Development and comparison of loop-mediated isothermal amplification and quantitative polymerase chain reaction assays for the detection of *Mycoplasma bovis* in milk

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ABSTRACT

Bovine mastitis is an economic burden for dairies worldwide. *Mycoplasma* species, and especially *Mycoplasma bovis*, are among the most important causative agents, and rapid, precise, and low-cost methods for *Mycoplasma* detection are urgently needed. For this purpose, loop-mediated isothermal amplification (LAMP) and quantitative PCR (qPCR) assays were developed and compared. The LAMP assay was designed and primer concentrations optimized to *M. bovis oppD*, encoding oligopeptide permease D. For qPCR, a Taqman assay (Applied Biosystems, Carlsbad, CA) targeting *M. bovis gltX*, encoding glutamate transfer RNA ligase, was optimized for primer concentration, annealing temperature, and DNA polymerase. Both assays were similarly sensitive, with a detection limit of approximately 10^4 to 10^5 *M. bovis* cells/mL. Both assays were also successful in confirming *M. bovis* identity in laboratory culture suspensions and in bovine milk. The LAMP and qPCR assays combined with the MoBio DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA) resulted in the correct detection of 13 out of 13 *M. bovis* isolates and 14 out of 16 *M. bovis*-positive milk samples collected from commercial dairies in California. When combined with the PrepMan Ultra reagent (Applied Biosystems), the qPCR assay resulted in confirming 21 out of 21 *M. bovis*-positive milk samples. Comparison of the assays to milk containing either *Mycoplasma arginini*, *Mycoplasma bovigenitalium*, *Mycoplasma californicum*, *M. alkalescens*, or *Acholeplasma laidlawii* or milk lacking any detectable *Mycoplasma* species or relatives resulted in 3 out of 17 (LAMP with MoBio), 1 out of 17 (qPCR with MoBio), and 2 out of 36 (qPCR with

PrepMan Ultra) false positives. Overall, the qPCR assay was more robust than LAMP and could be used on DNA recovered from milk prepared with the PrepMan Ultra reagent, a method that does not include a DNA purification step. The use of this qPCR method enables *M. bovis* detection in bovine milk in 40 to 55 min, and therefore provides new opportunities to accelerate and simplify *M. bovis* detection in unpasteurized milk to reduce the incidence of *M. bovis* mastitis outbreaks.

Key words: mastitis, *Mycoplasma*, milk, molecular detection

INTRODUCTION

Inflammation of the udder, also known as mastitis, is the most prevalent disease on commercial dairies and is a significant economic burden to the dairy industry worldwide (Halasa et al., 2007; Bar et al., 2008). Of the numerous recognized mastitis pathogens, *Mycoplasma bovis*, *Staphylococcus aureus*, and *Streptococcus agalactiae* are contagiously transmitted during milking and may cause subclinical or clinical chronic mastitis (Bayoumi et al., 1988; Fox and Gay, 1993; Bar-Gal et al., 2015).

Among these pathogens, species of *Mycoplasma* are of greatest concern because they are resistant to β -lactam antibiotics and are consequently difficult to treat (Jasper, 1977; Caswell and Archambault, 2007; Fox, 2012). *Mycoplasma bovis* is the most common *Mycoplasma* species associated with bovine mastitis, although numerous other species, including *Mycoplasma californicum*, *Mycoplasma alkalescens*, and *Mycoplasma bovigenitalium*, are also known to cause this disease (Jasper, 1977; Jasper et al., 1979; González et al., 1988; Kirk et al., 1997; Fox, 2012). *Mycoplasma* have been isolated from mastitic cattle worldwide (Fox et al., 2005). In the United States, the prevalence of *Mycoplasma* is estimated at 14.4% in larger herds (500 or more cows), 4.2% in medium herds (100 to 499 cows),

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and 1.8% in herds smaller than 100 cows (González et al., 1988; Kirk et al., 1997; USDA, 2008). *Mycoplasma* spp. have a higher prevalence in the western states in the United States, as compared with the eastern states, and in larger herds (500 or more cows; USDA, 2008).

To prevent the further spread of *M. bovis* in dairy herds, rapid, precise, and low-cost methods for detection are urgently needed. We estimated that testing for *Mycoplasma* spp. according to laboratory culture-based methods requires at least 7 d and costs approximately \$6 per milk sample (Murai et al., 2014). Species identification in private milk quality laboratories is commonly based on the assessment of colony morphology using selective media and requires specialized training to distinguish between phenotypically similar colonies. This approach renders identification challenging and sometimes even impossible. Such methods have long been regarded as the gold standard for *Mycoplasma* detection (Baas and Jasper, 1972; Parker et al., 2018); however, it is not possible to distinguish between different *Mycoplasma* species by colony morphology alone, and other non-pathogenic milk contaminants such as *Acholeplasma* species can be falsely identified as *Mycoplasma*. To address this shortcoming, immunofluorescence-based techniques have been used for species verification of single colony isolates (Baas and Jasper, 1972). The use of immunofluorescence is laborious, costly, difficult to perform, and the necessary reagents are not widely available. Overall, the use of laboratory culture to detect *M. bovis* is very time-consuming, expensive, and labor-intensive.

Previous research has developed DNA-based, loop-mediated isothermal amplification (**LAMP**; Bai et al., 2011; Higa et al., 2016; Ashraf et al., 2018) and quantitative (**q**) PCR (Cai et al., 2005; Clothier et al., 2010; Rossetti et al., 2010; Sachse et al., 2010; Boonyayatra et al., 2012; Naikare et al., 2015; Gioia et al., 2016; Parker et al., 2017; Behera et al., 2018) assays to detect *Mycoplasma*. The LAMP is of interest because it does not require expensive laboratory equipment (Notomi et al., 2000; Li et al., 2017). Quantitative PCR assays have advantages over conventional PCR methods because qPCR enables more sensitive and precise detection of multiple strains or species as well as the enumeration of total cell numbers (Naikare et al., 2015; Kralik and Ricchi, 2017). Although both LAMP and qPCR were shown to be applicable for the accurate detection of *M. bovis* and other *Mycoplasma* species in milk (Clothier et al., 2010; Rossetti et al., 2010; Naikare et al., 2015; Parker et al., 2017; Ashraf et al., 2018; Behera et al., 2018), direct comparisons of LAMP and qPCR methods are lacking, as are efforts to optimize the assays to accelerate and simplify detection.

The objective of our study was to develop molecular detection methods for *M. bovis* in raw milk that are accurate, low-cost, and with known detection limits. For this purpose, we designed real-time PCR and LAMP assays and tested them on *Mycoplasma* isolates and milk samples positive or negative for *M. bovis* according to standard laboratory approaches.

MATERIALS AND METHODS

Milk Samples and Bacterial Isolates

A total of 68 milk samples were collected from 9 individual cow mastitic quarters ($n = 9$) and bulk tanks from 59 dairies ($n = 59$) in the Central Valley of California and received by the University of California, Davis, Veterinary Medicine Teaching & Research Center (VMTRC), Milk Quality Laboratory (Tulare, CA). A fraction of each of these milk samples was preserved by freezing at -20°C and the remaining fraction was analyzed for *Mycoplasma* using VMTRC Milk Quality Laboratory culture protocols adapted from the National Mastitis Council (NMC, 2017). For *Mycoplasma* species identification, an established fluorescent staining procedure was used (Baas and Jasper, 1972). Species identity was also confirmed by 16S rRNA gene sequencing as previously described (Lane, 1991). Based on these assessments, the milk samples were either negative for *Mycoplasma* (15 samples) or contained *M. bovis* (23 samples), *M. bovis genitalium* (13 samples), *M. californicum* (8 samples), *M. alkalescens* (5 samples), *Mycoplasma arginini* (2 samples), or *Acholeplasma laidlawii* (2 samples; Supplemental Table S1, <https://doi.org/10.3168/jds.2018-15306>). Additionally, genomic DNA (**gDNA**) from *M. bovis* type strain Donetta PG45 ATCC 25523 (Hale et al., 1962; Wise et al., 2011) was purchased from the American Type Culture Collection (ATCC, Manassas, VA).

The milk samples and the bacterial isolates enriched from those samples were examined in various ways. A total of 21 of the bacterial isolates were selected for direct examination by the LAMP and qPCR assays developed here (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15306>). The milk samples were also distributed for LAMP and qPCR testing with different DNA extraction methods (Supplemental Table S1).

Preparation of Heat-Killed *Mycoplasma* Isolates

For molecular analysis to be performed in the Marco laboratory (Department of Food Science and Technology, University of California, Davis), *Mycoplasma* isolates were grown at the VMTRC Milk Quality

Laboratory in Modified Hayflick agar medium (Veterinary Medicine Biological Media Services, UC Davis, Davis, CA) and incubated at 37°C in 5% CO₂ for 3 d. To facilitate transport between the Tulare and Davis laboratories, the bacterial cells were next collected by centrifugation at 13,800 × *g* for 5 min at 21°C, suspended in 2 mL of PBS, and then incubated for 30 min at 60°C. Heat-inactivation was confirmed by plating 50 µL of the cell suspension onto modified Hayflick agar and incubating at 37°C for 7 d. Samples were held at −20°C until confirming that the *Mycoplasma* cells were inactivated and then shipped to the Marco laboratory at the Davis campus for further testing. These samples were used directly for LAMP and qPCR assays because DNA extraction and purification steps were deemed to be unnecessary.

Genomic DNA Extraction

Cells in 2 mL of milk were collected by centrifugation at 8,000 × *g* for 10 min at 4°C, followed by washing the cell pellets in 3% of tri-sodium citrate buffer (Costa et al., 1996, Quigley et al., 2012) to remove clots or flakes. The DNA was then extracted using the MoBio Power Food Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) or the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Carlsbad, CA) following the manufacturers' instructions. Thirty-three of the milk samples for which DNA was extracted with the MoBio kit were tested by both LAMP and qPCR. For a more comprehensive qPCR analysis with DNA isolated by the PrepMan reagent, 57 milk samples were tested, including 22 samples that were also tested with the MoBio kit (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15306>).

The DNA concentration and purity was measured with the Qubit 3.0 Fluorometer using the Qubit ds-DNA HS Assay Kit (Life Technologies, Carlsbad, CA) and on a NanoDrop 2000c instrument (Thermo Scientific, Waltham, MA). A total of 1 to 2 µL, ranging from between 5 to 100 ng/µL of DNA, was used as template in the subsequent assays. The DNA extractions and

all pre- or postamplification steps were performed in working areas separated from each other to prevent in-laboratory contamination.

LAMP Primers Designed for the Detection of *M. bovis*

To identify genes with high levels of DNA sequence conservation to use in the LAMP assay, 12 *M. bovis* genes required for basic cellular functions (*fusA*, *gyrB*, *gspA*, *rpoB*, *lepA*, *glpQ*, *gltx*, *tkl*, *adh1*, *pta2*, *oppD*, and *oppF*; Register et al., 2015) were compared. All available *M. bovis* DNA sequences for these genes (ranging between 6 to 43 sequences per gene) were obtained from the NCBI Nucleotide database (www.ncbi.nlm.nih.gov/nucleotide). The DNA sequence alignments were performed using the ClustalW alignment tool (Thompson et al., 1994); LAMP primers for *oppD* were designed with the PrimerExplorer V4 program (PrimerExplorer, Eiken Chemical Co. Ltd., Shanghai, China). The LAMP primers included outer primers (MycoF3; MycoB3), 2 inner primers (MycoFIP; MycoBIP), and 2 loop primers (MycoLA; MycoLB) with melting temperatures at 58°C (Table 1). To minimize the possibility of hairpin structures, the 3' ends of MycoF3 and MycoB3 and the 5' ends of MycoFIP and MycoBIP were designed such that they exhibit a change in Gibbs free energy below −4 kcal/mol. The presence of secondary structures and possible formation of primer-dimers were tested with the OligoAnalyzer 3.1 software (OligoAnalyzer, Integrated DNA Technologies Inc., Skokie, IL). The specificity of the primers was assessed by a primer-BLAST search against the NCBI nucleotide (nr) database (Ye et al., 2012). Primers were regarded to be acceptable if they contained a minimum of 2 mismatches to unintended targets and at least 2 mismatches within the last 5 bp of the 3' end.

Customization of LAMP Reaction Conditions

The LAMP was performed in a final volume of 25 µL, containing 1.4 mM of each deoxynucleotide triphos-

Table 1. Loop-mediated isothermal amplification primers targeting *Mycoplasma bovis oppD*

Primer	Sequence primer 5'→3'	Concentration ¹ (nM)
MycoF3	ACTAATCCAGCTCACCCTTA	0.025
MycoB3	CGTTGCTGCTTTATGATGAC	0.025
MycoFIP	TGGGGTTCCTTGAATTGAGAATAAT-TACATGAGCGCTTATCTCG	0.20
MycoBIP	CAGATATGGCAAACCTTACCTATCGG-GGTGGTTCCTTTTCATAGTCAA	0.20
MycoLA	TCTCATCATCATTTTCAGGTATAGC	0.075
MycoLB	TGACCCTTTTGCACCTAGAA	0.075

¹Final concentrations used for testing *Mycoplasma* isolates and milk samples.

phate (Promega, Madison, WI), 8 mM MgSO₄ (New England BioLabs Inc., Ipswich, MA), 1 M betaine (Sigma-Aldrich, St Louis, MO), 0.32 U/μL of Bst 2.0 DNA polymerase (New England BioLabs Inc.), and Bst 2.0 DNA polymerase buffer (New England Biolabs Inc.). For assay development, final concentrations of primers MycoF3 and MycoB3 ranged from 0.0125 to 0.2 μM. Concentrations for the inner primers (MycoFIP and MycoBIP) ranged from 0.1 to 1.6 μM. The loop primer (MycoLA and MycoLB) concentrations ranged from 0.0375 to 0.6 μM. Template DNA was denatured for 3 min at 95°C and then placed on ice for 30 s before addition; LAMP was performed at 58°C with reaction times of 15, 25, 35, 45, or 55 min. Amplification was stopped by inactivating the Bst-polymerase (95°C for 3 min). For each run, positive controls using gDNA equivalent to 1,000 copies of the *M. bovis* type strain Donetta PG45 ATCC 25523 genome were included and nontemplate reactions were used as negative controls. The LAMP products were detected by screening for fluorescence under UV light upon adding 3 μL of SYBR Green I (100×; Life Technologies) into the reaction tubes. Amplification products were also subjected to capillary electrophoresis with the Bioanalyzer DNA 1000 kit (Agilent Technologies, Santa Clara, CA) or to 1.5% agarose gel electrophoresis to check for the typical, ladder-like band pattern generated during LAMP (Notomi et al., 2000; Tomita et al., 2008).

The analytical range, including the lower limit of *M. bovis* detection by the LAMP assay, was determined using 10-fold serial dilutions of *M. bovis* ATCC 25523 gDNA. Estimates of cell numbers and genome equivalents were based on the 1,003,404 bp *M. bovis* ATCC 25523 genome (Wise et al., 2011) and the average molecular weight of a base pair of 617.96 g/mol. Because of the poor utility of the LAMP assay, the analytical range of spiked milk samples was not assessed.

qPCR Primer Design for Detection of *M. bovis*

Mycoplasma bovis genes *gltX* (encoding glutamate tRNA ligase), *gyrB* (DNA gyrase subunit B), and *gspA* (glycerol-3-phosphate dehydrogenase) previously used for multilocus sequence typing (Register et al., 2015) were selected as qPCR targets. All qPCR primer and probes designed in the study are listed in Table 2. Primers were designed using Primer3 version 4.0.0 (Koressaar and Remm, 2007; Untergasser et al., 2012; Ye et al., 2012). All primers were designed to have a melting temperature at 58°C to 60°C and an amplicon size between 82 to 90 bp. The presence of secondary structures and possible primer-dimers were evaluated using OligoAnalyzer 3.1 software (OligoAnalyzer, Integrated DNA Technologies Inc.). The specificity of the primers and probes was assessed by a primer-BLAST search against the NCBI nucleotide (nr) database (Ye et al., 2012). Primers were regarded to be acceptable if they contained a minimum of 2 mismatches to unintended targets and at least 2 mismatches within the last 5 base pairs of the 3' end.

qPCR Assay Conditions

Real-time PCR amplification was performed in 100-μL, 96-well plates on an ABI 7500 Fast Real-time PCR system (Applied Biosystems). Each well contained a final volume of 10 μL of the reaction mix, with TaqMan Universal PCR Master Mix containing the AmpliTaq enzyme (Applied Biosystems) and 250 nM of each primer and Taqman probe. Amplification was performed under standard conditions by incubating at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and then annealing and elongation at either 55, 58, or 60°C for 1 min. Once primer and probe concentration as well as annealing

Table 2. Quantitative PCR primers and probes for *Mycoplasma bovis*

Gene ¹	Function	Primer/probe identification	DNA primer and probe 5'→3' ²
<i>gltX</i>	Glutamate transfer RNA ligase	<i>gltXF</i> <i>gltXR</i> <i>gltXPrD</i>	GAGGCGAAGAACACATTACAAAT TGGCCAAATTCAGGAGATTTTCA 6'FAM-ACACCTAGACAACCTCTCRATT-TAMRA
<i>gspA</i>	Glycerol-3-phosphate dehydrogenase	<i>gspAF</i> <i>gspAR</i> <i>gspAPrD</i>	ACTACTGCTCAATATTAGGCCCA ATTGCTCATTGTGCCCTACA 6'FAM-TGCTRCTGAAGTATTTGAAAATGCAY-TAMRA
<i>gyrB</i>	DNA gyrase subunit B	<i>gyrBFD</i> <i>gyrBR</i> <i>gyrBPr</i>	TGGATCCTGAGCARTTGTGAG GCCTTGGCTGCATCATCAAT 6'FAM-ACTACAATGAATCCTGAGAATAGAAA-TAMRA

¹The *gltX* assay was selected for further testing on *Mycoplasma* isolates and milk samples.
²The concentration of each primer was 250 nM.

temperatures were chosen, DNA polymerases were also tested including the Kapa Probe fast qPCR kit Master Mix (2×) Universal (Kapa Biosystems, Wilmington, MA), the Takara Premix Ex Taq (2×; Clontech Laboratories Inc., Takara Bio Company, Shiga, Japan), and the iTaq Supermix (Bio-Rad Laboratories, Hercules, CA). After 40 PCR cycles, melting curves were generated by increasing the temperature from 60 to 95°C at 0.2°C/10 s and recording the fluorescence. For each run, reactions containing gDNA equivalent to 1,000 copies of the *M. bovis* type strain Donetta PG45 ATCC 25523 were included as positive controls and nontemplate reactions were used as negative controls.

Amplification efficiencies and the analytical range, including the lower limit of *M. bovis* detection, were determined using 10-fold serial dilutions of *M. bovis* ATCC 25523 gDNA. Estimates of cell numbers and genome equivalents were quantified as described for the LAMP assay [based on the 1,003,404-bp *M. bovis* ATCC 25523 genome (Wise et al., 2011) and the average molecular weight of a base pair of 617.96 g/mol]. The analytical range of *M. bovis* detection by qPCR was also determined after spiking PBS containing a known number of heat-killed *M. bovis* NM2012 cells into milk followed by using 10-fold serial dilutions of this suspension for gDNA extraction with the MoBio Power Food Kit and PrepMan Ultra reagent.

Analytical and Diagnostic Accuracies

The lower limits of detection were investigated for the LAMP and qPCR assays. Growth of *Mycoplasma* spp. in laboratory culture in combination with subsequent immunofluorescence staining was used as the reference for comparison. Laboratory culture results were used to confirm whether the sample was positive or negative, presuming that the reference method was 100% accurate. The following data were assessed for each of the LAMP and qPCR tests using culture as the reference test: *M. bovis* prevalence in the milk samples tested, assay accuracy and inaccuracy, sensitivity defined as the proportion of culture-positive samples that have a test-positive status, specificity defined as the proportion of culture-negative samples that have a test-negative status, and the type I and II errors indicating the possibility to obtain false positive (α) or false-negative (β) test results (Parikh et al., 2008; Drobatz, 2009; World Organisation for Animal Health, 2010). A 2-sample test for proportions (Z-test) was used to compare the sensitivity of qPCR using both DNA extraction methods. Similarly, a Z-test was used to compare the sensitivity of qPCR using the PrepMan Ultra reagent to the sensitivity of LAMP assay following DNA extraction with

the MoBio kit. In addition, McNemar's test was used to compare the sensitivities of qPCR and LAMP following DNA extraction with the MoBio kit. Specificities of the assays were compared in a similar manner.

RESULTS

LAMP Assay to Detect *M. bovis*

Comparisons of the DNA sequences for 12 *M. bovis* genes required for basic cellular functions (*fusA*, *gyrB*, *gspA*, *rpoB*, *lepA*, *glpQ*, *gltx*, *tkt*, *adh1*, *pta2*, *oppD*, and *oppF*; Register et al., 2015) resulted in the identification of *oppD* as a suitable target for LAMP assay development. The *oppD* gene, encoding an oligopeptide transport ATP-binding protein (oligopeptide permease D), was selected because it had the highest level of nucleotide conservation between the genes examined (100% nucleotide sequence identity) and was amenable to primer design for the LAMP assay.

Different *oppD* primer concentrations were compared and 0.025 μ M outer primers (MycoF3; MycoB3), 0.2 μ M inner primers (MycoFIP; MycoBIP), and 0.075 μ M loop primers (MycoLA; MycoLB) yielded the most consistent results (Figure 1 and 2). The use of higher primer concentrations (MycoF3 and MycoB3, between 0.05 to 0.2 μ M; MycoFIP and MycoBIP, between 0.4 to 1.6 μ M; and MycoLA and MycoLB, between 0.15 to 0.6 μ M) resulted in false-positive outcomes [e.g., Supplemental Figure S1 (top panel); <https://doi.org/10.3168/jds.2018-15306>], most likely due to the formation of primer dimers [Supplemental Figure S1 (bottom panel)].

The LAMP products were obtained after 45 and 55 min when 1,000 genome equivalents of *M. bovis* ATCC 25523 were used as a template (Supplemental Figure S2; <https://doi.org/10.3168/jds.2018-15306>). No amplification was observed after 15, 25, or 35 min; therefore, 55 min was used to assess the analytical specificity and sensitivity of the assay.

Accuracy of the LAMP Assay

The lower detection limit of the *oppD* LAMP assay when *M. bovis* ATCC 25523 gDNA was used as a template was 10 genome equivalents per reaction, corresponding to 10^4 cells/mL, assuming 1 genome copy per cell (Figure 2). The LAMP assay correctly detected all 13 *M. bovis* isolates and was negative for 6 out of the 8 non-*bovis* *Mycoplasma* tested (Table 3). Two *M. bovis genitalium* isolates (15M0599_27 and 15M0701_17) were found to be positive with the LAMP assay.

For DNA extracted from 33 milk samples using the MoBio Power Food kit, 14 out of 16 samples found to contain *M. bovis* according to standard laboratory culture-based methods were also positive by LAMP (Table 4). Milk harboring *M. arginini* (1 sample) and *M. californicum* (3 samples) were negative for *M. bovis* with LAMP. The LAMP assay was also negative for 3 out of 4 samples containing *M. bovisgenitalium* and 7 out of 9 samples that tested negative for any *Mycoplasma* species (Table 4). For the 3 potentially false-positive results (samples 15M0599_27, AM15, and KM15), DNA extractions were repeated and LAMP positive reactions were confirmed. It was notable that the *M. bovis* LAMP assay was positive for both the whole milk sample 15M0599_27 (Table 4) and the *M. bovisgenitalium* isolate enriched from that sample (Table 3).

Figure 1. Visualization of loop-mediated isothermal amplification (LAMP) products. The LAMP products were separated by (A) capillary gel electrophoresis or stained with SYBR Green (Life Technologies, Carlsbad, CA) for observation under (B) visible and (C) UV light. 1 = positive control, containing 1,000 copies of genomic DNA of *Mycoplasma bovis* type strain Donetta PG45 ATCC 25523; 2 = negative control. The LAMP was performed using a final concentration of 0.025 μ M outer primers (MycF3; MycB3), 0.2 μ M inner primers (MycFIP; MycBIP), and 0.075 μ M loop primers (MycLA; MycLB).

Although the PrepMan Ultra Reagent was also used to extract DNA from milk for LAMP testing, that method was not pursued further because its application yielded inconsistent results (data not shown).

qPCR Assay to Detect *M. bovis*

To develop qPCR assays for *gltX* (encoding glutamate tRNA ligase), *gyrB* (DNA gyrase subunit B), and *gspA* (glycerol-3-phosphate dehydrogenase), different primer concentrations and annealing temperatures were tested using the TaqMan Universal PCR Master Mix (containing the AmpliTaq enzyme). The most consistent amplification efficiencies were obtained with the *gltX* primer-probe pair, at a concentration of 250 nM and amplification performed at 58°C. Next, we compared different qPCR enzymes and found that the iTaq Master Mix required shorter run times and yielded high amplification efficiencies (Supplemental Figure S3; <https://doi.org/10.3168/jds.2018-15306>). Therefore, this enzyme, in combination with the *gltX* qPCR assay, was used in subsequent tests on DNA extracted from milk or heat-killed *Mycoplasma* isolates (Table 3).

Accuracy of the *gltX* qPCR Assay

The lower detection limit for the *gltX* assay was set at a cycle threshold of 37, and this constituted between 10 to 100 genome equivalents per reaction. Those numbers are approximately equivalent to 1×10^4 to 1×10^5 cells/mL, assuming 1 genome copy per cell (data not shown). The lower detection limit of *M. bovis* cells in milk was also equivalent to approximately 1×10^4 to 1×10^5 cfu/mL for both DNA extraction methods (Supplemental Figure S4; <https://doi.org/10.3168/jds.2018-15306>).

The *gltX* qPCR assay correctly identified 13 heat-inactivated *M. bovis* isolates (Table 3). Out of the 8 non-*bovis* isolates examined, 1 isolate of *M. bovisgenitalium* (15M0701_17) and 1 isolate of *M. californicum* (150620_89) tested positive by qPCR (Table 3).

For DNA extracted from 33 milk samples with the MoBio Power Food kit, the qPCR assay was positive for 14 out of 16 *M. bovis*-positive samples (Table 4). The 2 samples (15M753_2 and 15M753_5) that were negative for *M. bovis* by qPCR were also negative according to the *oppD* LAMP assay. One of 4 milk samples containing *M. bovisgenitalium* was positive in the *gltX* qPCR assay (15M0616_40). The remaining 16 *M. bovis* negative milk samples were also negative for *M. bovis* according to qPCR (Table 4).

For testing the PrepMan Ultra reagent with the qPCR assay, a total of 57 milk samples were included

Table 3. Evaluation of loop-mediated isothermal amplification (LAMP) and quantitative PCR (qPCR) assays on heat-inactivated *Mycoplasma* cultures

Culture result ¹	Milk sample identification used for cultivation	LAMP ²	qPCR ²
<i>Mycoplasma arginini</i>	15M0599_19	—	—
<i>Mycoplasma bovigenitalium</i>	15M0599_27	+	—
<i>M. bovigenitalium</i>	15M0616_40	—	—
<i>M. bovigenitalium</i>	15M0616_105	—	—
<i>M. bovigenitalium</i>	15M0701_17	+	+
<i>Mycoplasma bovis</i>	15M0599_28	+	+
<i>M. bovis</i>	15M0616_70	+	+
<i>M. bovis</i>	15M0620_12	+	+
<i>M. bovis</i>	15M0620_23	+	+
<i>M. bovis</i>	15M0620_45	+	+
<i>M. bovis</i>	15M0620_116	+	+
<i>M. bovis</i>	15M0620_137	+	+
<i>M. bovis</i>	15M0620_165	+	+
<i>M. bovis</i>	15M0649_90	+	+
<i>M. bovis</i>	15M0671_98	+	+
<i>M. bovis</i>	15M0681_1	+	+
<i>M. bovis</i>	15M0693_11	+	+
<i>M. bovis</i>	15M0697_1	+	+
<i>Mycoplasma californicum</i>	15M0616_34	—	—
<i>M. californicum</i>	15M0620_89	—	+
<i>M. californicum</i>	15M0693_15	—	—

¹*Mycoplasma* species were identified by culture and fluorescent staining (Baas and Jasper, 1972).

²DNA was amplified from cells that were heat-killed at 60°C and then frozen at –20°C, for which subsequent DNA extraction and purification steps were not performed. Positive test results are indicated by a plus (+) and negative test results by a minus (–).

(Table 5). All 21 of the samples that tested positive for *M. bovis* according to laboratory culture were also positive with the *gltX* qPCR assay (Table 5). Out

of the remaining 36 milk samples that were found to lack any viable *M. bovis* or contained other *Mycoplasma* (or related) species, 2 samples (15M718_22

Figure 2. Analytical sensitivity of the loop-mediated isothermal amplification (LAMP) assay. The LAMP products were stained by SYBR Green I (Life Technologies, Carlsbad, CA) for visual observation under (A) visible and (B) UV light. (C) The LAMP products were also separated by gel electrophoresis. The numbers indicate genome equivalents of *Mycoplasma bovis* type strain Donetta PG45 ATCC 25523 used for LAMP: (1) 10^4 , (2) 10^3 , (3) 10^2 , (4) 10^1 , (5) 10^0 , and (6) negative control.

Table 4. Evaluation of loop-mediated isothermal amplification (LAMP) and quantitative PCR (qPCR) assays on DNA extracted from milk using the MoBio Power Food kit (MoBio Laboratories Inc., Carlsbad, CA)

Culture result ¹	Sample identification	LAMP ²	qPCR ²
<i>Mycoplasma arginini</i>	15M0599_19 ³	—	—
<i>Mycoplasma bovigenitalium</i>	15M0599_27 ³	+	—
<i>M. bovigenitalium</i>	15M0616_40 ³	—	+
<i>M. bovigenitalium</i>	15M0616_105 ³	—	—
<i>M. bovigenitalium</i>	15M0701_17 ³	—	—
<i>Mycoplasma bovis</i>	15M753_2	—	—
<i>M. bovis</i>	15M753_5	—	—
<i>M. bovis</i>	15M0599_28 ³	+	+
<i>M. bovis</i>	15M0616_70 ³	+	+
<i>M. bovis</i>	15M0620_12 ³	+	+
<i>M. bovis</i>	15M0620_23 ³	+	+
<i>M. bovis</i>	15M0620_45 ³	+	+
<i>M. bovis</i>	15M0620_116 ³	+	+
<i>M. bovis</i>	15M0620_137 ³	+	+
<i>M. bovis</i>	15M0620_165	+	+
<i>M. bovis</i>	15M0649_90 ³	+	+
<i>M. bovis</i>	15M0671_98 ³	+	+
<i>M. bovis</i>	15M0681_1 ³	+	+
<i>M. bovis</i>	15M0693_11 ³	+	+
<i>M. bovis</i>	15M0697_1 ³	+	+
<i>M. bovis</i>	15M0697_2	+	+
<i>Mycoplasma californicum</i>	15M0616_34 ³	—	—
<i>M. californicum</i>	15M0620_89 ³	—	—
<i>M. californicum</i>	15M0693_15 ³	—	—
Negative for <i>Mycoplasma</i>	AM15 ⁴	+	—
	BM15 ⁴	—	—
	CM15 ⁴	—	—
	EM15 ⁴	—	—
	FM15 ⁴	—	—
	IM15 ⁴	—	—
	JM15 ⁴	—	—
	KM15 ⁴	+	—
	LM15 ⁴	—	—

¹*Mycoplasma* species were identified by culture and fluorescent staining (Baas and Jasper, 1972).

²Positive test results are indicated by a plus (+) and negative test results by a minus (—).

³*Mycoplasma* milk samples with single culture isolates shown in Table 3.

⁴The milk sample was collected from a single cow. All other samples were from bulk tanks.

and 15M0616_40) were positive for *M. bovis* by qPCR (Table 5). Sample 15M0616_40 was also positive according to qPCR following DNA extraction with the MoBio Power Food kit (Table 4). These results were confirmed in repetitions of the assay following repeated DNA extractions.

Comparison of Assay Accuracy

Assuming that enrichment in laboratory culture medium followed by species identification by immunological methods is the reference test for *Mycoplasma*, the overall accuracy of the qPCR assay when the MoBio Power Food kit was applied for DNA extraction was higher (90.9%) than the LAMP assay (84.8%; Table 6). Although we noted some differences between the samples tested (Supplemental Table 1; <https://doi.org/10.3168/jds.2018-15306>), qPCR after DNA extraction

using the PrepMan Ultra reagent was also useful for the accurate detection of *M. bovis* (96.5%, accuracy). By comparison, LAMP was unsuccessful on DNA extracted from milk with the PrepMan Ultra reagent method; hence, test accuracy is only available for qPCR using this extraction method. The probability of test-negative given culture-negative status was higher for qPCR using the MoBio Power Food and the PrepMan Ultra DNA extraction methods (94.1 and 94.4%, respectively) compared with LAMP using the MoBio extraction method (82.4%; Table 6). With the exception of the identical sensitivity estimates for LAMP and qPCR following DNA extraction using the MoBio kit, we observed only numerical differences ($P > 0.05$) between the accuracy (sensitivity or specificity) of qPCR following either DNA extraction methods and between the latter and LAMP following DNA extraction using the MoBio kit.

Table 5. Evaluation of the *gltX* quantitative PCR (qPCR) assay on DNA extracted from milk using the PrepMan Ultra reagent (Applied Biosystems, Carlsbad, CA)

Culture result ¹	Sample identification	qPCR ²
<i>Acholeplasma laidlawii</i>	15M730_44	—
<i>A. laidlawii</i>	15M753_59	—
<i>Mycoplasma alkalescens</i>	15M720_13	—
<i>M. alkalescens</i>	15M720_20	—
<i>M. alkalescens</i>	15M753_80	—
<i>M. alkalescens</i>	15M753_52	—
<i>M. alkalescens</i>	15M753_119	—
<i>Mycoplasma arginini</i>	15M0599_19 ³	—
<i>M. arginini</i>	15M753_57	—
<i>Mycoplasma bovis</i>	15M0599_27 ³	—
<i>M. bovis</i>	15M0616_40 ³	+
<i>M. bovis</i>	15M0616_105 ³	+
<i>M. bovis</i>	15M0701_17 ³	—
<i>M. bovis</i>	15M720_118	—
<i>M. bovis</i>	15M730_47	—
<i>M. bovis</i>	15M746_49	—
<i>M. bovis</i>	15M753_56	—
<i>M. bovis</i>	15M753_60	—
<i>M. bovis</i>	15M753_88	—
<i>M. bovis</i>	15M863_14	—
<i>M. bovis</i>	15M863_63	—
<i>M. bovis</i>	15M866_32	—
<i>Mycoplasma bovis</i>	15M0599_28 ³	+
<i>M. bovis</i>	15M0616_70 ³	+
<i>M. bovis</i>	15M0620_12 ³	+
<i>M. bovis</i>	15M0620_23 ³	+
<i>M. bovis</i>	15M0620_45 ³	+
<i>M. bovis</i>	15M0620_116 ³	+
<i>M. bovis</i>	15M0620_137 ³	+
<i>M. bovis</i>	15M0620_165 ³	+
<i>M. bovis</i>	15M0649_90 ³	+
<i>M. bovis</i>	15M0671_98 ³	+
<i>M. bovis</i>	15M0681_1 ³	+
<i>M. bovis</i>	15M0693_11 ³	+
<i>M. bovis</i>	15M0697_1 ³	+
<i>M. bovis</i>	15M0697_2 ³	+
<i>M. bovis</i>	15M720_25	+
<i>M. bovis</i>	15M720_46	+
<i>M. bovis</i>	15M730_46	+
<i>M. bovis</i>	15M863_24	+
<i>M. bovis</i>	15M863_50	+
<i>M. bovis</i>	15M863_62	+
<i>M. bovis</i>	15M866_150	+
<i>Mycoplasma californicum</i>	15M0616_34 ³	—
<i>M. californicum</i>	15M0620_89 ³	—
<i>M. californicum</i>	15M0693_15 ³	—
<i>M. californicum</i>	15M753_58	—
<i>M. californicum</i>	15M753_90	—
<i>M. californicum</i>	15M746_48	—
<i>M. californicum</i>	15M863_39	—
<i>M. californicum</i>	15M866_103	—
Negative for <i>Mycoplasma</i>	15M718_4	—
	15M718_22	+
	15M720_51	—
	15M720_85	—
	15M730_45	—
	15M783_14	—

¹*Mycoplasma* species were identified by culture and fluorescent staining (Baas and Jasper, 1972).

²Positive test results are indicated by a plus (+) and negative test results by a minus (—).

³DNA was also recovered using the MoBio Power Food kit (MoBio Laboratories Inc., Carlsbad, CA) and used as template for loop-mediated isothermal amplification and qPCR (Table 3). The MoBio Power Food kit DNA extraction method was not applied to the other milk samples included in this table.

DISCUSSION

The goal of our research was to provide low-cost, simple methods that could be widely adopted by milk quality laboratories, practitioners, and dairy producers for *Mycoplasma* disease prevention and pathogen containment. We developed LAMP and qPCR assays that enable rapid and accurate detection of *M. bovis* in laboratory culture suspensions and in raw bovine milk. These assays were validated on milk and corresponding isolates collected from dairies in the Central Valley of California. The findings indicate the utility of the assays for detection of *M. bovis* in that important dairy production region of the United States. Moreover, because both methods are rapid (completed in <2 h) and relatively low cost, they have benefits over standard *Mycoplasma* laboratory culturing and identification methods.

The *gltX* qPCR assay performed better than LAMP on raw milk. This conclusion was based on the finding that qPCR was possible on gDNA extracted from milk when either the MoBio Power Food kit or PrepMan Ultra reagent was applied and the higher proportion of correctly identified positive and negative samples using culture-based identification as the reference. Furthermore, qPCR had a faster total reaction time with the PrepMan Ultra reagent combined with the iTaq polymerase compared with LAMP. The time required to perform the assay, including the DNA extraction step and qPCR amplification, was within 1 h, a period of time that is lower compared with other *M. bovis* PCR assays (Rossetti et al., 2010; Gioia et al., 2016; Parker et al., 2017; Behera et al., 2018). Several other qPCR assays have been developed for *M. bovis* and targeted other genes, including *uvrC* (coding for deoxyribodipyrimidine photolyase, enzyme in the DNA-repair system; Clothier et al., 2010; Rossetti et al., 2010; Naikare et al., 2015), *oppD* (coding for an oligopeptide permease; Sachse et al., 2010), and *fusA* (coding for elongation factor G; Boonyayatra et al., 2012). Comparisons between these assays could result in the identification of combinations that result in an increased accuracy of detection. Notably, the assays performed equally well or were more sensitive for the detection of low quantities of *M. bovis* cells compared with the *gltX* assay described here. However, outcomes can depend on milk composition, DNA extraction methods, and qPCR conditions used. Additional steps to improve this method could include multiplex qPCR to identify a broader range of *Mycoplasma* and contaminant species (e.g., Parker et al., 2017) and more extensive tests on pooled milk samples and milk collected from other geographic locations.

Table 6. Test accuracy estimates for loop-mediated isothermal amplification (LAMP) and quantitative PCR (qPCR) assays to detect *Mycoplasma bovis* using culture as the reference method¹

Item	LAMP (MoBio)	qPCR (MoBio)	qPCR (PrepMan Ultra)
Prevalence of positive samples in the milk sample pool, % (no.)	48.5 (16/33)	48.5 (16/33)	36.8 (21/57)
Assay accuracy, %	84.8	90.9	96.5
Assay inaccuracy, %	15.2	9.1	3.5
Sensitivity, % (95% CI)	87.5 (71.3, 100)	87.5 (71.3, 100)	100 (100, 100)
Specificity, % (95% CI)	82.4 (64.2, 100)	94.1 (82.9, 100)	94.4 (87, 100)
False-positive rate—type I error (α), %	17.6	5.9	5.6
False-negative rate—type II error (β), %	12.5	12.5	0

¹Estimates are calculated on the basis of a total of 33 samples tested with the MoBio extraction kit (MoBio Laboratories Inc., Carlsbad, CA) for both LAMP and qPCR assays (Table 4) and 57 (Table 5) samples tested by qPCR following DNA extraction with the PrepMan Ultra reagent (Applied Biosystems, Carlsbad, CA). See also Supplemental Table S1 for a summary of all samples used for each test (<https://doi.org/10.3168/jds.2018-15306>).

The LAMP uses a DNA polymerase and sets of primers that result in a synchronized sequence of hybridization and synthesis, leading to isothermal amplification (Notomi et al., 2000; Tomita et al., 2008). The LAMP is reported to be more sensitive than PCR, and because LAMP is performed at a single temperature there is no need for specialized equipment (Fu et al., 2011; Notomi et al., 2000; Poon et al., 2005; Tomita et al., 2008; Fu et al., 2011; Martínez-Valladares and Rojo-Vazquez, 2016). Overall, LAMP performed well on purified DNA; however, even under optimal conditions, the assay was highly susceptible to contamination and DNA preparation and LAMP had to be performed in separate locations in the laboratory, with only a limited number of replicates per day. Moreover, LAMP was unsuccessful on DNA extracted from milk with the PrepMan Ultra reagent, a method that does not include a DNA purification step.

Three LAMP assays for *M. bovis* were previously developed and used to detect *Mycoplasma* in clinical (nasal, tracheal, lung) samples and on milk positive for *M. bovis*, *Staphylococcus aureus*, and *Streptococcus agalactiae* (Bai et al., 2011; Higa et al., 2016; Ashraf et al., 2018). However, those assays were not tested on milk from healthy or asymptomatic cows or used to distinguish between different *Mycoplasma* species. The LAMP assay developed herein exhibited an equal or higher sensitivity and lower specificity compared the LAMP assays published previously (Bai et al., 2011; Higa et al., 2016; Ashraf et al., 2018). The lower specificity was the result of milk samples that were positive or negative for *M. bovis* according to LAMP amplification, but yielded different results according to bacterial culture. Comparing sensitivity or specificity estimates in our study yielded only numerical differences between

the assays and DNA extraction methods. A larger study should be employed to further explore differences in assay accuracy given the different extraction methods.

Notably, 1 *M. bovis* isolate was identified as *M. bovis* by both LAMP and qPCR assays. This potentially indicates the presence of a *M. bovis* co-culture or taxonomic misassignment. Among the milk samples, several that were designated as negative for *M. bovis* yielded positive amplification results by either assay. However, to be out of compliance with culture results is possible due to the detection of nucleic acids from dead or nonculturable *M. bovis* cells by amplification in culture-negative samples (Bradbury and McClenaghan, 1982; Fu et al., 2011). Alternatively, fastidious *Mycoplasma* spp. might have been overgrown by other bacteria present in the milk (Bradbury and McClenaghan, 1982).

In conclusion, we developed and compared 2 assays for *M. bovis* detection in bovine milk. Although the qPCR assay was more robust than the LAMP assay, validation studies on individual and pooled milk samples are still required. Moreover, the qPCR approach can be improved by the incorporation of multiple target detection (multiplex) methods, such as those for the detection for *Mycoplasma* species broadly and *M. bovis* specifically.

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