Research Square

Preprints are preliminary reports that have not undergone peer review. They should not be considered conclusive, used to inform clinical practice, or referenced by the media as validated information.

# A Novel Polymerase Chain Reaction Assay for the Detection of Seven *Mycoplasma* Species of Cattle Origin

**Jyhmirn Lai** ( jyhmirn@mail.ncyu.edu.tw )
National Chiayi University https://orcid.org/0000-0003-2237-1526
**Hengching Lin**
National Chiayi University - Sinmin Campus
**Peichuan Hsu**
Tainan University of Technology
**Satoshi Gondaira**
Rakuno Gakuen University: Rakuno Gakuen Daigaku
**Hidetoshi Higuchi**
Rakuno Gakuen University: Rakuno Gakuen Daigaku
**Hajime Nagahata**
Okayama University: Okayama Daigaku

**Research Article**

**Keywords:** Mycoplasma, Polymerase chain reaction, bovine

**Posted Date:** August 23rd, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-551853/v2

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

# Abstract

The aim of this study was to develop a pair of primers for detecting ruminant mycoplasma pathogens. We designed a set of primers based on the most similar sequences within 16sRNA 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. bovisgenitalium* within the annealing temperature range of 46°C to 48°C. The minimum amount of DNA that can be detected using the protocol is 250 ng, which is equivalent to 2,000 cfu/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*, *Klebiella pneumonia*, *Korucia rosea*, and *Acholeplasma* spp. 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.

# 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 that 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 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 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 to detect some *Mycoplasma* spp. have been developed; for example, primers for *M. hominis*, *M. fermentans*, *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. muris*, and *M. collis* were developed in 1992 (van Kuppeveld et al. 1992); *M. bovirhinis*, *M. alkalescens*, and *M. bovigenitalium* (Kobayashi et al. 1998); and *M. bovis* (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

Standard isolates were provided by Dr. Higuchi, Rakuno Gakuen University, Hokkaido, Japan. Isolates were cultured in mycoplasma culture broth (Kanto Kagaku, Japan) at 37°C for 72 h. 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 25523), 0.6 ng/μL of *M. arginini* (ATCC 23838), $10^{-1}$ ng/μL of *M. bovigenitalium*, $10^{-3}$ ng/μL of *M. bovirhinis* (ATCC, 27748), $10^{-3}$ ng/μL of *M. alkalescens* (ATCC 29103), $10^{-3}$ ng/μL of *M. canadense* (ATCC 29418), $10^{-2}$ ng/μL of *M. californicum*, and $10^{-3}$ ng/μL of *M. dispar* (ATCC 27140). In this study, $10^{-1}$ ng/μL was equal to 0.76 pmol/tube. The DNA from all the positive control isolates were extracted according to the procedure for the AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Corning Inc., USA).

## 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, Frey et al. 2005, were collected from Taiwan 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 was collected. DNA from the milk samples were 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 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. bovisrhinis* PG43 (Accession: LC158834.1), and the sequence was 90% to 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 bacteria and animal (including human) genomes, using the U.S. National Library of Medicine, BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, the size of the specific amplicon, based on *Mycoplasma bovis* 2093 (taxid: 28903, *rr*13 and *rr*14 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 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 seconds, 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, 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*, *Klebiella* spp., *Korucia rosea*, *Staphylococcus aureus*, and coagulase-negative staphylococci, including *Staphylococcus epidermitis*, *S, chromogenes*, *S. lugdunensis*, *S. xylous*, 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*.

## 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 able detect *M. bovisrhinis*. 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). The limit of detection for the primer set can be as low as 0.005 pmol. According to the following equation:

$0.05 \text{ pmol} \times 5 \text{ μL} = 250 \text{ ng}$,

1,000 cfu/mL was equal to 5 ng of DNA, and the limit of detection was 2,000 cfu/mL.

# Sensitivity And Specificity Of The Developed Primers

In this study, 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* DNAin all positive samples (Figs. 3–8). The primers did not falsely detect any DNA in the negative samples or any DNA from other bacteria (data not shown). Therefore, sensitivity was 100% and specificity was 100%.

# 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. From 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 (Parker et al. 2018) described the limits of detection for other PCR assays. For example, the limit of detection for PCR was 400 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 hours of incubation (Pfutzner and Sachse 1996). Other assays have been developed to detect *Mycoplasma* spp. and *Acholeplasma* spp. contaminants in cell cultures or in samples from other animals (Anton, Martinez-Murcia 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.

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. bovisgenitalium*, within an annealing temperature range of 46°C 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.

# Declarations


### Funding:

This study was funded by the Council of Agriculture, Executive Yuan of the Republic of China, Taiwan. No. 110AS-5.1.2-BQ-B1 and Rakuno Gakuen University International Guest Researcher Program in 2017.


### Conflicts of interest:

JML declares that he has no conflict of interest. HCL declares that he has no conflict of interest. PCH declares that she has no conflict of interest. SG declares that he has no conflict of interest. HH declares that he has no conflict of interest. HN declares that he has no conflict of interest.

### Availability of data and material:

All data are shown in the manuscript.

### Authors’ contributions:

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

### 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.

### Acknowledgements

The authors would like to thank all members of the Extension Center and all members of the laboratory managed by Dr. Nagahata and Dr. Higuchi at Rakuno Gakuen University. The authors also thank Rakuno Gakuen University International Guest Researcher Program for their support. This manuscript was edited by Wallace Academic Editing.

## References

1. Anton, A.I., Martinez-Murcia, A.J., Rodriguez-Valera. F., 1998. Sequence diversity in the 16S-23S intergenic spacer region (ISR) of the rRNA operons in representatives of the *Escherichia* coli ECOR collection. Journal of Molecular Evolution, 47,62–72
2. Bashiruddin, J.B., Frey, J., Konigsson, M.H., Johansson, K.E., Hotzel, H., Diller, R., de Santis, P., Botelho, A., Ayling, R.D., Nicholas, R.A., Thiaucourt, F., Sachse, K., 2005. Evaluation of PCR systems for the identification and differentiation of *Mycoplasmaagalactiae* and *Mycoplasmabovis*: a collaborative trial. Veterinary Journal, 169,268–275
3. Chavez Gonzalez, Y.R., Ros Bascunana, C., Bölske, G., Mattsson, J.G., Fernandez Molina, C., Johansson, K.E., 1995. In vitro amplification of the 16S rRNA genes from *Mycoplasmabovis* and *Mycoplasmaagalactiae* by PCR. Veterinary Microbiology, 47,183–190
4. Cheng, C.J., 2020, Seroprevalence and molecular detection of *Mycoplasma* spp. in dairy goat milk in Taiwan, (unpublished Master thesis, National Chung Ching University)
5. Higa. Y., Uemura, R., Yamazaki, W., Goto, S., Goto, Y., Sueyoshi, M., 2016, An improved loop-mediated isothermal amplification assay for the detection of *Mycoplasmabovis*. The Journal of Veterinary Medicine Science, 78,1343–1346
6. Higuchi, H., Iwano, H., Kawai, K., Ohta, T., Obayashi, T., Hirose K, Ito N, Yokota H, Tamura Y, Nagahata H (2011) A simplified PCR assay for fast and easy mycoplasma mastitis screening in dairy cattle. Journal of Veterinary Science, 12,191–193
7. Hotzel, H., Sachse, K., Pfutzner, H., 1996, Rapid detection of *Mycoplasmabovis* in milk samples and nasal swabs using the polymerase chain reaction. Journal of Applied Bacteriology, 80,505–510
8. Kobayashi, H., Hirose, K., Worarach, A., Paugtes, P., Ito, N., Morozumi, T., Yamamoto, K., 1998, In vitro amplification of the 16S rRNA genes from *Mycoplasmabovirhinis*, *Mycoplasmaalkalescens* and *Mycoplasmabovigenitalium* by PCR. Journal of Veterinary Medical Science, 60,1299–1303
9. McAuliffe, L., Ellis, R.J., Lawes, J.R., Ayling, R.D., Nicholas, R.A., 2005, 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. Journal of Medical Microbiology, 54, 731–739
10. Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House. J.K., 2018, A review of mycoplasma diagnostics in cattle. Journal of Veterinary Internal Medicine, 32,1241–1252
11. Pfutzner, H., Sachse, K., 1996, *Mycoplasmabovis* as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. Revue scientifique et technique, 15,1477–1494

12. van Kuppeveld, F.J., van der Logt, J.T., Angulo, A.F., van Zoest, M.J., Quint, W.G., Niesters, H.G., Galama, J.M., Melchers, W.J., 1992, Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. Applied Environmental Microbiology, 58,2606–2615

# Figures

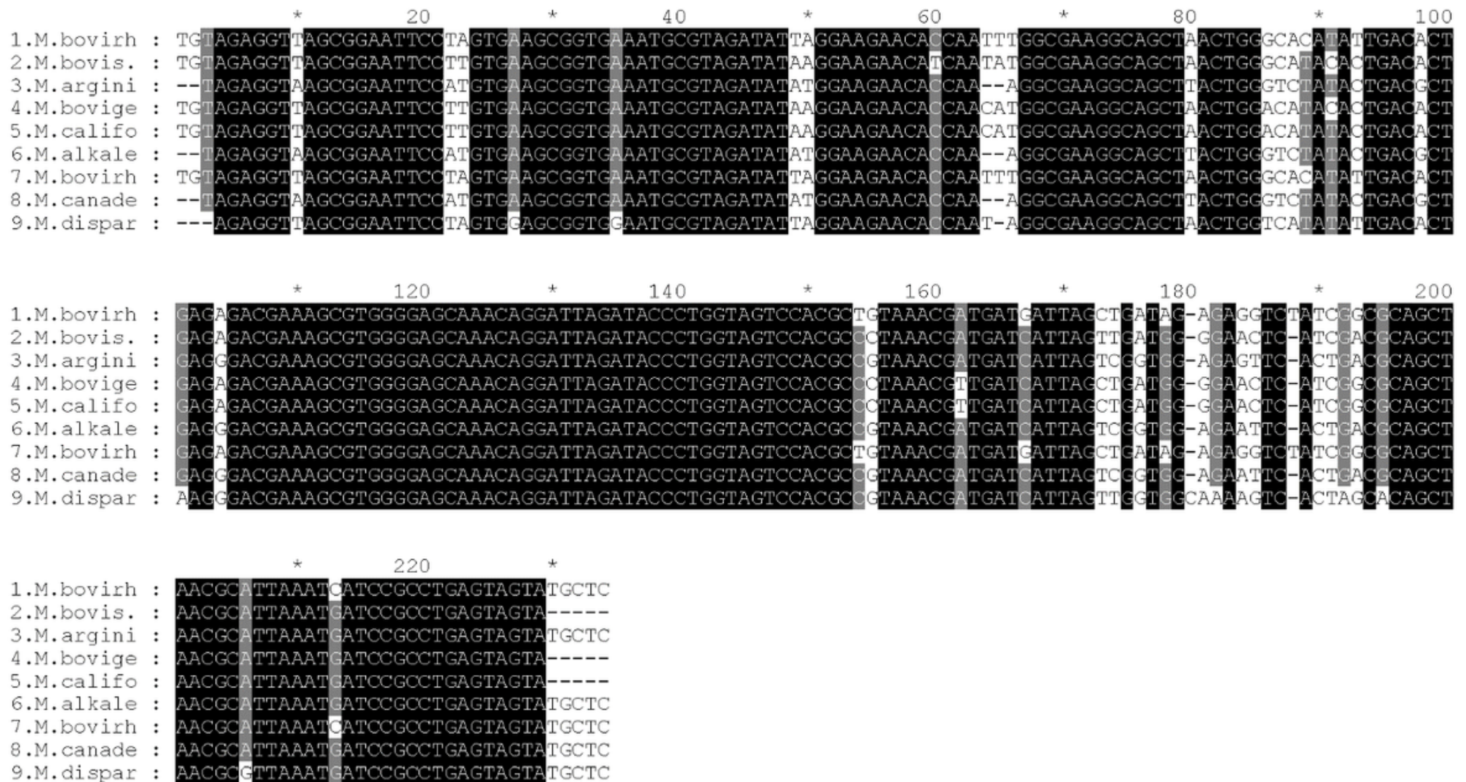


**Figure 1**

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 16S rRNA gene, partial sequence. Sequence ID: MK789491.1; (4) M. bovigenitalium strain MYCO6 16S rRNA gene, partial sequence. Sequence ID: MK789480.1; (5) M. californicum strain HAZ160_1lincomycin Sequence ID: AP018944.2; 6. M. alkalescens gene for 16S 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 16S rRNA, complete sequence, strain: 275C Sequence ID: LC158835.1; (9) M. dispar strain GS01 chromosome, complete genome Sequence ID: CP024161.1

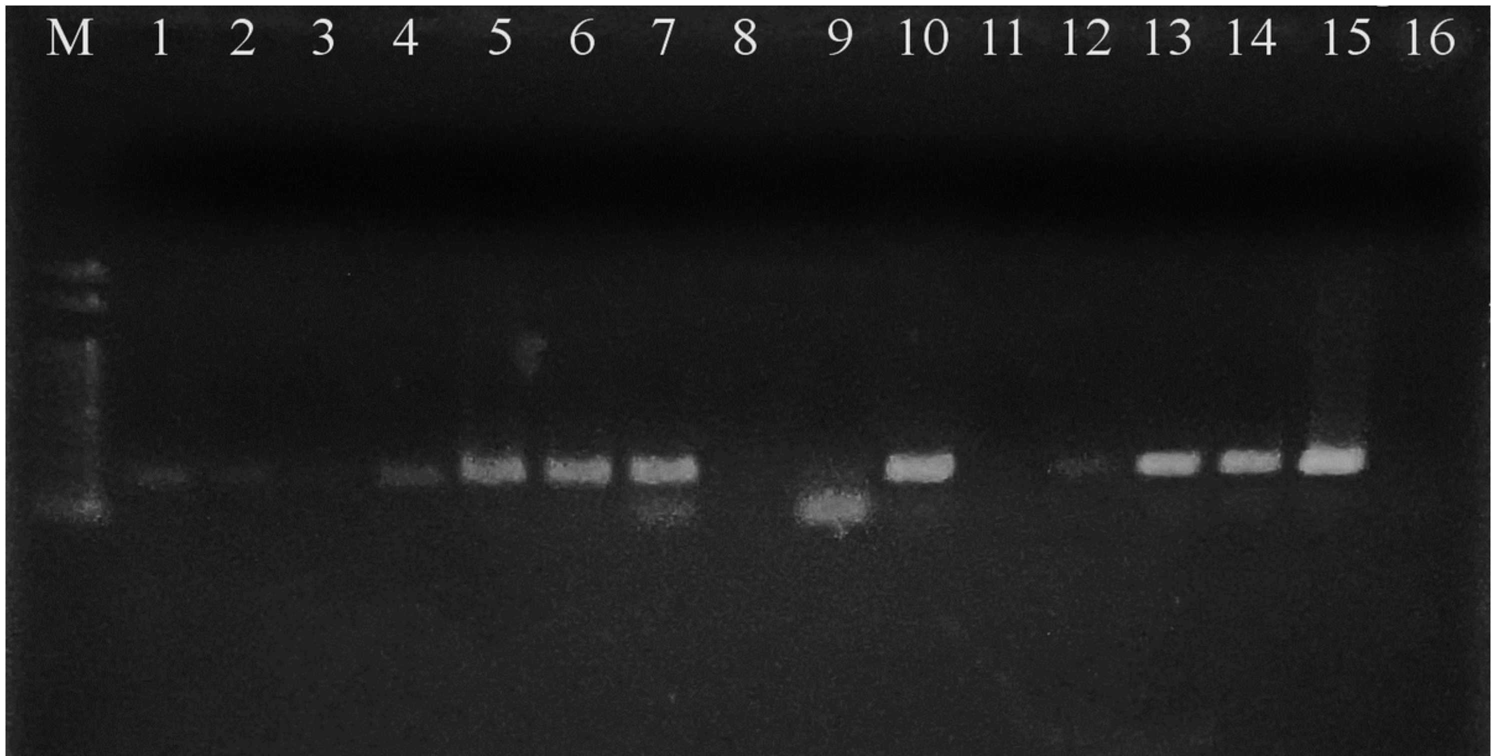


**Figure 2**

PCR results of Mycoplasma spp. at different DNA concentrations. Line M, marker; Lines 1 and 9, Mycoplasma dispar; Lines 2 and 10, M. bovisgenitalium; Lines 3 and 11, M. bovirhinis; Lines 4 and 12, M. bovis; Lines 5 and 13, M. alkalescens; Lines 6 and 14, M. californicum; Lines 7 and 15, M. arginine; Lines 8 and 16, M. canadense. Lines 1–8 contained 5 pg DNA; Lines 9–16 contained 0.5 pg DNA.

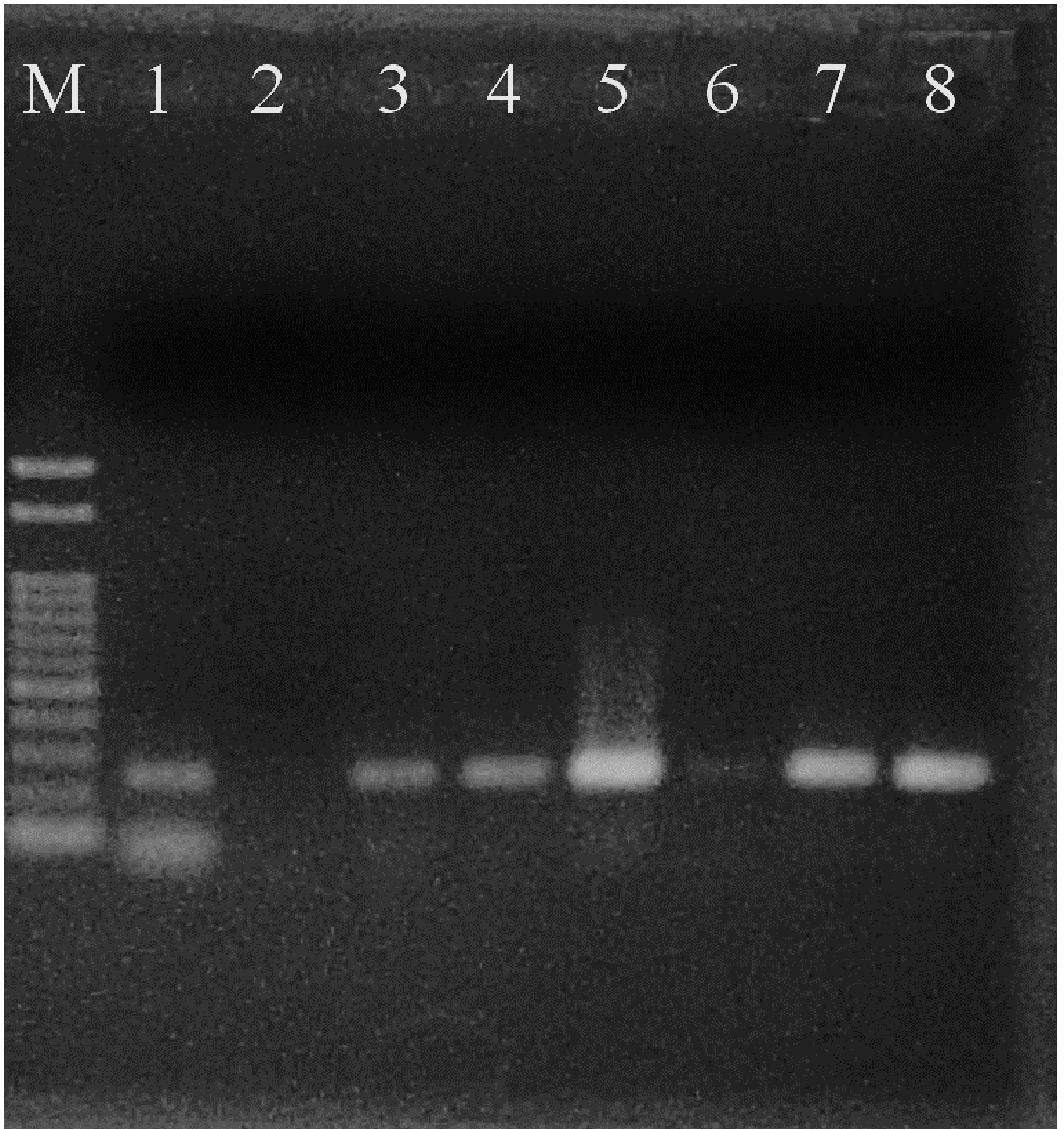


**Figure 3**

PCR results of Mycoplasma spp. Line M, marker; Line 1, M. dispar ; Line 2, M. borvirhinis; Line 3, M. alkalescens; Line 4, M. bovis; Line 5, M. arginini; Line 6, M. bovisgenitalium; Line 7, M. californicum; Line 8, M. canadense

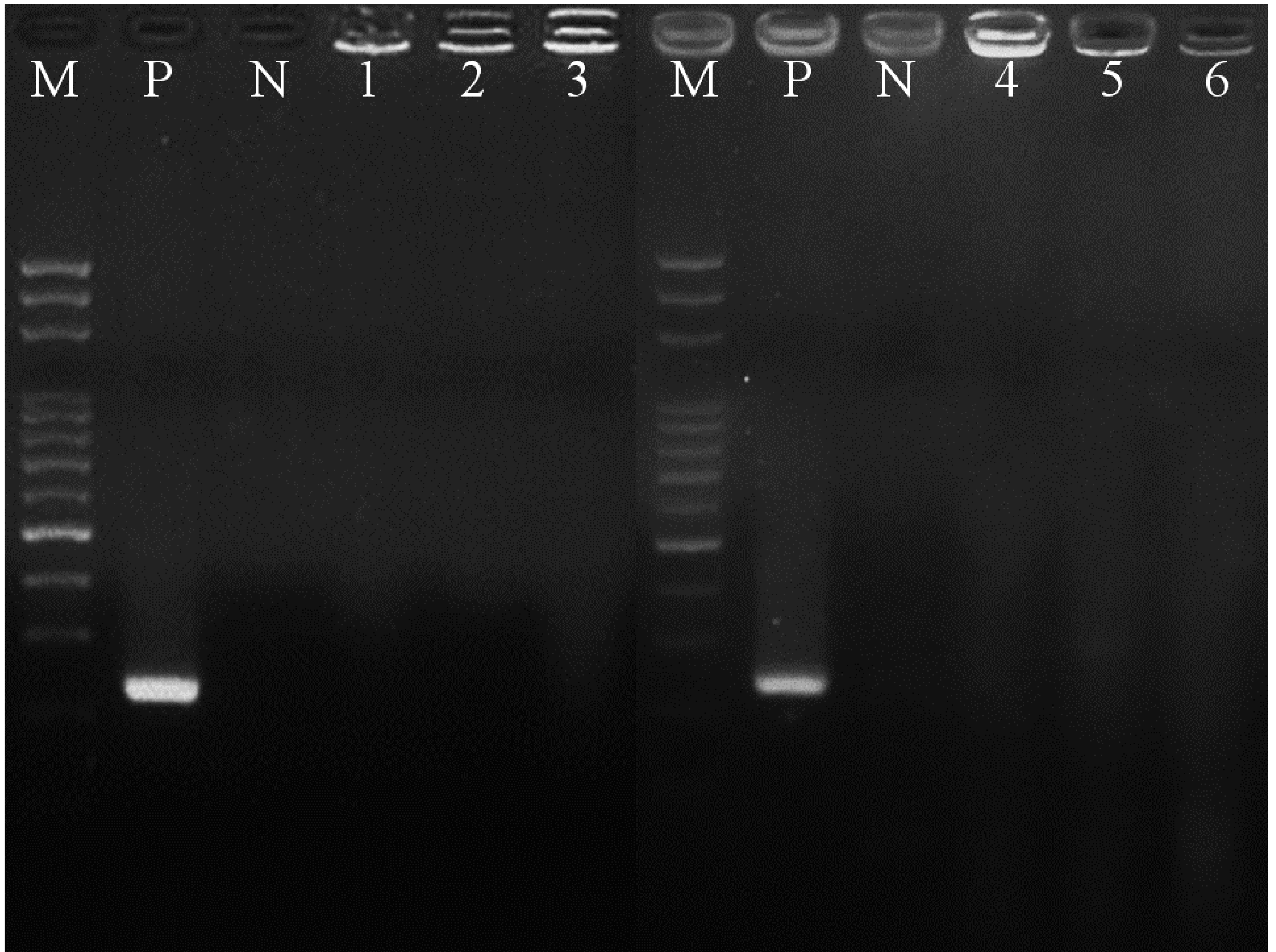


**Figure 4**

Specificity test of the uni primers. Line M, marker; Line P, positive control; Line N, negative control; Line 1, Staphylococcus epidermitis ; Line 2, Staphylococcus aureus; Line 3, Staphylococcus chromogenes; Line 4, Staphylococcus lugdunensis; Line 5, Staphylococcus xylosus; Line 6, Staphylococcus simulans.

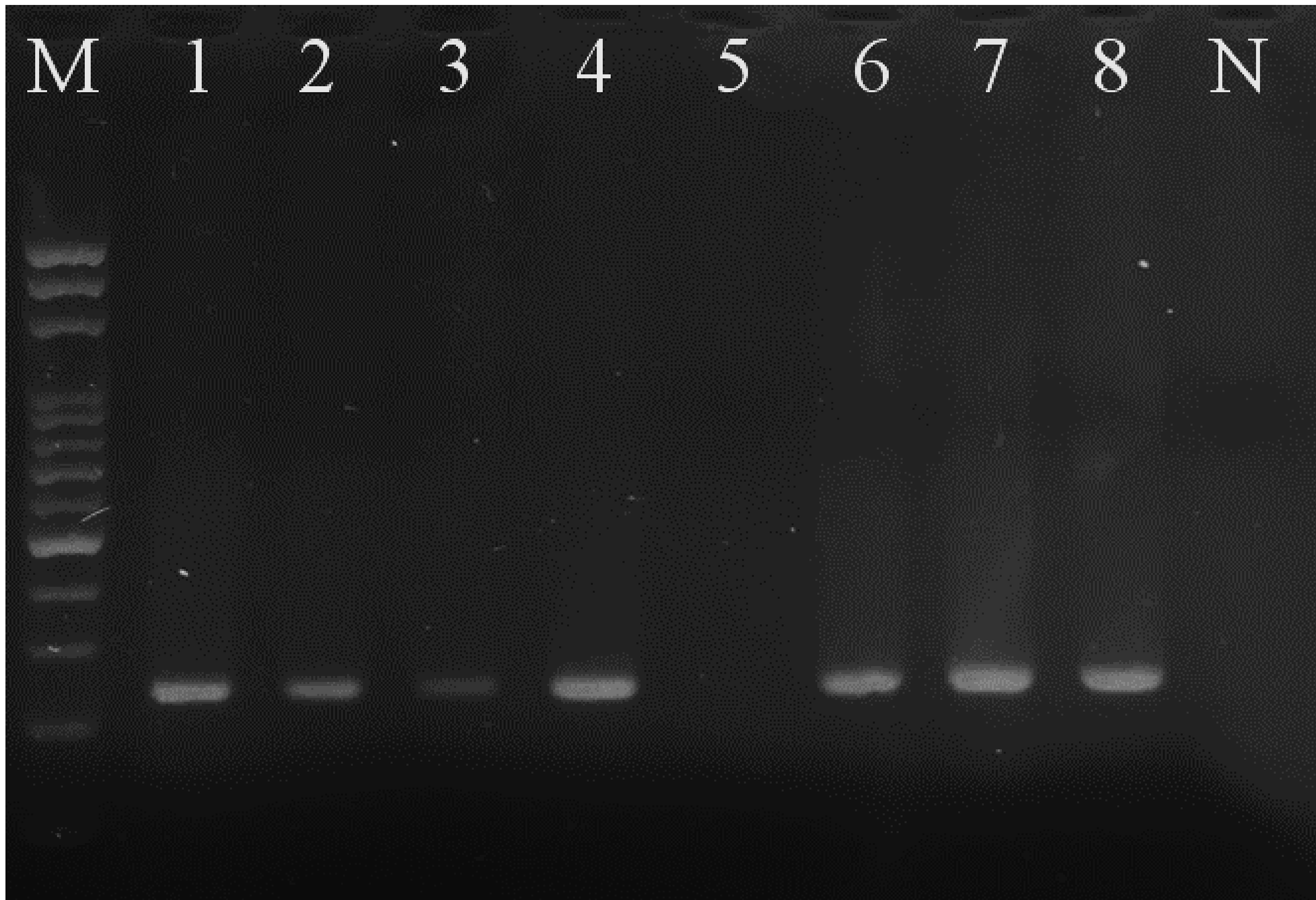


**Figure 5**

Field samples by cotton swab to M. conjuctivae. Line M, marker; Line N, negative control; Line 1-8, swab samples.

**Figure 6**

Field samples by milk. Line M, marker; Line P, positive control; Line N, negative control; Lines 1-10, negative milk samples; Lines 11-14, positive milk samples.

Figure 7

Sensitivity of the uni primer. Line M, marker; Line 1, M. dispar 0.5 pg; Line 2, M. dispar 0.005 pg; Line 3, M. bovisgenitalium 0.005 pg; Line 4, M. borvirhinis 0.005 pg; Line 5, M. bovis 0.005 pg; Line 6, M. alkalescens 0.005 pg; Line 7, M. californicum 0.005 pg; Line 8, M. arginine 0.005 pg; Line 9, M. canadense 0.005 pg.

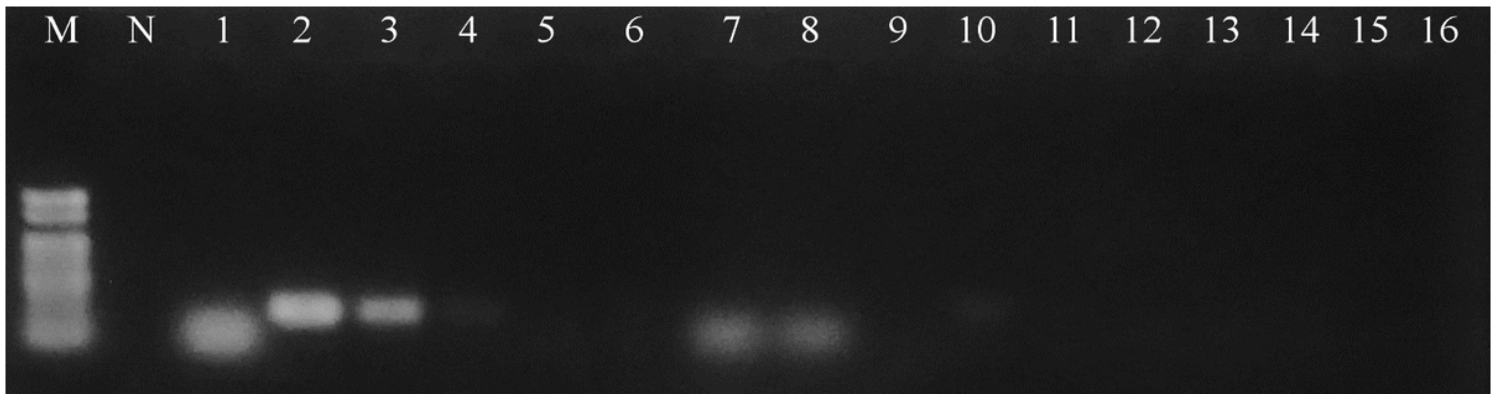


Figure 8

Field samples by milk to M. conjuctivae. Line M, marker; Line N, negative control; Line 1, M. borvirhinis; Lines 2-8, M. canadense 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7 in 46°C; Lines 9-16, M. canadense 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7 in 51°C.