Validation of a mycoplasma molecular diagnostic test and distribution of mycoplasma species in bovine milk among New York State dairy farms

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ABSTRACT

Mycoplasma mastitis is a contagious and costly disease of dairy cattle that significantly affects animal health and milk productivity. *Mycoplasma bovis* is the most prevalent and invasive agent of mycoplasma mastitis in dairy cattle, and early detection is critical. Other mycoplasma have been isolated from milk; however, the role and prevalence of these species as mastitis pathogens are poorly understood. Routine screening of milk for mycoplasma by bacteriological culture is an important component of a farm control strategy to minimize a herd mycoplasma outbreak, but phenotypic methods have limited ability to speciate mycoplasma, affecting how farms and practitioners can understand the role and effect of species other than *M. bovis* in herd health. Fastidious mycoplasma culture can be lengthy and inconclusive, resulting in delayed or false negative reports. We developed and validated a multitarget PCR assay that can in the same day confirm or reject a presumptive positive mycoplasma culture found upon bacteriological testing of clinical specimens, further discriminate between *Acholeplasma* and *Mycoplasma*, and identify *M. bovis*. Coupled with sequence analysis isolates can be further identified as bovine mycoplasma *Mycoplasma arginini*, *Mycoplasma alkalescens*, *Mycoplasma canadense*, *Mycoplasma bovirhinis*, *Mycoplasma bovis*, *Mycoplasma californicum*, *Acholeplasma laidlawii*, and *Acholeplasma oculi*. Assay validation included analysis of 845 mycoplasma representing these species and 30 additional bacterial species obtained from routine milk submissions to the Quality Milk Production Services from New York State farms and veterinary clinics between January 2012 and December 2015. Among 95 herds, we found 8 different *Mycoplasma* species and 3 different *Acholeplasma* species, with an overall prevalence of *M. bovirhinis* of 1%, *A. oculi* of 2%, *M. arginini* of 2%, *M. californicum* of

3%, *M. canadense* of 10%, *M. bovis* of 10%, *A. laidlawii* of 11%, *M. alkalescens* of 17%, and *M. bovis* of 78%. More than one mycoplasma was found in 14% of the herds tested, and both *M. bovis* and *Acholeplasma* were found in 6% of the farms. Incorporation of the validated molecular diagnostic assay into routine bacteriological screening as a supportive confirmation and identification tool will lead to an improved assessment of *Mycoplasma* and *Acholeplasma* prevalence data, which will facilitate increased knowledge about the role of these mycoplasma in mastitis.

Key words: *Mycoplasma*, *Acholeplasma*, bovine, mastitis, molecular diagnostic assay

INTRODUCTION

Mycoplasma IMI of dairy cattle is a serious, highly contagious disease that can have a significant financial effect on a farm through loss of animals and reduced milk quality and production (Wilson et al., 1997; Maunsell et al., 2011). Mycoplasma are resistant to antibiotic treatment, and carriage in the animal can be persistent and fluctuate broadly (Jasper, 1982; Biddle et al., 2003). Readily transmitted through blood and lymph, mycoplasma transmission between cows commonly occurs through direct contact or via equipment during milking (USDA APHIS, 2003, 2008; Fox, 2012). Because of these factors, control strategies to reduce spread and effect rely on regular bacteriological culture screening of bulk-tank milk or pooled animal milk. When positive cultures are identified, smaller pools or individual cows are tested to pinpoint infection, and positive animals are culled or segregated (USDA APHIS, 2008; Maunsell et al., 2011; Fox, 2012).

After initial isolation of mycoplasma from clinical samples by bacteriological culture, in some cases positive confirmation can be a lengthy and inconclusive process that can increase the response time of a farm to a mycoplasma outbreak. Fastidious mycoplasma require specialized media and incubation conditions for isolation and often grow slowly or exhibit atypical colony morphology that can confound a definitive culture

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report or lead to an erroneous identification (Freundt, 1983; Gourlay and Howard, 1983; Nicholas et al., 2008). Atypical colonies on mycoplasma media can feasibly be due to growth of unstable L-form bacteria induced by penicillin included in the media, cellular material present in a clinical sample, or insoluble precipitates produced by older media (Razin, 1983); however, few bacteriological tools exist that can discriminate between these and mycoplasma. The National Mastitis Council recommends incubating cultures 7 to 10 d before a negative report (NMC, 1999), and subculture of atypical growth is commonly recommended (Nicholas et al., 2008). However, failure of a suspect mycoplasma to subculture may be due to the fastidious nature or uncultureability of a particular mycoplasma strain, resulting in a false negative report.

Several different mycoplasma species have been reported to be isolated from bovine milk including *Mycoplasma bovis*, *Mycoplasma arginini*, *Mycoplasma alkalescens*, *Mycoplasma canadense*, *Mycoplasma bovirhinis*, *Mycoplasma bovigenitalium*, *Mycoplasma californicum*, *Mycoplasma dispar*, *Acholeplasma laidlawii*, *Acholeplasma oculi*, and *Acholeplasma granularum* (USDA APHIS, 2003; Nicholas et al., 2008; Justice-Allen et al., 2011). These mycoplasma have been implicated in causing mastitis as well as other reproductive and respiratory diseases in dairy cattle (Jasper, 1994; Nicholas et al., 2008). The frequently researched *M. bovis* has been considered the most prevalent and clinically important mycoplasma species in dairy cattle, associated with acute mastitis, arthritis, otitis, and pneumonia (Gonzalez and Wilson, 2003; Maunsell et al., 2011; Fox, 2012). Not all species have been as comprehensively studied as *M. bovis*, and less genetic data exists to contribute to our understanding of the significance of each species as pathogens. Serological and biochemical tests have been standardized for further identification of mycoplasma; however, both approaches are time consuming and difficult to perform accurately, reliably, and unambiguously (Volokhov et al., 2012). Indeed, the scarcity of available diagnostic tools for routine, reliable identification of mycoplasma species has contributed to the historical lack of knowledge about individual species. In particular, very little has been reported about the distribution among herds and role of *Acholeplasma* species in mastitis, and it is unclear whether *Acholeplasma* are simply environmental contaminants (Jasper, 1982). In the future, new knowledge of the clinical relevance of different *Mycoplasma* and *Acholeplasma* along with information gained from routine differentiation of mycoplasma to genus or species level may be an important contribution to a farm developing a treatment and disease management response.

Alternative molecular methods that can, within the same day of detection, confirm a bacteriological diagnosis of mycoplasma as well as further speciate are of value to the mastitis laboratory. Several research groups have developed for culture DNA various PCR-based strategies with and without additional culture strategies to discriminate between *Mycoplasma* and *Acholeplasma* (Boonyayatra et al., 2012a), to speciate bovine mycoplasma (Eissa et al., 2013), or to rapidly identify a few species of concern, particularly *M. bovis* (Baird et al., 1999; Bashiruddin et al., 2005; Boonyayatra et al., 2012b). All of these approaches have distinct merit; however, we have found no single strategy that can within the same day of detection confirm a culture positive mycoplasma and identify as *M. bovis*, *Mycoplasma*, or *Acholeplasma* and that can allow for further speciation of the mycoplasma targeted in the current research work.

Our objective for the current study was to develop and validate a molecular diagnostic assay combining conventional PCR and sequencing to quickly confirm and identify mycoplasma isolated from bacteriological culture of milk routinely submitted by farms and practitioners to the Animal Health Diagnostic Center, Quality Milk Production Services laboratories (Cornell University, Ithaca, NY). Within the same day as detection in culture, the assay can confirm or reject a presumptive positive mycoplasma found upon bacteriological testing of clinical specimens, where a single colony or atypical growth prevents a conclusive identification as mycoplasma. At the same time, the assay can discriminate between *Mycoplasma* and *Acholeplasma*, and identify the most significant mycoplasma pathogen, *M. bovis*. Through subsequent sequence analysis, other bovine *Mycoplasma* and *Acholeplasma* can be identified to species level, adding to our knowledge about the prevalence of these species among dairy farms. This new knowledge will contribute to a better understanding of the role of these species in bovine mycoplasma mastitis.

MATERIALS AND METHODS

Method Description

The molecular diagnostic assay developed includes a multiplex conventional PCR followed by optional sequencing. Composing the assay are 3 separate PCR amplifications that use separate amplification mixes but run concurrently in the same thermocycler under the same running conditions. The first assay amplifies *M. bovis* *wvrC* (**uvrC**) and *Mycoplasma* and *Acholeplasma* 16S rDNA (**16S**) gene targets and incorporates

Table 1. Bacterial species used in testing-method analytical performance, including origin and sources

Microorganism	Origin	Source ¹
<i>Mycoplasma bovis</i>	Bovine mastitis milk	ATCC 25523
<i>Mycoplasma canadense</i>	Bovine genital tract	ATCC 29418
<i>Mycoplasma alkalescens</i>	Bovine nasal cavity	ATCC 29103
<i>Mycoplasma bovigenitalium</i>	Bovine genital tract	ATCC 19852
<i>Mycoplasma bovirhinis</i>	Bovine respiratory tract	ATCC 27748
<i>Mycoplasma arginini</i>	Mouse brain	ATCC 23838
<i>Acholeplasma laidlawii</i>	Sewage	ATCC 23206
<i>Mycoplasma californicum</i>	Bovine mastitis	ATCC 33461
<i>Streptococcus agalactiae</i>	Quality control strain	ATCC 13813
<i>Streptococcus agalactiae</i>	Bovine udder infection	ATCC 27956
<i>Streptococcus dysgalactiae</i>	Bovine udder infection	ATCC 27957
<i>Streptococcus oralis</i>	Bovine milk	QMP B4-273
<i>Streptococcus uberis</i>	Bovine udder infection	ATCC 27958
<i>Listeria monocytogenes</i>	Bovine milk	FSL K2-017
<i>Lactococcus lactis</i> ssp. <i>lactis</i>	Unknown	ATCC 11454
<i>Enterococcus gallinarum</i>	Bovine milk	QMP B4-277
<i>Pediococcus pentosus</i>	Bovine milk	QMP W1-051
<i>Staphylococcus epidermidis</i>	Quality control strain	ATCC 12228
<i>Bacillus subtilis</i>	Bovine milk	QMP W1-127
<i>Staphylococcus aureus</i>	Bovine milk	QMP S1-027
<i>Staphylococcus xylosus</i>	Human skin	ATCC 29971
<i>Staphylococcus simulans</i>	Unknown	ATCC 11631
<i>Bacillus cereus</i>	Bovine milk	QMP W1-126
<i>Streptococcus infantarius</i> ssp. <i>infantarius</i>	Bovine milk	QMP AY1-066
<i>Shigella sonnei</i>	Bovine milk	QMP S1-003
<i>Enterococcus faecalis</i>	Bovine milk	QMP AY1-061
<i>Enterococcus casseliflavus</i>	Bovine milk	QMP AY1-064
<i>Pseudomonas aeruginosa</i>	Bovine milk	QMP W1-182
<i>Salmonella enterica</i> ssp. <i>enterica</i> serovar Typhimurium	Bovine milk	FSL S3-908
<i>Enterobacter cloacae</i>	Unknown	ATCC 23355
<i>Klebsiella pneumoniae</i>	Unknown	ATCC 13883
<i>Serratia marcescens</i>	Bovine milk	QMP Z4-137
<i>Escherichia coli</i>	Bovine milk	QMP S1-212
<i>Pasteurella multocida</i>	Bovine milk	QMP W1-183
<i>Raoultella terrigena</i>	Bovine milk	QMP M2-741
<i>Raoultella planticola</i>	Bovine milk	QMP M2-351
<i>Klebsiella variicola</i>	Bovine milk	QMP M2-348
<i>Clostridium perfringens</i>	Bovine milk	QMP Z3-591

¹ATCC = American Type Culture Collection (Manassas, VA); QMP = Quality Milk Production Services (Cornell University, Ithaca, NY); FSL = Food Safety Laboratory (Cornell University, Ithaca, NY).

2 forward and reverse primer sets. A second assay amplifies the *Mycoplasma* 16S to 23S rDNA intergenic transcribed spacer region (**MITs**), and a third assay amplifies the *Acholeplasma* 16S to 23S rDNA intergenic transcribed spacer region (**AITs**).

Amplification of a combination of 2 or more targets is used to identify a specimen DNA as *M. bovis* (*uvrC*, 16S, MITs positive; AITs negative), *Mycoplasma* (not *M. bovis*; 16S, MITs positive; *uvrC*, AITs negative), *Acholeplasma* (16S, AITs positive; *uvrC*, MITs negative), or not a mycoplasma (negative 16S, MITs; negative *uvrC*, 16S, MITs; negative 16S, AITs; any single gene target alone positive, or all gene targets negative).

Beyond identification of *M. bovis*, further discrimination of *Mycoplasma* and *Acholeplasma* to species level can be made by looking at amplification products of 16S and AITs (*Acholeplasma*) and MITs (*Mycoplasma*).

Method Design

Organisms. *Mycoplasma* and *Acholeplasma* type strains used for assay development and validation were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and are listed in Table 1.

Mycoplasma and *Acholeplasma* field isolates included in the diagnostic validation were obtained from routine bacteriological culture of milk obtained from bulk tank, single pen or animal pools, or individual cow quarter samples. These milks were originally routinely submitted to Quality Milk Production Services laboratories by New York State dairy farms or veterinarians between January 2012 and December 2015 and represented 95 different farms. Using these geographically distinct isolates provided the greatest available degree of clonal diversity for the validation. Milks were cultured for my-

Table 2. Sequences, targets, and expected amplicon sizes for forward and reverse primer pairs used in the study

Primer name	Primer sequence	Target ¹	Amplicon size, bp
MycouvrCF364	5'-TTA CGC AAG AGA ATG CTT CA-3'	<i>uvrC</i>	171
MycouvrCR545	5'-TCA TCC AAA AGC AAA ATG TTA AA-3'		
Myco16SF772	5'-GGG AGC AAA CAG GAT TAG ATA CCC T-3'	16S	269
Myco16SR1041	5'-TGC ACC ATC TGT CAC TCT GTT AAC CT-3'		
MycoITSF9	5'-ACA CCA TGG GAG CTG GTA AT-3'	MITs	236–374, varies by species
MycoITSR383	5'-CCT CAT CGA CTT TCA GAC CCA AGG CAT-3'		
AcholITSF	5'-AAA GTG GGC AAT ACC CAA CGC-3'	AITs	426 and 219, 2 amplicons
AcholITSR	5'-CCA CTG TGT GCC CTT TGT TCC T-3'		

¹16S = 16S rDNA; MITs = *Mycoplasma* 16S to 23S rDNA intergenic transcribed spacer region; AITs = *Acholeplasma* 16S to 23S rDNA intergenic transcribed spacer region.

coplasma using Hayflick Media and incubation for up to 7 d at 37°C with CO₂ enrichment (NMC, 1999). All 845 mycoplasma isolated during the study were analyzed by our PCR assay and further sequenced to species level.

Genomic DNA Extraction for PCR. The Qia-gen DNeasy Blood and Tissue Kit (Life Technologies, Carlsbad, CA) was used for DNA extraction from mycoplasma colonies according to the manufacturer's instructions with the following modifications. Only a single, well-defined, presumptive positive mycoplasma colony was selected from a representative area of the culture plate for DNA extraction. During the DNA elution step, 100 µL of kit Buffer AE was used. All DNA aliquots were stored at –20°C until analysis.

Assay and Primer Design. Three separate PCR reactions were designed to occur within 3 different tubes but also run under the same amplification protocol in the same thermocycler. One of the reactions amplifies 2 targets, *uvrC* and 16S, and the other 2 reactions separately amplify the MITs and AITs targets. After PCR amplification, products are analyzed by electrophoresis on an ethidium bromide agarose gel and can also be purified for sequencing and sequence analysis.

For the *uvrC*–16S assay, previously published mycoplasma 16S primers (van Kuppeveld et al., 1992) were used to amplify *Mycoplasma* and *Acholeplasma* positive isolate DNA. We used *uvrC* as a gene target for *M. bovis*, because this not only readily distinguishes *M. bovis* from other bovine mycoplasma, including the closely related *Mycoplasma agalactiae*. The *uvrC* forward primer (Thomas et al., 2004) was used, and the *M. bovis uvrC* reverse primer was designed using National Center for Biotechnology Information nucleotide and genome databases and Lasergene DNASTAR Primerquest software (Madison, WI). For the MITs and AITs assays, previously published primer sets were used with only slight modification to increase specificity for bovine mycoplasma (Nakagawa et al., 1992; Harasawa et al., 1993; Tang et al., 2000).

The utility of all primer sets was confirmed for *M. bovis*, *Mycoplasma*, and *Acholeplasma* using Lasergene

Megalign software (DNASTAR Inc.) and alignment data created from the National Center for Biotechnology Information gene and nucleotide databases and from the SILVA rRNA database using acquisitions of bovine MITs, AITs, 16S, and *uvrC* sequences. After a broad search BLAST analysis of primers and resulting amplicons, alignments of all sequences and primers were made against specific eubacteria (listed among those in Table 1) that can be found in bovine milk. Species phylogenetically related to mycoplasma, including members of the genera *Bacillus*, *Clostridium*, *Lactobacillus*, and *Streptococcus* (Woese et al., 1984–1985; van Kuppeveld et al., 1992; Tang et al., 2000), were part of this alignment group.

Alignment results of the ITS sequence of eubacteria against our MITs and AITs primers and practical assessment showed that some cross reactivity with primers is possible, although overall sequence homology is poor. However, complementation of a single eubacteria DNA to both 16S and either MITs or AITs primers has not been found by either alignment or practical PCR analysis. Because the assay should also confirm true positives and true negatives, amplification of multiple gene targets was required in the assay design to increase confidence in these results. Primers and sequences are listed in Table 2.

Conventional PCR. Three different 23-µL PCR master mixes were created for the *uvrC*–16S duplex, the MITs, and the AITs reactions using GoTaq Green (Promega, Madison, WI), equimolar concentrations of forward and reverse primers (800 nM of *uvrC* and 250 nM of 16S; 400 nM of MITs; 400 nM of AITs; IDT, Coralville, IA), and nuclease-free molecular biology grade water (IDT). Two microliters of test DNA were added to each of 3 reaction tubes. The PCR amplification profile was performed on a T100 thermal cycler (BioRad, Hercules, CA) with the following conditions: 1 cycle of 94°C for 4 min; 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s; 1 cycle of 72°C for 7 min; and a final hold at 12°C until stop. Amplicons are run on an ethidium bromide gel, with expected amplicon

Figure 1. Gel electrophoresis image of PCR assay amplicons for each amplification reaction [*uvrC*–16S, *Acholeplasma* 16S to 23S rDNA intergenic transcribed spacer region (ITS), and *Mycoplasma* 16S to 23S rDNA ITS] with each group flanked by 100-bp DNA ladder size standards. Column 1 is a known positive *Mycoplasma bovis*, column 2 is a known positive *Mycoplasma* (not *M. bovis*), column 3 is a known positive *Acholeplasma*, and column 4 is a specimen DNA that is negative for *M. bovis*, *Mycoplasma*, and *Acholeplasma*. Sample/column 1 = *M. bovis* (+*uvrC*, +16S, –*Acholeplasma* ITS, +*Mycoplasma* ITS); Sample/column 2 = *Mycoplasma* species, not *M. bovis* (–*uvrC*, +16S, –*Acholeplasma* ITS, +*Mycoplasma* ITS); Sample/column 3 = *Acholeplasma* species (–*uvrC*, +16S, +*Acholeplasma* ITS, –*Mycoplasma* ITS); Sample/column 4 = negative for bovine *Mycoplasma* and *Acholeplasma* species (–*uvrC*, –16S, –*Acholeplasma* ITS, –*Mycoplasma* ITS).

sizes shown in Figure 1. Evaluation of PCR results is summarized in Table 3, and gel results are illustrated in Figure 1.

Sequence Analysis. Isolate DNA found *M. bovis*, *Mycoplasma*, or *Acholeplasma* positive were sequenced using PCR amplicons. The PCR product cleanup was performed by ExoSAP-IT treatment per manufacturer instructions (Affymetrix/USB, Santa Clara, CA). To sequence *Mycoplasma*, the MITS product was used and analyzed as a double strand consensus sequence, whereas for *Acholeplasma*, the AITS 2 band product was used and analyzed as a mixed single strand. In

a comprehensive sequence analysis of the ITS region of all known *Acholeplasma*, Volokhov et al. (2007) reported that most species DNA when amplified produced 2 bands of distinctly different molecular weights. This difference in length was due to the insertion of 1 or 2 tRNA genes into a single ITS allele. The amplified ITS DNA of *Acholeplasma* included in the current study would be expected to and does show this 2-band pattern. Sequence analysis of this mixed amplicon can occur either by excising a single band and performing a 2-strand consensus, or by analyzing the mixed amplicon specimen as a single but overlapping strand, which is expected to be complementary.

For all amplicon analysis, Sanger sequencing was performed by the Cornell Biotechnology Resource Center Genomic Facility (Ithaca, NY). Sequencing was performed in 2 directions using Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA) on a PRISM 3700 DNA analyzer (Applied Biosystems). Raw results were analyzed using Lasergene SeqMan Pro software (Lasergene 10; version 5.08; DNASTAR Inc.). Edited consensus sequences were entered into the National Center for Biotechnology Information BLAST nucleotide search engine, and matched data were interpreted and reported using the Clinical Laboratory Standards Institute–approved guideline of interpretive criteria for identification of bacteria and fungi by DNA target sequencing (CLSI, 2008). Briefly, using Clinical

Table 3. Expected PCR amplification results for a *Mycoplasma bovis* positive, *Mycoplasma* positive (inclusive of *Mycoplasma alkalescens*, *Mycoplasma arginini*, *Mycoplasma bovigenitalium*, *Mycoplasma bovirhinis*, *Mycoplasma californicum*, and *Mycoplasma canadense*), and *Acholeplasma* positive (inclusive of *Acholeplasma laidlawii*, *Acholeplasma granularum*, and *Acholeplasma oculi*) isolates

Microorganism	Gene target ¹			
	<i>uvrC</i>	16S	MITS	AITS
<i>Mycoplasma bovis</i>	+	+	+	–
<i>Mycoplasma</i>	–	+	+	–
<i>Acholeplasma</i>	–	+	–	+

¹16S = 16S rDNA; MITS = *Mycoplasma* 16S to 23S rDNA intergenic transcribed spacer region; AITS = *Acholeplasma* 16S to 23S rDNA intergenic transcribed spacer region. Amplification of the given gene target is indicated by +, whereas no amplification or amplification of an incorrect size DNA is indicated by –.

Laboratory Standards Institute guidelines, identification to the species level was defined as sequence similarity of $\geq 99\%$ with that of the type strain sequence (or other strain published in peer-reviewed literature) in GenBank, with percent identity $>0.8\%$ divergence from other species.

Method Validation

Assay analytical and diagnostic performance validation followed recommendations of AOAC International Microbiological Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis (Feldsine et al., 2002), the World Health Organization Codex Alimentarius International Food Standard CAC/GL 74-2010 (FAO/WHO Codex Alimentarius Commission, 2010), and the OIE World Organization of Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2010).

Sequencing and sequence analysis of *uvrC* (*M. bovis*; Subramaniam et al., 1998; Thomas et al., 2004) and 16S to 23S rDNA ITS were used as the gold standard. For characterization to the species and strain level, sequencing of variable and hypervariable regions of the *Mycoplasma* and *Acholeplasma* 16S to 23S ITS genetic region within the rRNA operon has been extensively used (Volokhov et al., 2006, 2007), often in concert with 16S analysis (Volokhov et al., 2012).

Analytical Performance. To test inclusivity we selected at random from our culture database 50 different *M. bovis* isolates, 50 isolates representing bovine *Mycoplasma* (*M. bovis*, *M. bovirhinis*, *M. canadense*, *M. californicum*, *M. alkalescens*, and *M. arginini*) and 50 isolates representing bovine *Acholeplasma* (*A. laidlawii*, *A. oculi*, *A. granularum*). Each isolate selected was from a different farm source and had been previously sequence analyzed to identify using published methods (Volokhov et al., 2007, 2012).

Exclusivity was tested by assessing 30 different bacterial species that might be naturally found in bovine milk or that are closely genetically related and thus potentially cross-reactive. Closely related genera included members of *Bacillus*, *Clostridium*, *Lactobacillus*, and *Streptococcus* (Woese et al., 1984–1985; van Kuppeveld et al., 1992; Tang et al., 2000). These bacterial strains were either acquired from the American Type Culture Collection as type strains or as field isolates from routine diagnostic laboratory submissions and culture of milk, with identity confirmed by 16S and *rpoB* sequencing (Drancourt et al., 2000, 2004). Isolates tested are listed in Table 1.

Diagnostic Performance. The sampling design created to assess diagnostic sensitivity (DSe) and specificity (DSp) was based on commonly used statistical formulas (Lesaffre et al., 2007). Briefly, DSe and DSp are calculated using true positive (TP), true negative (TN), false positive (FP), and false negative (FN) results, where $DSe = TP / (TP + FN) \times 100\%$ and $DSp = TN / (TN + FP) \times 100\%$. For each of the assay sets (*uvrC*-16S, MITS, AITS) for each of the target groups (*M. bovis*, *Mycoplasma*, and *Acholeplasma*), estimates of DSe and DSp were first established as performance indicators. We set a 99% confidence interval for DSe and DSp parameters as a reference. Because it is anticipated that the likely values of DSe and DSp for each assay pair for each target group will be close to the reference set, a standard 95% confidence interval and a 2% allowable error were set for the estimate or predictive value of each of these parameters in practice. Using these indicator values and limits, and the table and recommendations presented in the OIE Terrestrial Manual (OIE, 2010), a minimum sample size of 95 known positives and 95 known negatives was established. The study design must consider that 3 different groups are discriminated (*M. bovis*, *Mycoplasma*, and *Acholeplasma*).

To test *uvrC*, 16S, MITS (known positive *M. bovis*) DSe, 95 field isolates were assayed. To test 16S, MITS (known positive *Mycoplasma*, not *M. bovis*) DSe, 95 field isolates representing 7 different bovine species (*M. arginini*, *M. alkalescens*, *M. canadense*, *M. californicum*, *M. bovirhinis*, *M. bovirhinis*, *M. bovirhinis*, *M. dispar*) were assayed. To test 16S, AITS (known positive *Acholeplasma*) DSe, 95 field isolates representing 3 different bovine *Acholeplasma* (*A. laidlawii*, *A. granularum*, and *A. oculi*) were selected.

To test DSp for *uvrC*, 16S, MITS (known negative *M. bovis*), 95 negative samples were selected among isolates of non-*M. bovis* *Mycoplasma* species (45), representing 20 different bovine (*M. arginini*, *M. alkalescens*, *M. canadense*, *M. californicum*, *M. bovirhinis*, *M. bovirhinis*, *M. bovirhinis*, *M. dispar*) and nonbovine (*M. agalactiae*, *Mycoplasma canis*, *Mycoplasma cynos*, *Mycoplasma edwardii*, *Mycoplasma falconis*, *Mycoplasma feliminutum*, *Mycoplasma felis*, *Mycoplasma gallinarum*, *Mycoplasma gypis*, *Mycoplasma leonicaptivi*, *Mycoplasma maculosum*, *Mycoplasma mucosicanis*, and *Mycoplasma spumans*) species and isolates of *Acholeplasma* species (50).

To test DSp for *uvrC*, 16S, MITS (known negative *Mycoplasma*), negative samples were selected among isolates of *Acholeplasma* (50) and nonmollicutes (45). To test DSp for *uvrC*, 16S, AITS (known negative

Acholeplasma), negative samples were selected among isolates of *Mycoplasma* (50) and nonmollicutes (45).

RESULTS

Method Validation

Analytical Performance. For all 50 *M. bovis* tested for analytical performance, the *uvrC*, 16S, and MITS gene targets all amplified with no amplification of AITS. For all 50 *Mycoplasma* species tested for analytical performance, both 16S and MITS amplified with no amplification of *uvrC* or AITS. For all 50 *Acholeplasma* species tested, both 16S and AITS amplified with no amplification of *uvrC* or MITS. All amplicon sizes were as anticipated: 171-bp *uvrC* (single band); 269-bp 16S (single band); 236- to 374-bp (single band) MITS; and 219- and 426-bp (2 bands) AITS. All *M. bovis* DNA produced a *uvrC*, 16S, and MITS amplicon. All other *Mycoplasma* produced a 16S and MITS amplicon. All *Acholeplasma* produced a 16S and 2 AITS amplicons.

For the *uvrC*-16S assay, it was found that *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus oralis* produced a single 269-bp amplicon, but neither MITS nor AITS assays showed DNA amplification. Amplification of *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Lactococcus lactis* DNA also occurred in the *uvrC*-16S assay, but the amplicons were of a distinctly different size than what is required to indicate a mycoplasma. *Streptococcus agalactiae*, *L. lactis*, and *Salmonella typhimurium* produced amplicons of size close to that expected for *uvrC* (171 bp) but either did not show a second band representing 16S amplification or had a second band of incorrect size. None of these bacteria DNA amplified when tested with the MITS or AITS primers.

Diagnostic Performance. The diagnostic sensitivity and specificity were calculated for each diagnostic performance data set: *uvrC*-16S and MITS (where *M. bovis* is the known positive); *uvrC*-16S and MITS (where *Mycoplasma* not *M. bovis* are the known positive); and *uvrC*-16S and AITS (where *Acholeplasma* is the known positive). For each data set there were 95 reference samples in each category (95 known positive and 95 known negative). Testing each diagnostic performance data set resulted in 95 true positives (TP), 0 false positives (FP), 0 false negatives (FN), and 95 true negatives (TN). From these values the DSe was calculated as $TP/(TP + FN) \times 100\%$, or 100%(96.1 – 100%) for each set. The DSp was calculated as $TN/(TN + FP) \times 100\%$, or 100%(96.1 – 100%) for each set.

Distribution and Prevalence of Mycoplasma Among New York Farms

Among the 845 mycoplasma isolates obtained during this study, the following total occurrence and distribution of each species among the farms, from highest level of prevalence to lowest level, was identified: 627 *M. bovis* from 68 farms (78% prevalence); 22 *M. alkalescens* from 15 farms (17% prevalence); 119 *A. laidlawii* from 10 farms (11% prevalence); 15 *M. bovigenitalium* from 9 farms (10% prevalence); 16 *M. canadense* from 9 farms (10% prevalence); 35 *M. californicum* from 3 farms (3% prevalence); 3 *M. arginini* from 2 farms (2% prevalence); 3 *M. bovirhinis* from 1 farm (1% prevalence); 5 *A. oculi* from 2 farms (2% prevalence); and 1 *A. granularum* from 1 farm (<1% prevalence).

More than one mycoplasma species was detected in 13 of the 95 farms (14% of all farms represented). In one case, more than one species was detected from a single quarter milk sample (*M. bovis* and *M. bovigenitalium*). In 12 of these 13 farms, *M. bovis* was identified as well as at least one other mycoplasma species. In 8 of these 13 farms (61% of multiple species positive farms and 6% of all 95 farms) the occurrence of both *M. bovis* and *A. laidlawii* was identified. Within the group of 13 farms, we identified an occurrence of 2 different mycoplasma species in 6 farms, 3 different species in 3 farms, 4 different species in 3 farms, and 8 different species in 1 farm.

DISCUSSION

The resulting analytical and diagnostic validation data collected indicate this is a reliable assay with excellent specificity and sensitivity and has passed internal criteria of the Animal Health Diagnostic Center for validation of newly developed clinical diagnostic assays to be routinely used for customer testing. Only minor optimizations were required during initial analytical validation exclusivity testing, where cross-reactivity results for some nontarget strains led to slight modifications to the assay. Specifically, exclusivity (ability of method to reject any unintended target) was improved by requiring that all 4 assay components (*uvrC*, 16S, MITS, and AITS) be run and assessed together. Thus, to confirm an isolate as *Mycoplasma* or *Acholeplasma*, or as *M. bovis*, no single gene target can be used and a combination of gene targets must be considered (16S and MITS; 16S and AITS; and 16S, MITS, and *uvrC*, respectively).

Validation results showed that used alone the 3-component PCR assay can reliably and within 1 d confirm bacteriological culture detection of bovine mycoplasma,

identify an isolate as the highly contagious *M. bovis*, and further discriminate between *Mycoplasma* and *Acholeplasma* species. Isolates can also be reported as culture negative for bovine *Mycoplasma* and *Acholeplasma* species when no DNA amplification exists or where the prerequisite combination of amplicons is not produced. The assay therefore is an excellent option for routine analysis of clinical samples where confirmation or rejection of a positive *Mycoplasma* result is needed, or perhaps where no other pathogen has been identified. It can offer additional information when low level mycoplasma is suspected, where atypical growth is present, or where the mycoplasma does not subculture. Furthermore, the most broadly clinically significant and ubiquitous mycoplasma, *M. bovis* (Maunsell et al., 2011), can be reported back to the farm or practitioner within the same day as culture detection.

To support phenotypic analysis of mycoplasma, such alternative methods of speciation that are reliable and discriminatory but also relatively quick and easy to perform and analyze are of value. Several research groups have developed PCR-based and other strategies for culture analysis to discriminate between *Mycoplasma* and *Acholeplasma*, to speciate mycoplasma, or to rapidly identify a few species of concern. However, no one published method accomplishes all these goals, and none of the methods are intended for culture confirmation. McAuliffe et al. (2003) reported identification of *Mycoplasma* using PCR amplification followed by denaturing gradient gel analysis; however, *Acholeplasma* identification was not included in the study and analysis is challenging. A method reported by Boonyayatra et al. (2012a) used amplification of 16S rDNA by PCR followed by complementary phenotypic assays to distinguish between *Mycoplasma* and *Acholeplasma*; however, the method did not further speciate and required additional culture for completion. Rebelo et al. (2011) reported a relatively rapid method that employed PCR amplification of ITS followed by high resolution melt analysis; however, this method only identified *Mycoplasma* and analysis is difficult. Recently, a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method for identification of ruminant *Mycoplasma* (Pereyre et al., 2013) and a rapid DNA microarray assay for identification of both *Mycoplasma* and *Acholeplasma* (Schnee et al., 2012) have been reported. However, both methods require specialized and expensive equipment, and the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method is intended for a high density mycoplasma specimen, requiring additional culture. Furthermore, in both cases a high level of skill is required for performance and analysis. Several various technologies to directly detect mycoplasma DNA in

milk with subsequent identification have been reported (Baird et al., 1999; Justice-Allen et al., 2011; Boonyayatra et al., 2012b); however, these methods were not intended to be confirmatory or identification tools to support culture analysis.

Initial investigation of the current assay as a direct detection method for milk was unsuccessful (data are not reported here). However, preliminary testing of the current assay used directly on other less complex clinical sample types including bovine tracheal swabs in Amies, lung tissue swabs in Amies, nasal swabs in Amies, and tracheal fluid has shown promise. Although these sample types are not used for mastitis testing, they are samples of choice for detection of mycoplasma associated with bovine pneumonia and arthritis (Maunsell et al., 2011). The DNA was extracted directly from fluid aliquots of clinical samples that previously tested positive for mycoplasma using bacteriological culture. The DNA was also extracted from colonies from the positive cultures. These DNA were further analyzed in parallel with the current assay. Direct testing of the 4 different sample types resulted in positive *Mycoplasma*, with an identification for all of *M. bovis*. The parallel culture analysis was identical. Therefore, the assay shows promise at least initially for direct analysis of simple clinical samples for mycoplasma in cases of bovine pneumonia or arthritis, and with further research and validation testing could feasibly replace the more lengthy bacteriological culture methods for these sample types.

Mycoplasma speciation data acquired during the validation of the current molecular diagnostic assay furthered our knowledge of the distribution and prevalence of bovine *Mycoplasma* or *Acholeplasma* within the New York State region. It has been discussed that the distribution and prevalence of bovine mycoplasma varies regionally across the United States practices (Fox, 2012), which may be influenced in part by herd size and differences in herd management (USDA APHIS, 2003); however, it is unclear whether prevalence of individual species also varies regionally. Herd level prevalence data for bovine *Mycoplasma* as a group has been reported for northeastern states, including New York, as recently as 2008 (USDA APHIS, 2008). However, the last published survey data for individual mycoplasma species among New York herds was reported in 1992 for the interval 1972 to 1990 (Gonzalez et al., 1992). This study employed a fluorescent antibody technique for mycoplasma identification (Stewart, 1967), a method that is not inclusive of all mycoplasma and can be difficult to interpret. Neither report included *Acholeplasma* prevalence data. Thus, these data may not accurately reflect prevalence of individual species. Among the 165 herds surveyed by Gonzalez et al. (1992), the major-

ity of mycoplasma recovered was identified as *M. bovis* with only a single instance of *M. californicum*. For the current report, a greater diversity of species was identified, with 8 different *Mycoplasma* species among 95 herds, as well as 3 different *Acholeplasma* species. The overall prevalence of each *Mycoplasma* species (*M. californicum* 3%; *M. alkalescens* 17%; *M. arginini* 2%; *M. canadense* 10%; *M. arginini* 2%; *M. bovirhinis* 1%; *M. bovis* 78%) was different from that reported by USDA APHIS (2003), who measured a generally higher prevalence of *M. bovis* (86%) and a small but equal number of *M. californicum*, *M. alkalescens*, *M. canadense*, and *M. bovis*. A summary of other surveys performed in western United States herds showed generally a very high prevalence of both *M. bovis* and *M. californicum*, followed by a moderate level of *M. bovis* and a low prevalence of other *Mycoplasma* (Fox, 2012), supporting the premise of regional species distribution differences.

As a result of the current study it was found that among 14% of the herds, a *Mycoplasma* other than *M. bovis* occurred alone or along with *M. bovis*. Others have similarly reported isolation of multiple mycoplasma species from bulk-tank milk, either alone or along with *M. bovis* (Justice-Allen et al., 2011). The current study also found that among 6% of the herds tested, both *M. bovis* and *A. laidlawii* were simultaneously isolated, a condition that remained relatively stable during the 4-yr sampling period. This occurrence of both *M. bovis* and *A. laidlawii* in a herd along with the lack of data to unequivocally document *A. laidlawii* as a primary mastitis pathogen may suggest that *A. laidlawii* may present as an opportunistic pathogen or secondary commensal microorganism in an already immunocompromised animal. It is worth further study to determine whether *A. laidlawii* can cause IMI in individual animals where no previous or current infection with another mycoplasma has occurred. Overall, these data suggest further investigation into the clinical significance of multiple *Mycoplasma* species and IMI.

CONCLUSIONS

Incorporation of the current validated molecular diagnostic assay into routine bacteriological screening as a supportive confirmation and identification tool will lead to an improved assessment of *Mycoplasma* and *Acholeplasma* prevalence, data that will facilitate increased knowledge about the role of these mycoplasma in IMI as primary or secondary pathogens. New knowledge will ultimately lead to improved farm management response times and strategies during a mycoplasma outbreak, and improvements in herd health management.

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