A novel polymerase chain reaction assay for the detection of seven Mycoplasma species of cattle origin

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Abstract
The study aimed to develop a pair of polymerase chain reaction primers for detecting ruminant mycoplasma pathogens. We designed a set of primers based on the most similar sequences within 16 S rRNA regions of seven Mycoplasma spp. These primers have high sensitivity for detecting Mycoplasma dispar, M. arginine, M. canadense, M. bovis, M. alkalescens, M. californicum, and M. bovigenitalium within the annealing temperature range of 46 to 48 °C. The minimum amount of DNA that can be detected using the protocol is 250 ng, which is equivalent to 2,000 colony-forming units per mL. The primers can detect mycoplasma from DNA extracted directly from milk samples. The common bovine mastitis pathogens of Staphylococcus aureus coagulase-negative staphylococci, Escherichia coli, Streptococcus uberis, Klebsiella pneumonia, and Kocuria rosea were not detected by the primers. We believe the high sensitivity and specificity of these primers make them useful for detecting infection with seven Mycoplasma species in ruminants, allowing the primers to be used in clinical settings.

Keywords Mycoplasma · Polymerase chain reaction · Bovine

Introduction
Mycoplasma species are highly contagious and cause conjunctivitis, arthritis, pneumonia, septicemia, mastitis, and reproductive disorders in ruminants (Parker et al. 2018). Mycoplasma bovis is also associated with other pathogens to cause bovine respiratory disease complex (BRDC) (Higa et al. 2016). However, the difficulty in culturing the pathogen limits the ability for farmers and specialists to apply effective control and preventive interventions. This results in veterinarians and farmers being unaware of an outbreak until the pathogen has spread further. Mass culling is an option, but this is an unfavorable decision considering the economic loss and animal welfare issues. Since 1997, various methods for detecting Mycoplasma spp., such as bacterial culture, molecular detection methods, and immunohistochemical staining of tissue specimens, have been introduced (Parker et al. 2018). However, excluding the molecular detection methods, the remaining aforementioned methods are time-consuming, and occasionally, the results are not reproducible (Higa et al. 2016). Many polymerase chain reaction (PCR) protocols are available for the detection of mycoplasma at the species level, which have been introduced by different researchers (van Kuppeveld et al. 1992; Kobayashi et al. 1998; Bashiruddin et al. 2005; Higuchi et al. 2011; Higa et al. 2016). Genus-level primers have been developed to detect some Mycoplasma spp. such as those developed in 1992 for M. hominis, M. fermentans, M. pulmonis, M. arthritidis, M. neurolyticum, M. muris, and M. collis (van Kuppeveld et al. 1992); for M. bovirhinis, M. alkalescens, and M. bovigenitalium in 1998 (Kobayashi et al. 1998); and for M. bovis in 2005 (Bashiruddin et al. 2005). One report described a successful method to detect M. bovis, M. arginini, M. bovigenitalium, M. californicum,
M. bovirhinis, M. alkalescens, and M. canadense (Higuchi et al. 2011); however, because of patent issues, the sequences of the primers have not been published. A loop-mediated isothermal amplification method was developed to detect M. bovis (Higa et al. 2016); however, no method has been developed to successfully detect the majority of ruminant mycoplasma pathogens.

In Taiwan, as no suitable PCR primers have been developed to detect ruminant mycoplasma, there is an urgent need to develop a new set of primers that will enable the investigation of the current prevalence of mycoplasma infection in ruminants. This study aims to develop a set of universal primers for the detection of M. bovis, M. arginini, M. bovigenitalium, M. californicum, M. alkalescens, M. dispar, and M. canadense through PCR.

Materials and methods

DNA extraction from standard isolates

This study was firstly conducted in a laboratory in Japan, and completed in a Taiwanese laboratory. The standard isolates from Japan were provided by Dr. Higuchi, Rakuno Gakuen University, Hokkaido, Japan. These isolates were cultured in mycoplasma culture broth (Kanto Kagaku, Japan) at 37 °C for 72 h. In Japan, DNA was extracted by boiling the isolates and then adjusting the concentrations of the respective isolates as follows: $10^{-2}$ ng/µL of M. bovis (ATCC 25,523), 0.6 ng/µL of M. arginini (ATCC 23,838), $10^{-1}$ ng/µL of M. bovigenitalium, $10^{-2}$ ng/µL of M. bovirhinis (ATCC, 27,748), $10^{-3}$ ng/µL of M. alkalescens (ATCC 29,103), $10^{-3}$ ng/µL of M. canadense (ATCC 29,418), $10^{-2}$ ng/µL of M. californicum, and $10^{-3}$ ng/µL of M. dispers (ATCC 27,140). In this study, $10^{-1}$ ng/µL was equal to 0.76 pmol/tube.

Sensitivity test using bulk and mastitis milk samples

Bulk milk samples, either infected with (+) or not infected with (−) Mycoplasma bovis, and milk samples from cows with mastitis, which had been previously identified using primers designed by Bashiruddin et al. 2005, were collected from Taiwan’s dairy farms to evaluate the sensitivity of the new set of primers. Milk samples (1 mL) were centrifuged at 3,000 rpm for 10 min, and 100 µL of the liquid supernatant

Fig. 1 The sequences (5’–3’) of all selected mycoplasma pathogens used to design the set of primers. (1) Mycoplasma bovirhinis strain PG43, Sequence ID: LC158834.1; (2) M. bovis PG45 chromosome clone MU clone A2, complete genome Sequence ID: CP002188.1; (3) M. arginini strain MYCO17 16 S rRNA gene, partial sequence. Sequence ID: MK789491.1; (4) M. bovigenitalium strain MYCO6 16 S rRNA gene, partial sequence. Sequence ID: MK789491.1; (5) M. californicum strain HAZ160_1lincomycin Sequence ID: AP018944.2; 6. M. alkalescens gene for 16 S rRNA, complete sequence, strain: PG51 Sequence ID: MK789480.1; (5) M. bovirhinis strain HAZ160_1lincomycin Sequence ID: AP018944.2; 6. M. alkalescens gene for 16 S rRNA, complete sequence, strain: PG51 Sequence ID: LC158831.2; (7) M. bovirhinis strain NCTC10118 chromosome 1 Sequence ID: LR214972.1; (8) M. canadense gene for 16 S rRNA, complete sequence, strain: 275 C Sequence ID: LC158835.1; (9) M. dispar strain GS01 chromosome, complete genome Sequence ID: CP024161.1
was collected. DNA from the milk samples was prepared according to the protocol described for the AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Corning Inc.).

Primer design

Highly specific primers were designed using NCBI’s Primer-BLAST tool based on the 16S rRNA gene sequences of *M. bovis* PG 45, *M. arginini*, *M. bovigenitalium*, *M. californicum*, *M. alkalescens*, *M. canadense*, *M. dispar*, and other *Mycoplasma* species. The main sequence was chosen from the partial 16S rRNA sequence (bases 618 to 850) of *M. bovirhinis* PG43 (Accession: LC158834.1), and the sequence was 90–99% homologous to the 16S rRNA sequence of *M. bovis*, between 316,750 bp and 316,976 bp (Fig. 1). The most similar part was then compared with the region with the highest dissimilarity in other bacterial and animal (including human) genomes, using the U.S. National Library of Medicine, BLAST® tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, the size of the specific amplicon, based on *Mycoplasma bovis* 2093 (taxid: 28903, rr13 and rr14 genes, GenBank: KX462439.1), was 233 bp, and the chosen oligonucleotide sequences (5’–3’) for the newly designed universal primers were as follows:

Forward primer, 5’-TGT AGA GGT TAG CGG AAT TCC-3’; reverse primer, 5’-GAG CAT ACT ACT CAG GC-3’.

Optimization of PCR for *Mycoplasma* spp.

PCR was performed in a total reaction volume of 20 µL containing 2 µL of each forward and reverse primer (10 pmol), 1 µL of double distilled water (DDW), 10 µL of 2X Ampdirect solution (Shimadzu, Japan), and 5 µL of the DNA sample. The PCR conditions used were as follows: initial denaturation at 94 °C for 7 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 46 °C for 40 s, extension at 72 °C for 1 min, and then a final extension step of 72 °C for 7 min. The PCR products were separated through electrophoresis on 2% (w/v) agarose gels, stained with ethidium bromide solution or with HealthyView™ Nucleic Acid Stain (JSB bio Inc., Taiwan), and then visualized with a UV transilluminator.

Limit of detection

To understand the limit of detection for each standard isolate, a 10-fold series dilution was conducted for the DNA of each standard isolate. Then, 5 µL of each serial DNA dilution was used in PCR. The minimum concentration that could be detected for each amplicon was recorded as the limit of detection.

Specificity tests

To test the primers’ specificity, DNA was extracted from *Escherichia coli*, *Streptococcus uberis*, *Klebsiella* spp., *Kocuria rosea*, *Staphylococcus aureus*, and coagulase-negative staphylococci, including *Staphylococcus epidermidis*, *S. chromogenes*, *S. lugdunensis*, *S. xylosus*, and *S. simulans*. The DNA samples were then used as templates to test the specificity of the primer pair. Based on the sequence of the positive amplicons, this set of primers could not detect any species of *Acholeplasma*.

![Fig. 2](image-url) PCR results of *Mycoplasma* spp. at different DNA concentrations. Lane M, marker; Lanes 1 and 9, *Mycoplasma dispar*; Lanes 2 and 10, *M. bovigenitalium*; Lanes 3 and 11, *M. bovirhinis*; Lanes 4 and 12, *M. bovis*; Lanes 5 and 13, *M. alkalescens*; Lanes 6 and 14, *M. californicum*; Lanes 7 and 15, *M. arginini*; Lanes 8 and 16, *M. canadense*. Lanes 1–8 contained 5 pg DNA; Lanes 9–16 contained 0.5 pg DNA.

![Fig. 3](image-url) PCR results of *Mycoplasma* spp. Lane M, marker; Lane 1, *M. dispar*; Lane 2, *M. bovirhinis*; Lane 3, *M. alkalescens*; Lane 4, *M. bovis*; Lane 5, *M. arginini*; Lane 6, *M. bovigenitalium*; Lane 7, *M. californicum*; Lane 8, *M. canadense*.
Results

Detection ability and limit of detection

The designed primers could detect DNA from seven standard isolates under the aforementioned PCR conditions. However, the primers could not detect \( M. \) bovirhinis. The following limits of detection for Mycoplasma species DNA segments for the primer set were estimated: \( M. \) canadense, \( M. \) alkalescens, and \( M. \) dispar: \( 10^{-3} \) µg/µL (0.0076 pmol/tube); \( M. \) californicum and \( M. \) bovis: \( 10^{-2} \) µg/µL (0.076 pmol/tube); \( M. \) bovigenitalium: \( 10^{-1} \) µg/µL (0.76 pmol/tube); and \( M. \) arginine: 0.6 µg/µL (4.56 pmol/tube) (Fig. 2).

Sensitivity and specificity of the developed primers

An established PCR assay for \( M. \) bovis and a newly developed PCR protocol using primers designed in this study were compared. The new primer set was able to detect all \( M. \) bovis DNA in all positive samples (Figs. 3, 4, 5 and 6). The primers did not falsely detect any DNA in the negative samples or any DNA from other bacteria. Therefore, based on our data, the specificity was 100% (Fig. 4).

Discussion

There is a severe lack of research and limited studies on mycoplasma infection in ruminants in Taiwan; \( M. \) bovirhinis, \( M. \) dispar, and \( M. \) alkalescens were first identified in ruminants using biochemical assays in Taiwan in 1982 (data not published); since then, the only research conducted has been a master’s degree–level research project that focused on \( M. \) capri, and the project was completed in 2020 (Cheng 2020). \( M. \) bovis is a severe problem; infection in cattle and goats with other mycoplasma pathogens is less known. Since 2008, an increasing number of outbreaks, which might be related to mycoplasma infections, have been recorded in our laboratory. Therefore, because of the need to establish a PCR assay that detects ruminant mycoplasma pathogens, we developed a useful and practical method for detecting ruminant mycoplasma pathogens. In addition, using these novel primers and the PCR assay, the prevalence of Mycoplasma spp. infection in Taiwan dairy farms could be determined.

The set of primers designed in the current study was based on the sequence of \( M. \) bovis, but after analyzing the resulting amplicon, we could differentiate seven Mycoplasma spp. of cattle origin and some Mycoplasma spp. of goat origin (data not published). The primers can detect Mycoplasma spp. in milk directly, without the need for preculturing. Therefore, an outbreak can be detected within half a day. Subsequently, an effective and efficient control and prevention program can be implemented. A recent report described the limits of detection for other PCR assays (Parker et al. 2018). For example, the limit of detection for PCR was 400 colony-forming units per mL (cfu/mL) in broth cultures (Chavez Gonzalez et al. 1995) and 500 cfu/mL in milk samples after DNA extraction (Hotzel et al. 1996). One study reported that infected cattle can shed \( 10^{5} \) to \( 10^{8} \) cfu/mL in milk, but \( 10^{3} \) to \( 10^{6} \) cfu/mL in milk can be detected in an infected cow before clinical signs become apparent. The same study described that the limit for detection using an ELISA kit was as low as 1,000 cfu/mL in milk after 48 h of incubation (Pfützner and Sachse 1996). Other assays have been developed to detect Mycoplasma spp. and Acholeplasma spp. contaminants in cell cultures or samples from other animals (Anton et al. 1998). However, many of these assays have disadvantages; for example, one assay requires an enzyme digest system to differentiate the pathogens. Some assays have more complex requirements, such as denaturing gradient gel electrophoresis (DGGE) to differentiate species (McAuliffe et al. 2005). Among the other assays developed so far, we believe that only a few are useful for the detection of mycoplasma pathogens in ruminants. The limit of detection for our set of primers is 250 ng of the DNA sample,
which is equal to 2,000 cfu/mL, and the entire operation can be performed within half a day. Therefore, we believe that our method can be used under any condition and can reasonably compete with other published assays.

The limitation of the set of primers must be noted. As this study only tested the main ruminant mastitis pathogens, some opportunistic pathogens, however, may have a chance to bind to the set of primers. Therefore, we would sequence the amplicon every time to avoid misdiagnosis. Although some people may query whether this pair of primers could detect other pathogens, for example, *Acholeplasma* spp. which has a similar sequence. But when the amplicon has been set, we could identify their differences through their DNA. Using above mentioned *Acholeplasma* spp. as an example, their similarity was only about 80% (Appendix I). Furthermore, it would be also helpful to culture the pathogen, or to use high resolution melting curve analysis to avoid the mistakes (Al-Farha et al. 2018). In the future, we hope to develop a real-time polymerase chain reaction technique to improve sensitivity and specificity.

In conclusion, the set of primers we designed has high sensitivity to detect seven *Mycoplasma* species of cattle origin, comprising *M. dispar*, *M. arginine*, *M. canadense*, *M. bovis*, *M. alkalescens*, *M. californicum*, and *M. bovigenitalium*, within an annealing temperature range of 46 to 48 °C. Some goat mycoplasma pathogens can also be detected. Based on its high specificity and sensitivity, we believe this novel set of primers can help farmers and veterinarians to detect outbreaks in the future.

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1007/s11274-022-03312-6.

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**Authors’ contributions** JML and HN conceived and designed the research. JML, SG, and HH conducted experiments. JML, HCL, and HPH contributed new reagents or analytical tools. JML, HPH, and HCL analyzed the data. JML wrote the manuscript. All authors read and approved the manuscript. JML and HN managed the funding.

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**Availability of data and material** All data are shown in the manuscript.

**Declarations**

**Conflict of interest** None of the authors have any conflicts of interest to declare.

**Ethics approval** This article does not contain any studies with animals performed by any of the authors.

**Consent for publication** This material is the authors’ own original work, which has not been previously published elsewhere. The paper is not currently being considered for publication elsewhere. The paper reflects the authors’ own research and analysis in a truthful and complete manner. The paper properly credits the meaningful contributions of co-authors and co-researchers. The results are appropriately placed in the context of prior and existing research. All sources used are properly disclosed. All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content. All authors agree to publish the manuscript in this journal.

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