A PCR assay and PCR-restriction fragment length polymorphism combination identifying the 3 primary *Mycoplasma* species causing mastitis

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ABSTRACT

The focus of the current research was to develop real-time PCR assays with improved sensitivity and the capacity to simultaneously speciate the 3 most common mycoplasma mastitis agents: *Mycoplasma bovis*, *Mycoplasma californicum*, and *Mycoplasma bovisgenitalium*. Real-time PCR was chosen because it provides rapid results. Partial 16S rRNA gene sequencing was used as the gold standard for evaluating candidate real-time PCR assays. To ascertain the real-time PCR assay specificity, reference strains of *Mycoplasma* species, *Acholeplasma axanthum*, and common gram-positive and gram-negative mastitis pathogens were tested. No cross-reactions were observed. *Mycoplasma* spp. isolated from bovine milk samples ($n = 228$) and other organ sites ($n = 40$) were tested by the real-time PCR assays and the partial 16S rRNA gene sequencing assay. Overall accuracy of this novel real-time PCR was 98.51%; 4 of 228 isolates identified as *M. bovis* by the partial 16S rRNA gene sequencing assay were identified as both *M. bovis* and *M. californicum* by real-time PCR. Subsequent amplicon sequencing suggested the presence of both *M. bovis* and *M. californicum* in these 4 samples. Using a cycle threshold of 37, the detection limits for real-time PCR were 10 copies of DNA template for both *M. bovis* and *M. bovisgenitalium*, and 1 copy for *M. californicum*. This real-time PCR assay is a diagnostic technique that may be used as a screening tool or as a confirmation test for mycoplasma mastitis.

Key words: *Mycoplasma* species, mastitis, intramammary infection, real-time PCR

INTRODUCTION

Mycoplasma species were first recognized as a cause of contagious mastitis in dairy cattle in the United States in 1961 (Hale et al., 1962). Since then, mycoplasma

mastitis has been reported throughout the country and in many regions of the world (Fox et al., 2005). *Mycoplasma* spp. are categorized as contagious mastitis pathogens (Fox and Gay, 1993), and mycoplasma mastitis appears to be an emerging problem in the United States, with approximately 10 to 20% of large (>500 cows) dairy herds affected by this agent (USDA, 2008). Because of the difficulty in culturing the pathogen, it is likely that mycoplasma mastitis is under-reported (Nicholas et al., 2007). Moreover, Nicholas et al. (2007) indicate that the cost of the disease has been estimated to exceed \$100 million annually in the United States.

Mycoplasma bovis is the most common *Mycoplasma* species associated with mastitis and accounts for 49 to 60% of isolates (Jasper, 1979; Kirk et al., 1997). *Mycoplasma californicum* and *Mycoplasma bovisgenitalium* are frequently reported as being associated with mastitis in dairy cows and appear to be the next most common causes of mycoplasma mastitis (Jasper, 1979; Kirk et al., 1997).

The slow growth and fastidious nutritional requirements of *Mycoplasma* spp. in vitro impede identification of this genus. Although culture is the primary method for mycoplasma identification, culture cannot distinguish between pathogenic and nonpathogenic species and requires up to 10 d to reach a definitive culture diagnosis (Hogan et al., 1999). Highly specific and sensitive PCR techniques can overcome these problems (Pinnow et al., 2001). Currently, nested PCR followed by RFLP analysis is used to identify suspected *Mycoplasma* spp. isolates (Tang et al., 2000). Although this technique is promising for identification of *Mycoplasma* species directly from biological samples, it requires 2 steps of PCR and a long period of incubation for enzyme digestion, requiring approximately 18 to 30 h to complete.

Real-time PCR techniques might overcome the difficulties associated with the in vitro culture of *Mycoplasma* spp. and reduce the time needed for speciation by conventional PCR. Real-time PCR methods have been available for more than a decade (Heid et al., 1996) and facilitate the screening of large numbers of

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samples within a few hours with excellent sensitivity and specificity. Fluorescence detection of specific PCR products and accumulation of this signal for each PCR cycle on a real-time basis enable quantification of template DNA (Leutenegger, 2001). Real-time PCR with fluorogenic probes (TaqMan, Qiagen, Valencia, CA) increases the specificity of real-time PCR assays because only a specific amplicon target sequence is recognized.

The purpose of this study was to develop 3 real-time PCR assays capable of detecting the 3 most common mycoplasma mastitis agents, *M. bovis*, *M. californicum*, and *M. bovisgenitalium*. To validate these newly developed real-time PCR assays, the conventional PCR described by Tang et al. (2000) was also performed on the same samples and results were compared using 16S rRNA gene partial sequencing as a gold standard basis of comparison.

MATERIALS AND METHODS

Organisms

A set of 13 representative *Mollicutes* and 15 other eubacterial species obtained from the American Type Culture Collection (ATCC, Manassas, VA) were used to determine the analytical specificity of the candidate real-time PCR assays (Table 1). A set of 268 isolates from bovine sources, primarily milk samples ($n = 228$) and samples from other organ sites ($n = 40$), isolated on modified Hayflick's agar, was used to determine the analytical sensitivity of candidate PCR reactions. Samples from other organ sites were obtained by using a polyester-tipped swab moistened with mycoplasma enrichment medium and swabbed over the mucosal surface of the tissues (Biddle et al., 2005). Organ systems included the eye, respiratory, and urogenital systems. Swab samples were also collected from the outer ear, buccal cavity, and rectum. To ensure purity, a filtration-cloning technique as described by Tully (1983) was performed on all *Mollicute* isolates. All isolates were cultured in pleuropneumonia-like organism (PPLO) broth (BBL, Franklin Lakes, NJ), in a 10% CO₂ environment for 4 d. Glycerol (30% vol/vol) was added to cultured broth before storage at -85°C until use.

Primers and Probes Designed for *M. bovis*, *M. californicum*, and *M. bovisgenitalium*

Primers and probes were designed using the Qiagen online tool (<http://www1.qiagen.com/products/pcr/quantitect/customassays.aspx>; Qiagen, Valencia, CA). Three different housekeeping genes, *fusA* encoding for elongation factor G, *rpoB* encoding for RNA polymerase β subunit, and 16S-23S rRNA intergenic spacer region

(IGS) were selected for *M. bovis*, *M. californicum*, and *M. bovisgenitalium*. Primers and probes were tested for interactions using the online Oligo Analysis tool (<http://www.operon.com/technical/toolkit.aspx>). The sequences were tested for their specificity by searching against the GenBank database using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990). The sequences of primers and probes are shown in Table 2.

Genomic DNA Extraction for PCR

Using PureLink Genomic DNA Kits (Invitrogen, Carlsbad, CA), a silica-based column purification method, gram-negative bacterial genomic DNA extraction was performed on all *Mollicute* and gram-negative cultures according to the manufacturer's instructions. For gram-positive bacteria, genomic DNA was extracted according to the protocol described by Pitcher et al. (1989). All genomic DNA extracts were frozen at -80°C until further use.

Real-Time PCR

The PCR reaction was initiated by combining 5 μL of the extracted DNA with 12.5 μL of 2 $\times$ Master Mix: HotStart Taq DNA polymerase, dNTP mix, ROX passive reference dye, and QuantiTect Probe real-time-PCR buffer [containing Tris-Cl, KCl, (NH₄)₂SO₄, 8 mM MgCl₂, pH 8.7], 10 pmol of each primer, and 5 pmol of probe according to the QuantiTect Probe PCR kit (Qiagen) instructions. All real-time PCR assays were performed on a Step One Plus (Applied Biosystems, Foster City, CA) real-time PCR instrument. The thermal cycling protocol included 15 min of predenaturation at 95°C , 45 cycles of 15 s of denaturation at 94°C , and 60 s of annealing at 60°C . Based on a cycle threshold (C_T) of 37, each reaction was classified as positive or negative for a target (Bustin, 2004).

Quantitative Testing of the Real-Time PCR Assays

To establish the quantitative ability of each real-time PCR assay, a standard curve was generated. Target sequences in *fusA*, *rpoB*, and ITS genes were cloned into pIDTSMART-AMP plasmids (Integrated DNA Technologies, Coralville, IA). All plasmids were diluted to concentrations of 20,000, 2,000, 200, 20, 2, and 0.2 copies of DNA template/ μL . Five microliters of each dilution was combined with primers, probes, and 12.5 μL of 2 $\times$ Master Mix as described previously. To generate standard curves for *M. bovis*, *M. bovisgenitalium*, and *M. californicum* real-time PCR assays, each dilution of each target DNA was tested in triplicate.

Table 1. *Mycoplasma* species and other bacterial species tested for specificity of the novel real-time PCR assays¹

Organism	Origin of isolates	Source ²	Real-time PCR		
			<i>M. bovis</i>	<i>M. californicum</i>	<i>M. bovis genitalium</i>
<i>Mycoplasma bovis</i>	Bovine mastitic milk	ATCC 25025	+	–	–
<i>M. bovis</i>	Bovine mastitic milk	ATCC 25523	+	–	–
<i>M. bovis</i>	Bovine mastitic milk	CU 22253	+	–	–
<i>Mycoplasma californicum</i>	Bovine mastitic milk	ATCC 33461	–	+	–
<i>Mycoplasma bovis genitalium</i>	Bovine genital tract	ATCC 14173	–	–	+
<i>Mycoplasma alkalescens</i>	Bovine nasal cavity	ATCC 29103	–	–	–
<i>M. alkalescens</i>	Bovine mastitic milk	CU 21146	–	–	–
<i>Mycoplasma agalactiae</i>	Mastitic goat milk	ATCC 35890	–	–	–
<i>Mycoplasma arginini</i>	Lung of sheep with pneumonia	ATCC 23243	–	–	–
<i>Mycoplasma bovirhinis</i>	Bovine respiratory tract	ATCC 27748	–	–	–
<i>Mycoplasma leachii</i> sp. nov.	Arthritic joint of a calf	IOM N29 Leach strains	–	–	–
<i>Mycoplasma ovipneumonia</i>	Ovine respiratory tract	ATCC 29419	–	–	–
<i>Acholeplasma axanthum</i>	Murine leukemia tissue culture	ATCC 25176	–	–	–
<i>Escherichia coli</i>	Clinical isolates	ATCC 25922	–	–	–
<i>Staphylococcus simulans</i>	Human	ATCC 11631	–	–	–
<i>Staphylococcus epidermidis</i>	Human	ATCC 12228	–	–	–
<i>Staph. epidermidis</i>	Human (catheter sepsis)	ATCC 35984	–	–	–
<i>Staphylococcus equorum</i>	Horse skin	ATCC 43958	–	–	–
<i>Staphylococcus hemolyticus</i>	Human skin	ATCC 29970	–	–	–
<i>Staphylococcus gallinarum</i>	Chicken nares	ATCC 35539	–	–	–
<i>Staphylococcus hyicus</i>	Pig with exudative epidermitis	ATCC 11249	–	–	–
<i>Staphylococcus intermedius</i>	Pigeon nares	ATCC 29663	–	–	–
<i>Staphylococcus caprae</i>	Goat milk	ATCC 35538	–	–	–
<i>Staphylococcus capitis</i>	Human	ATCC 35661	–	–	–
<i>Staphylococcus chromogenes</i>	Pig skin	ATCC 43764	–	–	–
<i>Staphylococcus aureus</i>	Bovine mastitic milk	ATCC 29740	–	–	–
<i>Streptococcus uberis</i>	Bovine udder infection	ATCC 27958	–	–	–
<i>Streptococcus dysgalactiae</i>	Bovine udder infection	ATCC 27957	–	–	–

¹Isolates with a + sign were positively identified by real-time PCR as that species and those with a – sign were not identified, using a cycle threshold value of 37 as the threshold of determination.

²ATCC = American Type Culture Collection (Manassas, VA); CU = Cornell University (Ithaca, NY); IOM = International Organization of Mycoplasma (West Lafayette, IN).

Detection Limit of Real-Time PCR Assays in Milk Samples

To determine the detection limit of each real-time PCR assay, 10-fold serial dilutions of 2 strains of *M. bovis*, *M. californicum*, and *M. bovis genitalium* were made in *Mycoplasma*-free milk. Two milliliters of inoculated milk was centrifuged at $14,000 \times g$ for 3 min. The supernatant was discarded and genomic DNA was extracted from milk cell pellet using PureLink Genomic DNA Kit (Invitrogen). One hundred microliters of the same inoculated milk was spread on Modified Hayflick's media, and plates were incubated for 7 to 10 d before enumeration of mycoplasma colonies.

PCR-RFLP

Adapted from Tang et al. (2000), a one-step PCR followed by RFLP was performed in a total volume of 50 μ L containing $1 \times$ PCR buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, and 50 mM KCl; pH 8.4), 50 μ mol of each of deoxynucleoside triphosphates (dNTP), 20 pmol of each primer, and 1 U of Taq DNA polymerase (Invitrogen).

A set of primers including F2, R2, and R34 was used to amplify the 16S-23S rRNA intergenic spacer regions of *Mycoplasma* and *Acholeplasma*. Sequences and targets of these primers are shown in Table 2. Five microliters of DNA extract was used as a template and added to 45 μ L of reaction mixture. The thermal cycling protocol was programmed as described previously (Tang et al., 2000) and products were digested by a restriction enzyme, *AseI*. Products were electrophoresed on a 2% agarose gel and patterns of DNA fragments were visualized by UV fluorescence.

16S rRNA Gene Partial Sequencing

A 16S rRNA gene region of approximately 1,500 bp was amplified using a universal primer pair, pH and pA, as listed in Table 2 (Stakenborg et al., 2005). The 50- μ L PCR reaction mixture included 3 U of Taq DNA polymerase (Invitrogen), $1 \times$ PCR buffer (20 mM Tris-HCl, 1.5 mM $MgCl_2$, and 50 mM KCl; pH 8.4), 50 μ M of each dNTP, 10 pmol of each primer, and 1 μ L of the genomic DNA as template. The thermal cycling protocol was programmed as described previously (Staken-

Table 2. Sequences of primers and probes used in the study and their targeted regions

Name	Sequence	Target
F2	5'-GTG(C/G)GG(A/C)TGGATCACCTCCT-3'	16S-23S rRNA intergenic spacer region of <i>Mycoplasma</i> species
R2	5'-GCATCCACCA(T/A)(A/T)AC(C/T)CTT-3'	
R34	5'-CCACTGTGTGCCCTTTGTTCCT-3'	16S-23S rRNA intergenic spacer region of <i>Acholeplasma</i> species
pA	5'-AGAGTTTGATCCTGGCTCAG-3'	16S rRNA gene
pH	5'-AAGGAGGTGATCCAGCCGA-3'	
MbF	5'-TAATGCAAGCAACTCTCGTAGT-3'	<i>fusA</i> gene of <i>Mycoplasma bovis</i>
MbR	5'-TGTCACCAAGTTGTGTGCTT-3'	
MbP	6FAM 5'-ACCAACAGCAGCAACAATATCACCTGC-3'BHQ1	
McF	5'-GCACCTTAGACGAAAGAGGGATT-3'	<i>rpoB</i> gene of <i>Mycoplasma californicum</i>
McR	5'-GGATTATCATCACTTTGGGACT-3'	
McP	6FAM5'-CGTGTGGTTCGGAAGTGGTTCAG-3'BHQ1	
MbvgF	5'-CTTCTACGGAGTACAAGCTAAT-3'	16S-23S rRNA intergenic spacer region of <i>Mycoplasma bovis</i>
MbvgR	5'-GAGAGAATTGTTCTCTCAAACTA-3'	
MbvgP	6FAM5'-TATCGTCATGGCTTGTTAGGTCCCA-3'BHQ1	

borg et al., 2005). The PCR products were sequenced using primer pA (Macrogen Corp., Rockville, MD) and the sequences were compared with the GenBank database using BLAST (Altschul et al., 1990). A hit was identified as the species giving the highest value but at least 98% identity.

Statistical Analysis

All isolate assays were performed in duplicate. If the replicates did not agree, a third replicate was performed. A C_T of 37 was used to define the presence or absence of a *Mycoplasma* species. Identification was regarded as definitive identification by real-time PCR if both replicates had $C_T < 37$.

For PCR-RFLP, identification was regarded as definitive if the DNA fragment patterns were identical. Results are presented as percentage agreement with 16S rRNA gene partial sequencing where the denominator was the number of isolates identified as *M. bovis*, *M. californicum*, or *M. bovisgenitalium* by the 16S rRNA gene partial sequencing technique and the numerator was the number of matched isolates identified as *M. bovis*, *M. californicum*, or *M. bovisgenitalium* by each PCR technique. The percentage accuracy is the proportion of field samples with *Mollicutes* that were classified correctly, with the denominator being the total number of field samples and the numerator being the number of samples in complete agreement.

RESULTS

Mycoplasma Isolates Identified by 16S rRNA Gene Partial Sequencing

Based on the identification using 16S rRNA gene partial sequencing, of the 268 isolates from milk and other organ sites, 228 (85.07%), 22 (8.21%), and 5 (1.87%) were identified as *M. bovis*, *M. californicum*, and *M. bovisgenitalium*, respectively. The distribution of *Mycoplasma* and *Acholeplasma* isolates is listed in Table 3.

Identification of *M. bovis*, *M. californicum*, and *M. bovisgenitalium* Using PCR-RFLP

The patterns of amplicons before and after *AseI* digestion of *M. bovis*, *M. californicum*, and *M. bovisgenitalium* are illustrated in Figures 1a and 1b. The sizes of digested amplicon for *M. bovis* were 140, 125, and 115 bp and for *M. bovisgenitalium* 265 and 115 bp. The amplicon of *M. californicum* (370 bp) could not be cut by *AseI* (Figure 1b). All *M. bovis* isolates (228/228) and all *M. californicum* isolates (22/22) were correctly

Table 3. Distribution of isolates of *Mycoplasma* spp. and *Acholeplasma* spp. as identified by 16S rRNA partial sequencing

Species identification using 16S rRNA partial sequencing	No. of isolates from milk samples ¹	No. of isolates from body sites and other organs	No. of reference strains	Total
<i>Mycoplasma bovis</i>	212	16	3	231
<i>Mycoplasma californicum</i>	11	11	1	23
<i>Mycoplasma bovigenitalium</i>	2	3	1	6
<i>Acholeplasma axanthum</i>	0	1	1	2
<i>Mycoplasma alkalescens</i>	2	0	2	4
<i>Mycoplasma arginini</i>	1	2	1	4
<i>Mycoplasma yeatsii</i>	0	2	0	2
<i>Mycoplasma cottewii</i>	0	1	0	1
<i>Mycoplasma bovirhinis</i>	0	0	1	1
<i>Mycoplasma felis</i>	0	1	0	1
<i>Mycoplasma leachii</i> (bovine serogroup 7)	0	0	1	1
<i>Mycoplasma agalactiae</i> ²	0	3	1	4
<i>Mycoplasma ovipneumonia</i>	0	0	1	1
Total	228	40	13	281

¹Two hundred twenty-one isolates were from milk samples collected from cows with clinical mastitis and 7 isolates were from bulk tank milk samples.

²Three *M. agalactiae* isolates were laboratory strains of unknown origin.

identified as shown in Table 4. None of the field strains of *M. bovigenitalium* were correctly identified by PCR-RFLP. Amplicons of 2 *M. bovigenitalium* isolates were not digested by *Ase*I, resulting in an electrophoretic pattern similar to those of *M. californicum* isolates as shown in Figure 2, lanes 2 and 4. These 2 isolates were misidentified by PCR-RFLP and thus would be considered as *M. bovigenitalium* false-negative results. Because these isolates were identified as *M. californicum* by PCR-RFLP, they would be considered as false-positive *M. californicum* results. The other 3 isolates identified as *M. bovigenitalium* by the 16S rRNA gene partial sequencing were not identified by PCR-RFLP (Figure 2, lanes 3, 5, and 6). Given that all 5 field isolates of *M. bovigenitalium* were misidentified, the test accuracy was 98.13% (263 of 268 samples accurately identified).

Test Specificity and Detection Limit of Real-Time PCR Assays

Genomic DNA of 15 eubacterial species and 13 representative *Mollicutes* were tested to define analytical specificity (Table 4). All *M. bovis*, *M. bovigenitalium*, and *M. californicum* strains produced the expected amplification signals, and log-linear dependences of C_T value on mycoplasma copy number were observed (Figures 3, 4, and 5). No specific products from other *Mycoplasma* species and eubacterial species were detected, and cross-reactivity was not observed.

The detection limit of all 3 assays was as low as 1 to 10 copies of targeted DNA (Figures 3–5). A C_T of 37 is the detection limit of 1 copy of *M. californicum* and approximately 10 copies of *M. bovis* and *M. bovigenitalium*. Moreover, the geometric means of the detection

limits of the genomic DNA extracted from milk were 10 ± 0 (SE) cfu/mL (C_T = 39.5 ± 0.6), 22.4 ± 20 cfu/mL (C_T = 41.7 ± 3.3), and 20 ± 0 cfu/mL (C_T = 33.7 ± 0.1) for *M. bovis*, *M. californicum*, and *M. bovigenitalium* real-time PCR assays, respectively.

Identification of *M. bovis*, *M. californicum*, and *M. bovigenitalium* Using Real-Time PCR Assays

For 228 isolates of *M. bovis*, 22 of *M. californicum*, and 5 of *M. bovigenitalium*, the real-time PCR assay and the 16S rRNA gene partial sequencing were in 100% agreement (Table 4). However, 4 isolates that were identified by the 16S rRNA gene partial sequencing as *M. bovis* were identified as both *M. bovis* and *M. californicum* by real-time PCR. Thus, the accuracy of the real-time PCR test relative to the 16S rRNA gene partial sequencing technique was 98.51% (264 of 268 samples were correctly identified). To investigate the possibility of cross amplification between the 2 species by the real-time PCR, those 4 isolates were amplified using the primers for *M. californicum*. Amplicons obtained from those 4 isolates were sequenced and searched for similarity against GenBank database. All 4 amplicons matched 100% with the target sequence of *M. californicum*, suggesting that both *M. bovis* and *M. californicum* were present and that the original 16S rRNA gene partial sequence analysis did not detect *M. californicum*.

DISCUSSION

The goal of the current research was to develop real-time PCR assays to detect the 3 most common mycoplasma mastitis agents: *M. bovis*, *M. californicum*, and

Figure 1. Electrophoretic gel of amplicons before digestion (A) and after digestion by *AseI* (B). (A) Lanes 1 and 6 = 100-bp molecular ladder, lane 2 = *Mycoplasma bovis* ATCC 25025, lane 3 = *M. bovis* ATCC 25523, lane 4 = *Mycoplasma californicum* ATCC 33461, lane 5 = *Mycoplasma bovis* ATCC 14173. (B) Lanes 1 and 5 = 100-bp molecular ladder, lane 2 = *M. bovis* ATCC 25025, lane 3 = *M. californicum* ATCC 33461, lane 4 = *M. bovis* ATCC 14173. ATCC = American Type Culture Collection.

M. bovis. All 3 assays correctly detected the specific target regions selected without cross amplification with other *Mycoplasma* spp. or other bacteria. The real-time PCR developed yielded results that were in 100% agreement with identification by the gold standard test, 16S rRNA gene partial sequence analysis. Relative test accuracy was 98.51%, because 264 of the 268 samples were classified similarly by both assays. The 4 samples that appeared to be misclassified might have been more accurately classified by the real-time PCR assay compared with the 16S rRNA gene partial sequence analysis. Further analysis of the real-time PCR amplicons suggested that in these 4 samples, both *M. bovis* and *M. californicum* were present. Isolates were obtained from milk and other body sites to determine whether strains that might colonize or infect one organ system might be significantly different from those at other sites, and thus would not be correctly identified with the assay system developed. The 100% agreement between the real-time PCR and the 16S rRNA gene partial sequence assays indicated that source of isolate did not affect correct identification.

Results described herein verify success in establishing a 16S rRNA gene partial sequencing assay to detect 3 common mycoplasma mastitis pathogens accurately and rapidly. Species identification of mycoplasma mastitis agents is generally done by serological or PCR assays (González and Wilson, 2003). Time needed to speciate *Mycoplasma* by serology is less than 1 h (Infante Martinez et al., 1990), much less than PCR, which may require 24 h (González and Wilson, 2003). However, serological testing is not as sensitive as conventional PCR, the former detecting 5,000 cfu/mL and the latter approximately 100 cfu/mL or less (Hirose et al., 2001; Pinnow et al., 2001; Infante et al., 2002). The described real-time PCR assay procedure can detect 10 to 30 cfu/mL *Mycoplasma*; such a test was made using milk as a diluent in an effort to determine if the assay could later be developed to identify mycoplasma mastitis agents directly in milk. Time to completion of the assay was approximately 4 h. Another advantage is that it detects the most prevalent species that cause *Mycoplasma* mastitis. The 3 most prevalent species of mycoplasma mastitis agents are *M. bovis*, *M. californicum*, and *M. bovis* (Jasper, 1980; Kirk and Lauerman, 1994). Our findings from cows with clinical mastitis sampled in Washington support that conclusion, 93% (212/228) being *M. bovis*, 5% (11/228) being *M. californicum*, and 1% (2/228) being *M. bovis*.

Mycoplasma bovis is regarded as the most pathogenic *Mollicute* to frequently cause mastitis. (Gonzalez and Wilson, 2003) but *M. californicum* and *M. bovis* are reported as significant causes of IMI (Jasper, 1980; Jurmanová et al., 1983; Mackie et al., 1986; Jack-

Table 4. Identification of *Mycoplasma* spp. and *Acholeplasma* spp. isolates isolated from field samples using PCR-RFLP and real-time PCR¹

Species	Total	PCR-RFLP				Real-time PCR			
		<i>M. bovis</i>	<i>M. calif</i>	<i>M. bovisgen</i>	Other species	<i>M. bovis</i>	<i>M. calif</i>	<i>M. bovisgen</i>	Other species
<i>Mycoplasma bovis</i>	228	228	0	0	0	228	4 ²	0	0
<i>Mycoplasma californicum</i>	22	0	22	0	0	0	22	0	0
<i>Mycoplasma bovisgenitalium</i>	5	0	2	0	3	0	0	5	0
<i>Mycoplasma alkalescens</i>	2	0	0	0	2	0	0	0	2
<i>Mycoplasma agalactiae</i>	3	0	0	0	3	0	0	0	3
<i>Mycoplasma arginini</i>	3	0	0	0	3	0	0	0	3
<i>Mycoplasma yeatsii</i>	2	0	0	0	2	0	0	0	2
<i>Mycoplasma cottewii</i>	1	0	0	0	1	0	0	0	1
<i>Mycoplasma felis</i>	1	0	0	0	1	0	0	0	1
<i>Acholeplasma azanthum</i>	1	0	0	0	1	0	0	0	1
Total	268	228	24	0	16	228	26	5	14

¹Species identified by 16S rRNA partial sequencing. *M. calif* = *Mycoplasma californicum*; *M. bovisgen* = *Mycoplasma bovisgenitalium*.

²Four field isolates identified by the gold standard as *M. bovis* were identified as *M. bovis* and *M. californicum* by real-time PCR.

son and Boughton, 1991). The inability to identify *M. californicum* and *M. bovisgenitalium* in a mastitic milk sample or to mistake a *Mycoplasma* as a nonpathogenic *Mollicute* may lead to excessive transmission of the disease given the contagious nature of these pathogens.

Figure 2. Digested amplicons obtained from 6 *Mycoplasma bovisgenitalium* isolates. Lane 1 = precision mass molecular ladder (bands from top to bottom represent 1,000, 700, 200, and 100 bp), lanes 2 to 6 = field isolates of *M. bovisgenitalium*, lane 7 = *M. bovisgenitalium* ATCC 14173. ATCC = American Type Culture Collection.

Thus, the development of the described real-time PCR assay technique with its advantages in more timely identification of the common mycoplasma mastitis agents is warranted. The next step is the development of a multiplex system that would work directly in milk.

Several real-time PCR assays have been reported to detect *M. bovis* in many kinds of biological samples. Cai et al. (2005) successfully developed a real-time PCR assay that uses fluorescence resonance energy transfer (FRET) probes to detect *M. bovis* in milk and lung samples. Their system targets a region in 16S rRNA gene that has a positive result with both *M. bovis* and *Mycoplasma agalactiae*. To distinguish between these species, Cai et al. (2005) measured the melting temperature of probes. The detection limits they reported were 550 cfu/mL of milk. Sachse et al. (2010) reported a novel real-time PCR assay detecting *M. bovis* in nasal and conjunctival swabs and from milk samples. This real-time PCR assay targets the 3'-terminal region of the *oppD* gene encoding for an oligopeptide permease, a member of the ABC-transporter family. Sachse et al. (2010) reported the detection limit of their assay to be 100 cfu/mL of milk. Recently, Rossetti et al. (2010) developed a real-time PCR assay to detect *M. bovis* from milk lysate. Their assay targets a region in *uvrC* gene encoding for a part of exinuclease. The detection limit they reported is 2,000 cfu/mL of milk (Rossetti et al., 2010). Our real-time PCR assay targeted the *fusA* gene encoding for elongation factor G of *M. bovis*. The real-time PCR assays detecting *M. bovis* targeting *oppD* (Sachse et al., 2010), *uvrC* (Rossetti et al., 2010), and *fusA* have the advantage that they appear to be free of cross-amplification with *M. agalactiae*. The real-time PCR assay described herein is the first to speciate the 3 *Mycoplasma* pathogens most commonly associated with mastitis.

Figure 3. Standard curve of *Mycoplasma bovis* real-time PCR assay using pIDTSMART-AMP plasmids cloned with the target sequence in *fusA* gene of *M. bovis*.

Several techniques have been developed to detect *Mycoplasma* species, primarily for detection of cell culture contamination (Hay et al., 1989; Harasawa et al., 1993). We have applied a PCR-RFLP technique (Tang et al., 2000) to speciate *Mycoplasma* species in

our laboratory. This technique was developed to detect cell culture contamination and heretofore has not been tested with *Mycoplasma* strains found in cattle.

In this study, we used a cut-off value of $C_T < 37$ to discriminate between detection and nondetection. This

Figure 4. Standard curve of *Mycoplasma bovis genitalium* real-time PCR assay using pIDTSMART-AMP plasmids cloned with the target sequence in 16S-23S intergenic spacer region of *M. bovis genitalium*.

Figure 5. Standard curve of *Mycoplasma californicum* real-time PCR assay using pIDTSMART-AMP plasmids cloned with the target sequence in *rpoB* gene of *M. californicum*.

cut-off value gave a detection limit for the *M. californicum* assay of 1 copy of target DNA (Figure 5), whereas the detection limits for *M. bovis* and *M. bovisgenitalium* assays were approximately 10 copies of target DNA (Figures 3 and 4). This choice is consistent with that used by Bustin (2004), who suggested this value to guard against false-positive results due to technical errors that are manifested by weak signals present in negative controls. Using this cut-off value, 4 *M. bovis* isolates that were thought to be of a single species after filter cloning were identified as positive for both *M. bovis* and *M. californicum*. Those isolates were later confirmed to have both *M. bovis* and *M. californicum*. Because 16S rRNA gene partial sequencing assay is a qualitative PCR-based technique, only the dominant species in a sample can be detected. Real-time PCR assays can detect and quantify very low amounts of DNA template in a sample even when more than one species is present. For that reason, this real-time PCR assays may be superior to conventional nucleic acid detection techniques when used on mixed infection samples.

CONCLUSIONS

The real-time PCR assay developed had a lower limit of detection of 10 to 30 cfu/mL of the tested *Mycoplasma* species, approximately 4 to 10 times more sensitive than the detection limits that have been reported for conventional PCR. Moreover, the real-time PCR assay developed can be completed in 20 fewer hours than the conventional PCR procedure. The conventional

PCR-RFLP technique evaluated failed to speciate *M. bovisgenitalium* and thus this real-time PCR assay technique is superior in that it correctly speciates the 3 most prevalent *Mycoplasma* spp. The use of primers and probes that are specific and that have excellent agreement with the 16S rRNA gene partial sequencing test for the 3 most prevalent mycoplasma mastitis species suggests its potential to be developed into a routine mastitis diagnostic technique. Tests of the real-time PCR using milk as a diluent indicates that it has promise to be developed further, combining 3 assays in a single, multiplex, real-time test that can be used directly with milk samples.

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