\mu\text{L}$ of template DNA, and $16.5\text{ }\mu\text{L}$ of DEPC water. Using the T100™ Thermal Cycler (Biorad, USA), the PCR amplification was done with 35 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 30 s, annealing at $58\text{ }^{\circ}\text{C}$ for 30 s, and elongation at $72\text{ }^{\circ}\text{C}$ for 1 min. The initial denaturation and final extension were $95\text{ }^{\circ}\text{C}$ for 3 min and $72\text{ }^{\circ}\text{C}$ for 5 min, respectively. *Mycoplasma bovis* ATCC 25523 and the reaction mixture with no-template were used as a positive and negative controls.

The PCR products were detected by electrophoresis on a 1.5% agarose gel and staining with Gel Red.

Real-time PCR and HRM

One set of genus level universal primers specific for the 16S rRNA gene was used for real time-PCR (Table 1). The real-time PCR mixture was prepared using HRM kit AccuMelt HRM SuperMix (Quantabio, UK). DNA amplification was conducted in a 48 microplate (Illumina, San Diego, CA, USA). Each well contained $10\text{ }\mu\text{L}$ reaction solution of $5\text{ }\mu\text{L}$ HRM SuperMix, $1\text{ }\mu\text{L}$ DNA template (approximately 15 ng), $1\text{ }\mu\text{L}$ each primer (0.2 nmol) and $2\text{ }\mu\text{L}$ 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 $95\text{ }^{\circ}\text{C}$ for 15 s, annealing at $58\text{ }^{\circ}\text{C}$ for 45 s then extension at $72\text{ }^{\circ}\text{C}$ for 15 s. HRM was performed at $55\text{--}95\text{ }^{\circ}\text{C}$ at the rate of $0.1\text{ }^{\circ}\text{C}$. Results were analysed via EcoStudy software (version 5.0, Illumina). PCR products were analysed with electrophoresis in 1.5% agarose gel and staining with Gel Red (Al-Farha et al. 2018). For each test, *Mycoplasma bovis* ATCC 25523

Table 1 Primers sequences used for the detection and species identification of *Mycoplasma* spp.

Primers	Sequence (5→3)
PCR	
Forward primer	GGGAGCAAACAGGATTAGATACCT
Reverse primer	TGCCACATCTGTCAGTCTGTTAACCTC
Real-time PCR and HRM	
Forward primer	GGCGAAYGGGTGAGTAACAC
Reverse primer	CATHGYCTTG GTR RGCYNTTA

and *Escherichia coli* ATCC 25922 were included as positive and negative controls, respectively and also a no-template control to exclude contaminations in the reaction mixture. The assay was carried out in triplicates for all the samples. Moreover, twofold serial dilutions made from DNA template per *Mycoplasma* isolate were subjected to real-time PCR to determine sensitivity of the test.

Sequencing

Amplified PCR products from the 16S rRNA gene were purified using a Gel Purification Kit (Qiagen, Germany) and were submitted to the Australian Genome Research Facility Ltd. (AGRF, Adelaide, South Australia) for Sanger sequencing. Each fragment was sequenced in both forward and reverse directions. The sequences were blasted against existing sequences in GeneBank using the basic local alignment search tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Al-Farha et al. 2018).

The sequencing results were considered as golden standard and were compared with the HRM results. All HRM curves were hundred percent matched with the sequences.

Results

A total of 350 lung samples from clinical cases of bovine and porcine respiratory disease were collected (38 bovine and 312 porcine). A total of 10 of the bovine and 42 of the porcine origin samples yielded putative mycoplasma-like colonies on selective culture, which were subsequently identified as *Mycoplasma* spp. by conventional PCR (Fig. 1).

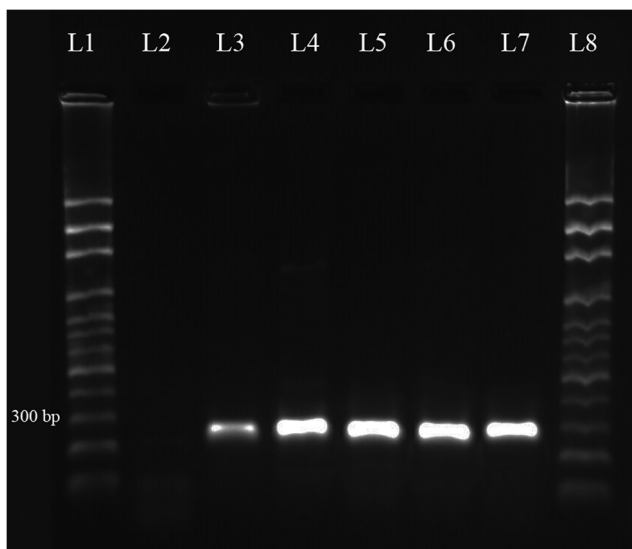


Fig. 1 Results of the PCR assay. 280-bp product in the 1.5% agarose gel. Lane L1 and L8, DNA marker (100-bp); L2, negative control; L3, positive control; L4, L5, L6 and L7; positive samples

Based on the Sanger sequencing results, all the isolates from bovine samples were *M. bovis* but the porcine origin samples belonged to other species of mycoplasma including *M. hyosynoviae*, *M. hyopneumoniae*, *M. hyorhinis* and uncultured *Mycoplasma* spp. Based on the HRM curve analysis, different mycoplasmas produced normalised and derivative HRM profiles consisted of melting temperatures and the overall shape of the melting curves (Fig. 2). *M. hyosynoviae* (Accession No. AF465777.1) generated one melting peak at 79.7; *M. hyopneumoniae* (Accession No. KY307831.1) generated two melting peaks at 79.7 and 83.7 °C; *M. hyorhinis* (Accession No. LT699248.1) generated two melting peaks 80.3 and 83.9 °C and *M. bovis* (Accession No. KX462439.1) generated 1 melting peak at 78 °C. Uncultured *Mycoplasma* spp. (Accession No. KY088285.1) generated one melting peak at 84 °C. There was no difference in the melting profiles of *Mycoplasma* isolate from 1:2, 1:4, and 1:8 fold differences in DNA template concentration.

Discussion

Since 2002, real-time high-resolution melting profile of amplified DNA has been extensively used for genotyping a wide range of bacteria (Miller et al. 2015; Zahidi et al. 2015; Ren et al. 2017). HRM-based assay defines the correlation between temperature and DNA extent of denaturation (Tong and Gifford 2012). According to the DNA length, nucleotide arrangement and GC content, the melting temperatures can consistently differentiate organisms at the species and sub-species level (Reed et al. 2007).

Recently, some bacterial isolates, including *Mycoplasma*, *Listeria*, *Brucella*, *Lactobacillus*, *Pasteurella* and *Staphylococcus* have been identified successfully using HRM analysis of the 16S rRNA gene (Miller et al. 2015, Zahidi et al. 2015, Ren et al. 2017). This approach has also been used for both speciation and strain typing of *Mycoplasma* (Ghorashi et al. 2010; Al-Farha et al. 2018). In one study, a RTPCR-HRM assay was used to identify *Mycoplasma* species isolated from ruminant, avian and canine origin samples. It was able to distinguish *M. arginini*, *M. bovigenitalium*, *M. bovis*, *M. bovirhinis*, *M. canadense*, *M. cynos*, *M. spumans*, *M. iowae*, *M. meleagridis* and *M. agalactiae* reference strains (Rebelo et al. 2011). In another study, RTPCR-HRM was used to discriminate between *Mycoplasma* spp. and *Acholeplasma laidlawii* isolated from bovine milk samples from mastitic dairy cows. By this approach, five different mollicutes, including *A. laidlawii*, *M. arginini*, *M. bovirhinis*, *M. bovis* and uncultured *Mycoplasma* were differentiated (Al-Farha et al. 2018). Ghorashi et al. 2010 also differentiated *Mycoplasma gallisepticum* strains using PCR and high-resolution melting curve analysis (Ghorashi et al. 2010). In a study conducted by Hashemi et al. 2018, high-resolution melting-curve analysis on *pvpA* gene was applied for

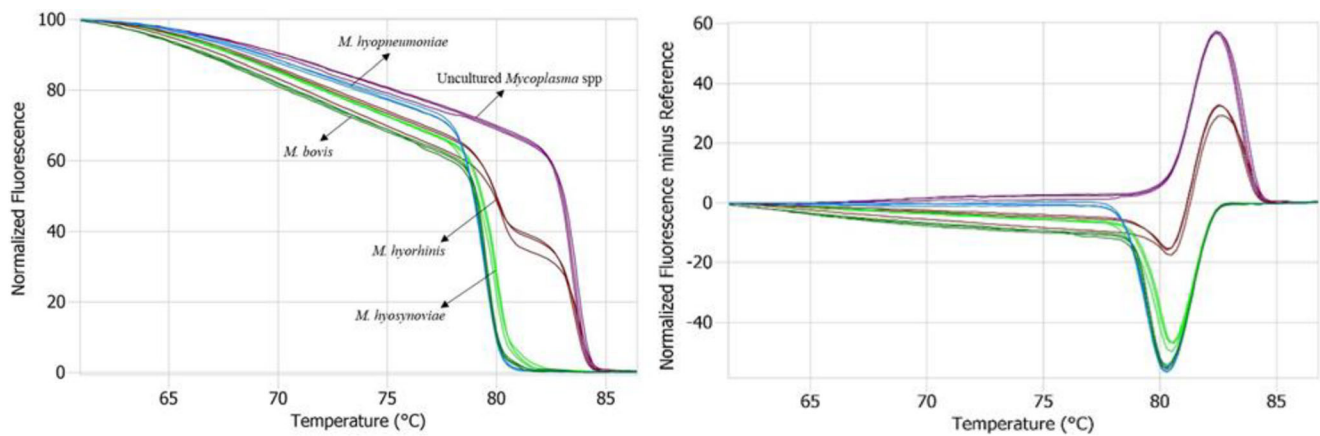


Fig. 2 Normalised and high-resolution melting curves of bovine and porcine *Mycoplasma* species determined by real-time polymerase chain reaction (PCR) coupled with HRM curve analysis (real-time PCR-HRM):

detection and classification of *Mycoplasma gallisepticum* strains (Hashemi et al. 2018). Landolt et al. 2019 also developed a new high-resolution melting (HRM) assay for identification and differentiation of *Mycobacterium tuberculosis* complex samples (Landolt et al. 2019). In this study, we used this approach to discriminate between *Mycoplasma* species isolated from bovine and porcine respiratory disease from different farms in South Australia. It was able to discern different *Mycoplasma* species including *M. bovis*, *M. hyosynoviae*, *M. hyopneumoniae*, *M. hyorhinitis* and uncultured *Mycoplasma* spp. The results indicated that real-time PCR-HRM assay is a rapid and cost-effective screening method that we can rely on to discriminate between different *Mycoplasma* species.

Based on these results, a real-time PCR-HRM assay is a simple alternative to sequencing and a great potential to become a useful diagnostic tool to differentiate *Mycoplasma* species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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red—*M. hyorhinitis*, light green—*Mycoplasma hyosynoviae*, blue—*M. hyopneumoniae*, dark green—*M. bovis*, purple—uncultured *Mycoplasma* spp

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