Running title: Mycoplasma identification from bovine isolates by Multiplex Conventional PCR Assays

Mycoplasma Identification by Multiplex Conventional PCR Assays and Sequence Analysis

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ABSTRACT

Three conventional PCR assays and a sequencing strategy were developed. These assays can confirm culture detection of bovine *Mycoplasma* and *Acholeplasma* species, including *Mycoplasma bovis, M. arginini, M. alkalescens, M. canadense, M. bovirhinis, M. bovigenitalium, M. californicum, Acholeplasma laidlawii, Acholeplasma oculi,* and *Acholeplasma granularum.* This assay has proven potential to identify other species of mycoplasma within the phylogenetic taxonomic clusters Bovis-lipophilum, Synoviae, and Hominis.

By PCR and gel analysis, the assay can identify any isolate as *Mycoplasma bovis*, the most important agent in bovine mastitis. This is indicated by a positive amplification of the *uvrC* gene target with concurrent amplification of *16S* and the *Mycoplasma* 16S-23S rRNA intergenic transcribed spacer regions (ITS). Isolates that show amplification of both the *16S* and the *Mycoplasma* ITS, but show no amplification of *uvrC,* can be reported as *Mycoplasma* species (not *M. bovis*). Isolates negative to *M. bovis* by the assay, and that show amplification of both the *16S* and the *Acholeplasma* ITS, can be reported as *Acholeplasma* species (not *Mycoplasma*). Isolates with negative *uvrC*, negative *Mycoplasma* ITS, and negative *Acholeplasma* ITS, but that are positive to the *16S* target are reported as negative for bovine *Mycoplasma* and *Acholeplasma* species. Further discrimination of *Mycoplasma* and *Acholeplasma* to species level can be made by looking at amplification products of the *16S* and *Mycoplasma* ITS, respectively.

Key words: Mycoplasma, intramammay infection, Multiplex Conventional PCR Assays

INTRODUCTION

Mycoplasma intramammary infection (IMI) of dairy cattle is a serious condition that results in financial losses and culling of infected animals. Mycoplasma mastitis is difficult to treat effectively with antibiotics and carriage in an animal may persist in a subclinical state. Mycoplasmas spread readily within a herd, thus early and accurate identification is critical in prevention and in reduction of the spread of this highly contagious disease. Clinical and pathological signs often are not characteristic for mycoplasma disease, thus laboratory diagnosis is commonly required.

Among the *Mollicute* bacteriological class, a number of *Mycoplasma* and *Acholeplasma* species have been isolated from bovine hosts and the farm environment. *Mycoplasma* species have been implicated in causing mastitis as well as other reproductive and respiratory diseases (Nicholas et al., 2008), with *Mycoplasma bovis* considered the most prevalent and clinically important mycoplasma species in dairy cattle, associated with acute mastitis, arthritis, otitis, and pneumonia. *Acholeplasma laidlawii* and other *Acholeplasma* species have been isolated from dairy cattle and are commonly found in the dairy environment, however due to a scarcity of research knowledge, are generally considered to be saprophytic microorganism and non pathogenic (Jasper, 1981).

The AHDC QMPS Bacteriology Laboratory employs Hayflick's Media under 10% CO_2 for routine culture of mycoplasmas from milk samples routinely submitted for analysis. This commonly used media incorporates sterol that is required by bovine *Mycoplasma* species for growth. Although *Acholeplasma* species do not require sterol for growth, they will also grow on this media but are indistinguishable from *Mycoplasma*. Unless a second sterol-free media is incorporated into analysis, the current routine culture method cannot discriminate between these genera.

Even under specialized conditions, mycoplasmas are often slow growing, or may exhibit atypical colony morphology that can confound a more definitive report. Some species or strains may not be

66 subculturable after initial detection on a plate from a primary clinical sample. To confirm a
67 diagnosis of mycoplasmas where such atypical growth is found, or where only a single colony
68 exists, QMPS employs subculture on Hayflick's Media for up to 7 days. This process can prolong
69 results reporting, but more importantly may fail to confirm a true positive mycoplasma if the
70 mycoplasma is resistant to subculture.

71

72 In some cases, differentiation of mycoplasmas to genus or species level is an important contribution
73 to the farm developing treatment and disease management response. Specific detection of
74 *Mycoplasma bovis*, identification of a mycoplasma detected as belonging to the genus of either
75 *Acholeplasma* or *Mycoplasma*, and/or further identification of *Acholeplasma* or *Mycoplasma* to the
76 species level may affect subsequent farm management and treatment strategies. Serological and
77 biochemical tests have been standardized for further identification of *Mycoplasma* species however
78 both approaches have limitations and neither has been defined for *Acholeplasma* species. Although
79 serological tests have the potential to speciate *Mycoplasma* isolates, these are lengthy procedures
80 and may be difficult to perform accurately and reliably. Mycoplasmas often share common surface
81 antigens, which can result in interspecies antisera cross reactivity. Reported antigentic heterogeneity
82 among some *Mycoplasma* species can add to the difficulties of arriving at unambiguous speciation.
83 Biochemical tests are discriminatory only to the genus level or to the fermentation group (Volokhov
84 et al, 2012).

85

86 To support phenotypic analysis of mycoplasmas, alternative methods of speciation that are reliable
87 and discriminatory are of value. A number of research groups have developed PCR-based strategies
88 to discriminate between *Mycoplasma* sp. and *Acholeplasma* sp., or to speciate mycoplasmas, or to
89 rapidly identify a few species of concern. Each of these strategies reported uses an initial

amplification of 16S and/or 16S-23S rRNA ITS DNA followed by a second or third step to gain additional information (Boonyayatra et al, 2012; Tang, et al., 2000; Volokhov et al, 2012; Volokhov, et al, 2005; Harawawa et al., 2005; van Kuppeveld et al, 1992; Lauerman, et al., 1995; Kong, et al., 1999; Baird et al., 1999). Examples reported include two-step nested conventional PCR with 1-2 gene target amplifications, single gene target PCR followed by complementary phenotypic assays, or amplification of 1-2 housekeeping gene targets by conventional PCR to complement 16S or 16S-23S ITS amplification results. For the current method, components of these commercial and research approaches have been utilized in order to arrive at a customized genetic assay that can be utilized with available resources, can be performed in a cost-effective manner for the customer, and that specifically achieves the following diagnostic goals:

1. To rapidly detect the most important bovine mastitis pathogen, *M. bovis*
2. To discriminate between *Mycoplasma* species and *Acholeplasma* species isolated from culture of clinical specimens
3. To allow for the further speciation of *Mycoplasma* or *Acholeplasma* if required
4. To enable confirmation or rejection of presumptive positive *Mycoplasma/Acholeplasma* found upon bacteriological testing of clinical specimens, where a single colony or atypical growth confounds a more definitive report

To functionally achieve these goals, three separate PCR assay amplifications have been developed that run concurrently in the same machine under the same running conditions. The first assay amplifies *uvrC* and *Mycoplasma* and *Acholeplasma 16S* gene targets. A second assay amplifies *Mycoplasma* 16S-23S rRNA ITS DNA. The third assay amplifies *Acholeplasma* 16S-23S rRNA ITS DNA. Each assay utilizes a PCR master mix optimal for the particular gene target(s), however all assays can be run in the same PCR machine under the same running conditions. After PCR

amplification and gel electrophoresis of amplicons, the expected results are as follows. A positive *M. bovis* isolate will produce *uvrC, 16S,* and *Mycoplasma* 16S-23S rDNA ITS amplicons. An isolate that is not *M. bovis* but is another *Mycoplasma* species will produce *16S* and *Mycoplasma* ITS amplicons. An isolate that is not *M. bovis* or another *Mycoplasma* species, but is an *Acholeplasma* species, will produce *16S* and *Acholeplasma* ITS amplicons. An isolate that is negative for any *Mycoplasma* or *Acholeplasma* species will either be negative for all three gene targets, or may show amplification of only a single target.

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To confirm an isolate as *Mycoplasma* or *Acholeplasma*, a combination of gene targets was selected in order to increase confidence in the true positive and true negative rates, and for the ability to further identify by sequencing if requested. The genetic assays described in the current report are based on the highly conserved mollicute *16S* sequences and on the highly conserved but more variable regions at the genus and species levels of the *Mycoplasma* and *Acholeplasma* 16S-23S rRNA ITS. Currently, the 16S rRNA gene sequence is the international standard for description and ranking of Mollicute class, and to date a substantial sequence database has been established (Brown et al., 2007). Although it is believed that mollicutes have evolved from clostridia, and are closely related to members of the genera *Bacillus, Clostridium, Lactobacillus,* and *Streptococcus* (Woese et al., 1984; van Kuppeveld et al, 1992; Tang et al, 2000), it is well researched that mollicute rRNA genes contain very few similarities to most common eubacterial rRNA genes (Woese et al., 1984; Weisburg et al., 1989; Razin et al, 1998). Significant work over the past 20 years or more has contributed to several well-established and publically available mycoplasma rRNA databases, including the National Center for Biotechnology Information (NCBI) nucleotide and genome databases and the SILVA rRNA database (Max Planck Institute for Marine Biology, Microbial Genomics and Bioinformatics Research Group, Bremen, Germany). These are dynamic

databases that are continually receiving sequence data contributions and can only improve as a resource for our assay.

The two sets of *Mycoplasma* sp. and *Acholeplasma* sp. ITS primers selected for the current assay have been previously validated and incorporated into several currently available commercial mycoplasma detection kits. Specifically, Invitrogen offers two different MycoSEQ Mycoplasma detections kits, both qPCR assays that detect over 90 *Mycoplasma* and *Acholeplasma* species in cell cultures or cell culture media. The American Type Culture Collection (ATCC) offers the Universal Mycoplasma Detection Kit (ATCC 30-1012K), a touchdown conventional PCR assay that similarly broadly detects over 60 different species of mollicutes in cell culture. Both kits reported ability to detect mollicutes found in bovine hosts and in the bovine environment, including *M. bovis*, *M. canadense*, *M. alkalescens*, *M. bovigenitalium*, *M. bovirhinis*, *M. arginini*, *M. californicum*, and *Acholeplasma laidlawii.* .

important to increase the confidence level for this parameter of a true positive.

After optimization of each of the three assays individually and together under identical running conditions, validation was performed on bovine diagnostic specimens submitted from the field to the AHDC QMPS Molecular Laboratory between July 2011 and October 2013. Our procedure and results are detailed in the sections to follow.

MATERIALS AND METHODS

2.0 Scope and Application (description of intended use)

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The *uvrC*/*16S*, *Mycoplasma* ITS, and *Acholeplasma* ITS PCR assays together can:

1. confirm a presumptive positive mollicute isolate (amplification of *16S* AND either *Mycoplasma* ITS OR *Acholeplasma* ITS) or reject a presumptive positive mollicute isolate (amplification of *16S* OR *Mycoplasma* ITS OR *Acholeplasma* ITS singly), where a single colony or atypical growth confounds a more definitive report.
2. discriminate between *Mycoplasma* sp. and *Acholeplasma* sp., by confirming an isolate as positive to *Mycoplasma* sp. (amplification of *Mycoplasma* ITS AND *16S*) or *Acholeplasma* sp. (amplification of *Acholeplasma* ITS AND *16S*).
3. identify specific mycoplasma isolate to species level beyond that of *M. bovis* using the amplicon of *16S* (to identify *Acholeplasma* species) or *Mycoplasma* ITS (to identify *Mycoplasma* species) in a subsequent sequencing reaction, for treatment/management purposes.
4. rapidly identify a positive mycoplasma isolate as *Mycoplasma bovis*, for treatment/management purposes (amplification of *uvrC* AND *16S* AND *Mycoplasma* ITS*)*.

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3.0 Description of Assay: Methodology

3.1 Materials and Methods

3.1.1 Materials:

GoTaq® Green Master Mix (Promega M7123)

nuclease-free molecular biology grade water, 2 ml aliquots (IDT 11-04-02-01)

Primers (IDT, Coralville, IA, USA):

uvrC primers:

MycouvrCF364: 5’-TTA CGC AAG AGA ATG CTT CA-3’

MycouvrCR545: 5’-TCA TCC AAA AGC AAA ATG TTA AA-3’

mycoplasma *16S* primers:

Myco16SF772: 5’-GGG AGC AAA CAG GAT TAG ATA CCC T-3’

Myco16SR1041: 5’-TGC ACC ATC TGT CAC TCT GTT AAC CT-3’ *Mycoplasma*

ITS primers:

MycoITSF9: 5’-ACA CCA TGG GAG CTG GTA AT-3’

MycoITSR383: 5’-CCT CAT CGA CTT TCA GAC CCA AGG CAT-3’

Acholeplasma ITS primers:

AcholITSF: 5'-AAA GTG GGC AAT ACC CAA CGC-3'

AcholITSR: 5'-CCA CTG TGT GCC CTT TGT TCC T-3'

ethidium bromide, 1% w/v (Fisher BP130210)

5X TBE (EMD 8800)

agarose-LE, ultrapure, molecular biology grade (USB Affymetrix 32802)

EXOSAP-IT® (USB Affymetrix 782011)

TrackIt® 100 bp DNA ladder (Invitrogen 10488-058)

One set of DNA extracts are prepared from the unknown isolate(s). DNA content is measured by Nanodrop spectrophotometery, and recorded. Three different PCR master mixes are created for the *uvrC/16S* duplex, the *Mycoplasma* ITS, and the *Acholeplasma* ITS reactions. A minimum Master Mix volume needed for 10 samples (including controls) is prepared. For more than 10 samples, Master Mix volume is calculated for the number of samples plus 1.

Each of the three PCR reaction runs under common thermocycler conditions of 4 min at 94°C, followed by 40 cycles of 30 sec at 94°C/ 30 sec at 56°C/30 sec at 72°C, with a final step of 72°C for 7 min and a hold at 12°C until stop. The assays have been validated using both the BioRad MyCycler and BioRad T100 thermal cyclers, which can hold up to 96 reaction tubes. Thus, 32 samples (including controls) can be run in a single instrument at one time. Each individual PCR reaction is carried out in a 25 µl volume. DNA is used at a concentration of 40-400 ng/reaction. Assays can be performed in 96-well plates or in 8-tube domed cap strips, with individual snap caps. If further sequence analysis is necessary, the tube strips should be used instead of plates to minimize lab contamination with PCR amplicon.

Controls employed for the *uvrC/16S* duplex PCR are: DNA from *M. bovis* previously amplified successfully with the duplex reaction (*uvrC* and mycoplasma *16S* positive amplification control), DNA from *M. bovis* extracted concurrently with DNA from unknown sample DNA (positive extraction control), and DNA from *Acholeplasma laidlawii* (*Acholeplasma 16S* positive amplification control).

Controls employed for the *Mycoplasma* ITS PCR are: DNA from *M. bovis* previously amplified successfully with the PCR reaction (*Mycoplasma* ITS positive control) and DNA from *M. bovis* extracted concurrently with DNA from unknown sample DNA (positive extraction control).

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Controls employed for the *Acholeplasma* ITS duplex PCR are: DNA from *Acholeplasma* previously amplified successfully with the duplex reaction (*Acholeplasma* ITS positive control), and DNA from *Acholeplasma laidlawii* extracted concurrently with DNA from unknown sample DNA (positive extraction control).

A negative DNA extraction control and a negative PCR reaction mix control are included for each of the three assays.

For optimal performance of these assays, clinical sample DNA must be adjusted to fall within 20-200 ng/ul. If the DNA is less than this, the water content of the PCR master mix can be reduced and the amount of DNA used in the assay increased accordingly.

PCR amplification products are stored at 4°C until further analysis can be performed. Analysis is by gel electrophoresis, where PCR amplicons from all assays are applied to wells of a 100 ml 2% w/v agarose gel containing 5 µg ethidium bromide. Loaded gels are electrophoresed in 0.5X TBE buffer on a BioRad mini-gel horizontal electrophoresis system at 105V for 50 minutes. DNA molecular weight markers of 100 bp are used in both the first and last lanes of the gel for sizing of amplicons of the unknowns and of the controls.

Expected amplicon sizes are as follows:

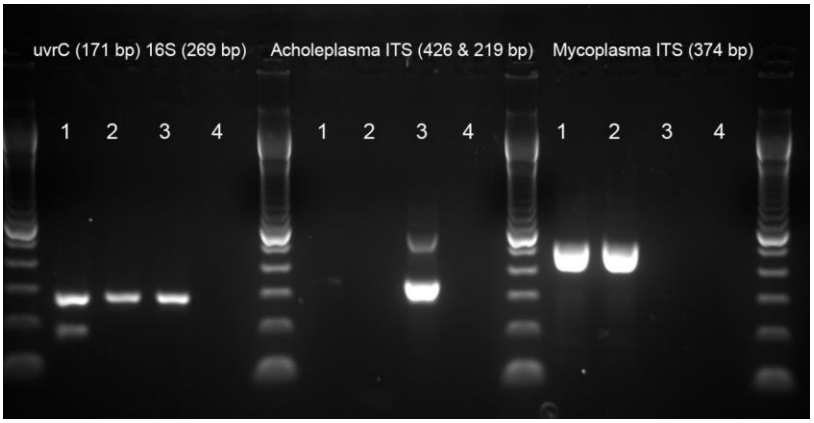
uvrC: 171 bp

16S: 269 bp

Mycoplasma ITS: 236-374 bp (variable, depending on species)

Acholeplasma ITS: 2 bands, 426 and 219 bp

The images below show a positive result for *M. bovis*, a positive result for *Mycoplasma* species (not *M. bovis*), and a positive result for *Acholeplasma* species:



Sample 1= *M. bovis* (+*uvrC*, +*16S*, - *Acholeplasma* ITS, +*Mycoplasma* ITS)

Sample 2 = *Mycoplasma* species, not *M. bovis* (-*uvrC*, +*16S*, -*Acholeplasma* ITS, +*Mycoplasma* ITS)

Sample 3= *Acholeplasma* species (-*uvrC*, +*16S,* +*Acholeplasma* ITS, -*Mycoplasma* ITS)

Sample 4 = negative for bovine *Mycoplasma* and *Acholeplasma* species (-*uvrC*, -*16S*, -*Acholeplasma* ITS, -*Mycoplasma* ITS)

Where isolates are found *M. bovis* negative and either *Acholeplasma* or *Mycoplasma* positive, and further speciation is required, amplicons for both duplex reactions are prepared for sequencing as follows. First PCR product cleanup is performed by ExoSAP-IT treatment (per manufacturer instructions; Affymetrix/USB, Santa Clara, CA). To sequence *Mycoplasma,* the *Mycoplasma* ITS product is used. To sequence *Acholeplasma*, the 16S product is used. To improve the quality and maximize the length of sequence available for analysis, both forward and reverse strands are sequenced. Following the protocol for Sanger Sequence analysis provided by the Cornell Biotechnology Resource Center DNA Sequencing Facility (BRC, Cornell University, Ithaca, NY), amplicons are mixed separately with 4 µM forward or reverse target primer and diluted to a final volume of 18 µl with molecular biology grade water, using either 8-tube strips or 96-well plates as sample size dictates. After placing a sequencing order with the BRC and obtaining an order identification number, samples are submitted to this facility for sequencing. Raw forward and reverse sequence data is provided by the BRC as a zip file. Data is imported into DNASTAR Lasergene 10 SeqMan Pro software, where the forward and reverse strands are aligned and then manually edited using our internal protocol. Edited consensus sequences are entered into the NCBI BLAST search engine, and matched data is interpreted and reported using the CLSI approved guideline of interpretive criteria for identification of bacteria and fungi by DNA target squencing (Clinical and Laboratory Standards Institute, 2008).

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3.2 Sample Volume; Sample Preparation

Isolate sources include raw milk swab agar plates, environmental swab agar plates, tissue swabs, pure cultures of mycoplasma on agar plates, or other sample types including tracheal swabs in Amies, lung tissue swabs in Amies, nasal swabs in Amies, or tracheal fluid. For isolates on an agar substrate, DNA is extracted from a presumptive positive mycoplasma colony. A well-defined colony is selected, and either a loop or needle tool is used to scrape or cut the colony cleanly from the agar plate upon which it is found. For mixed cultures or where a single mycoplasma colony exists, it is important to excise the full colony without including exterior surface media and potential contaminant DNA. For smaller colony growth, 2-3 colonies may be extracted if microscopic examination reveals uniformity in colony morphology. Multiple mycoplasmas and acholeplasmas may be found in a specimen, and so it is best to perform identification on a single colony if possible.

After excision or extraction, the colony is placed in 150 ul of molecular biology grade water in a 1.5 ml snap-cap microtube, and vortexed at highest speed. Alternatively, for fluid samples where mycoplasma is suspect, 150 ul of fluid is placed asceptically in a 1.5 ml microtube. For all sample/isolate types, the tube is placed in a previously equilibrated 98°C heat block for 10 minutes to lyse the cells and release DNA. Tubes are immediately cooled to room temperature, and centrifuged at 1200xg briefly for 1 min to remove lid condensate. DNA content is assessed by a NanoDrop™ 1000 UV/Vis spectrophotometer and associated software (Thermoscientific Products, Wilmington, DE) and recorded. Samples are stored at -20°C until use. Positive control mycoplasmas are concurrently processed.

3.3 Equipment and Supplies

- BioRad MyCycler and BioRad T100 thermal cycler
- PCR reaction tubes, domed caps, individual snap caps, strips of 8
- pipette tips, sterile filter, molecular biology grade (1-10 µl; Axygen TXLF 10LRS)(10-100 µl; VWR 53510-106)(100-1000 µl; VWR 16466-008 or Fisher 02-707-431)
- 1.5 ml molecular biology grade microtubes with locking lids; Eppendorf 0030108051
- heat block
- centrifuge
- PCR coldblock
- Gel electrophoresis apparatus
- Gel imaging system
- trays and combs for casting gels

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- microwave
- electrophoresis power supply

3.4 Description of Controls or Standards

Mycoplasma and *Acholeplasma* species isolates used for this validation study were purchased from the American Type Culture Collection of Maryland (ATCC), and are listed in the table below:

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INTERNAL ID #	STRAIN DESCRIPTION
QMP CG1-001	*Mycoplasma bovis* ATCC 25523
QMP CG1-002	*Mycoplasma canadense* ATCC 29418
QMP CG1-003	*Mycoplasma alkalescens* ATCC 29103
QMP CG1-004	*Mycoplasma bovigenitalium* ATCC 14173
QMP CG1-005	*Mycoplasma bovirhinis* ATCC 27748
QMP CG1-006	*Mycoplasma arginini* ATCC 23243
QMP CG1-008	*Acholeplasma laidlawii* ATCC 14089
QMP CG1-009	*Mycoplasma californicum* ATCC 33461

Following the protocol provided by ATCC, the freeze-dried cultures received were first rehydrated in Mycoplasma Broth (Hardy Diagnostics, Santa Maria, CA) and incubated at 37°C with 10% CO_2 and 80% humidity for 2-3 days. A second subculture into broth was performed to complete the revival of the cultures. The different species were streaked onto Hayflick's Mycoplasma Media and incubated at 37°C with 10% CO_2 and 80% humidity for 2-3 days. Single colonies were extracted aseptically for DNA extraction as described in section 3.3. DNA extracts were aliquotted and are stored at -20°C until use as positive controls.

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The ATCC stock cell suspensions are stored at −80°C in sterile 50% v/v glycerol/Mycoplasma media solution.

3.5 Choice of Gold Standard and Justification

At the AHDC, mycoplasmas are isolated by bacterial plate culture and subsequently identified visually. Culture methods are traditionally the most commonly used detection method for mycoplasmas at the AHDC and can be considered to be the current gold standard. However, mycoplasma speciation cannot be performed using this method, and the genera *Mycoplasma* and *Acholeplasma* cannot be differentiated.

Sequencing and sequence analysis of 16S and 16S-23S rDNA ITS genes were used as the gold standard. As mentioned previously, both *16S* and *16S-23S* ITS sequences are highly conserved for *Mycoplasma* and *Acholeplasma*, and have been used extensively in sequence analysis of these genera. Currently, the 16S rDNA gene sequence is the international

standard for description and ranking of the Mollicute class, and sequencing of this gene can be discriminatory to the genus and often species level (Brown et al., 2007; Stackebrandt and Rainey, 1995; van Keppeveld et al., 1992; Weisburg et al., 1989). For characterization to the species and strain level, sequencing of variable and hypervariable regions of the *Mycoplasma* and *Acholeplasma 16S-23S* ITS genetic region within the rRNA operon has been extensively used (Normand et al., 1996; Volokhov et al., 2006), often in concert with *16S* analysis (Volokhov et al, 2012; Brown et al., 2007). For our purposes, much data exists to facilitate sequence analysis. Many excellent and well-established sequence data repositories exist that are free-access and well maintained, including a mycoplasma *16S* database collection (Brown et al., 2007), the Universidad Miguel Hernandez Ribosomal Intergenic Spacer Sequence Collection (RISSC; Garcia-Martinez et al., 2001), the SILVA rRNA database, and the NCBI BLAST nucleotide and gene database collections.

4.0 Description of Assay Performance Include the Following Information

4.1 Overview of Assay Validation

The following validation resources were used to determine our process: AOAC International Microbiological Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis, the World Health Organization Codex Alimentarus International Food Standard CAC/GL 74-2010, and the World Organization of Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Chapter 1.1.4/5 (refer to references).

Each assay was independently optimized and assessed for specificity and sensitivity. Once it was apparent that each assay could be run concurrently under the same thermocycler parameters, further work with specificity, sensitivity, limit of detection, and repeatability was performed with all assays running together in the same machine.

4.2 Description of Assay Optimization (acceptance criteria)

4.2.1 Optimization of reagents

For the *uvrC/16S* assay, the previously published *16S* primers (Van Kuppeveld et al., 1992; Van Kuppeveld et al., 1994) were tested. The forward primer for *uvrC*, as described by Thomas et al., (2004), and a newly designed reverse primer were tested. Alignment data

from Lasergene DNASTAR Megalign software and Lasergene DNASTAR Primerquest software were used to create the *uvrC* reverse primer. To increase the specificity of the primers for the target sequence, a relatively short target length was designed. An additional benefit of a short target sequence is the likely probability for the sequence to remain intact under conditions of moderate DNA degradation. The target sequence and primer set for the *uvrC* is also being used for a molecular beacon-based qPCR assay, currently under development.

For the *Mycoplasma* ITS and *Acholeplasma* ITS assays, previously published primer sets were utilized (Harasawa et al., 1993; Nakagawa et al., 1992; Tang et al., 2000). The utility of the primer sets was confirmed for *M. bovis, Mycoplasma*, and *Acholeplasma* using alignment data created from the NCBI gene and nucleotide databases and from the SILVA rRNA database. Acquisitions of bovine *Mycoplasma*/*Acholeplasma* ITS*, 16S,* and *uvrC* sequences found in these database collections were utilized. To determine the possibility of non-specific amplification using any of the primer sets, alignments of ITS*, 16S,* and *uvrC* sequences were made against eubacteria that can be found in bovine milk. The list of those eubacteria and the alignment data is housed in the validation research binders in A2111E. The final primers selected for the assay are as follows:

uvrC primers:

MycouvrCF364: 5'-TTA CGC AAG AGA ATG CTT CA-3'
MycouvrCR545: 5'-TCA TCC AAA AGC AAA ATG TTA AA-3'

mycoplasma *16S* primers:

Myco16SF772: 5'-GGG AGC AAA CAG GAT TAG ATA CCC T-3'
Myco16SR1041: 5'-TGC ACC ATC TGT CAC TCT GTT AAC CT-3'

Mycoplasma ITS primers:

MycoITSF9: 5'-ACA CCA TGG GAG CTG GTA AT-3'
MycoITSR383: 5'-CCT CAT CGA CTT TCA GAC CCA AGG CAT-3'

Acholeplasma ITS primers:

AcholITSF: 5'-AAA GTG GGC AAT ACC CAA CGC-3'
AcholITSR: 5'-CCA CTG TGT GCC CTT TGT TCC T-3'

Assays were tested on BioRad MyCycler and BioRad T100 thermal cyclers, using a standard 3 step program (intial denaturation, annealing/amplification/extension, final extension and hold). The *uvrC/16S* assay was tested first, since it was initially thought that this duplex would be sufficient to confirm a positive mycoplasma isolate. The number of amplification cycles, and the annealing temperatures, extension times, initial denaturation time, relative primer concentrations (both *uvrC* and *16S* primer sets, forward and reverse), and DNA content were modified and tested until an optimum set of conditions was obtained. A range of $MgCl_2$ concentrations (1.5mM-5mM) in the GoTaq Green Master Mix was tested.

This master mix was utilized because it has been used successfully in this lab for all of the assay development work for conventional PCR, and also contains a gel electrophoresis running dye, a feature which saves steps later on in the process of sample analysis.

The Mycoplasma ITS assay was then incorporated with the intention of complementing 16S results for confirmation of *Mycoplasma* and to enable further sequencing and sequence analysis for identification to the species level. Later on the *Acholeplasma* ITS assay was incorporated to complement *16S* results for identification/confirmation of *Acholeplasma.* Since the amplicons produced among assays were very close in size, and there was the need to easily separate *16S* or *Mycoplasma* ITS amplicons for sequencing, the *Mycoplasma* ITS and *Acholeplasma* ITS were kept as uniplex assays.

Using thermocycler running conditions initially optimal for the *uvrC/16S* duplex, a range of primer concentrations and also annealing temperatures were tested for the individual *Mycoplasma* ITS and *Acholeplasma* ITS assays, since the optimal annealing temperature that can be used for all 3 assays together may be different from what is optimal for each assay individually.

An amplification running profile was selected that was optimal for all three assays, enabling us to run them concurrently in the same machine. Each assay required its own unique concentration of primers. Details of the final protocols and master mixes used can be viewed in the standard operating procedure drafts submitted with this validation report.

Optimization of the gel electrophoresis procedure was necessary in order to improve visualization of the *uvrC* amplicon. It was determined that a 2% w/v agarose gel containing 5 ng/ml ethidium bromide, run at 105V for 50 minutes in freshly prepared 0.5X TBE running buffer provided the best delineation of this small DNA band.

4.2.2 Normalization

Since the target group of mycoplasmas and acholeplasmas are of a wide range of genome sizes, it was necessary to determine the appropriate DNA concentration in order to be inclusive of the smallest genome species and to normalize the concentration range of DNA used in the assay. All three assays were run three times with a DNA dilution series of *M. bovis* (1003 kbp genome, the largest genome size among target *Mycoplasma* species), *Acholeplasma laidlawii* (1497 kbp genome, representative of *Acholeplasma* genome size), and *M. arginini* (610 kbp genome, the smallest genome size among target *Mycoplasma*

species). Initial DNA concentrations were adjusted to 200 ng/µl and 8 successive 10-fold dilutions were prepared in molecular biology grade water.

Results from each PCR run were comparable, as viewed on a gel. To be inclusive of the smallest genome organism, and taking into account the results of all three assays, it was discovered that the lowest concentration of DNA that can be consistently used (where the PCR amplification products are visible on a gel) is 20 ng/µl (or 40 ng/PCR reaction). It was found that DNA concentrations above 200 ng/µl can inhibit PCR amplification, and so this was determined to be the upper limit for functional use. Thus the operational range of DNA that applies to all three assays is 20-200 ng/µl.

To normalize sample DNA within and between assays, the concentration is adjusted to fall within this operational range. If the DNA is less than this, and within the measurable range of the Nanodrop spectrophotometer (2-3700ng/µl), the water content of the PCR master mix can be reduced and the amount of DNA used in the assay increased accordingly.

4.2.3 Establishment of cutoff values for neg/pos

A minimum DNA concentration cutoff value was determined for the assays used together in the identification of either *Mycoplasma* or *Acholeplasma.* For *Acholeplasma,* the *16S* and *Acholeplasma* ITS assays produce visible amplicon when as little as 2 pg/µl DNA is present. For *Mycoplasma* species with genome sizes above 1000 kbp, the *16S* and *Mycoplasma* ITS assays produce visible amplicon when as little as 20 pg/µl is present. Thus, when initial isolate sample size is very small, or where the isolate is not available but a body specimen or sample is (and subsequently target DNA content may be very low), these assays have valued utility as diagnostic tools, but with reduced sensitivity for some species. In the case were the DNA extract is below 20 ng/µl and below the measurable limit of the Nanodrop, the sample can still be tested but without the capability of reporting a sample as negative or refuting an initial diagnosis as positive.

Statistical analysis

RESULTS

Evaluation of Assay Performance

Formattato: Inglese (Regno Unito)

5.1 Precision

The precision parameters for qualitative methods such as conventional PCR are sensitivity (proportion of positive samples correctly identified) and specificity (proportion of negative samples correctly identified) while precision parameters for quantitative methods such as real-time PCR are repeatability (variation of results when tests are performed in a single lab by a single technician using the same resources) and reproducibility (variation of results when tests are performed in different labs). Since this is a multi-component assay with different levels of detail required, repeatability testing was included to insure optimization of each step of the process. Parameters of sensitivity and specificity will be discussed at length in section 5.5

To determine intra- and inter-assay variation when the method is performed by a single operator in a single lab, three different DNA extractions of *M. bovis* and *Acholeplasma laidlawii* were created by a single analyst. After adjusting the extracts to fall within the 20-200 ng/µl optimal test range, three replicates of each extraction were tested in the three different assays to determine intra-assay repeatability. The assays were repeated two additional times to determine inter-assay repeatability. The amplicons of each reaction were viewed on a gel and visually compared as present or absent. As discussed in the previous section 4.2.4, the three different DNA dilution series were tested three times in the assay to determine repeatability in terms of lower detection limit.

To determine assay performance and variation in relation to analyst or operator skill level, these assays have been performed independently by 3 different analysts, to the final sequence analysis step. Two of the analysts produced similar and consistent results while the third analyst produced inconsistent and at times incorrect results. Due to the number and intricacies of each step, and the level of detail and care required throughout the process, it is recommended that this method be performed by technical personnel who are already well trained in basic molecular methodology and who are more senior in experience level.

Reproducibility or variation relating to assay performance in another laboratory with other personnel has not been determined.

5.2 Accuracy

An established reference standard with which accuracy could be determined is not available for this assay.

5.3 Limit of Detection (LOD)

Diagnostic LOD: It was discovered that the lowest concentration of DNA that can be consistently used functionally for all three assays, inclusive of the smallest genome mycoplasma (where the PCR amplification products are visible on a gel) is 10-20 ng/µl (or 20-40 ng/PCR reaction). It was found that DNA concentrations above 200 ng/µl can inhibit PCR amplification, and so this was determined to be the upper limit for functional use. Thus the operational or diagnostic range of DNA that applies to all three assays is 10-200 ng/µl or 20-400 ng/rxn.

Experimental LOD: For *Acholeplasma* analysis, the *16S* and *Acholeplasma* ITS assays produce visible amplicon when as little as 2 pg/µl DNA is present. For *Mycoplasma* species with genome sizes above 1000 kbp, the 16S and *Mycoplasma* ITS assays produce visible amplicon when as little as 20 pg/µl is present. It is possible to perform the three assays with as little as 20 pg/µl DNA, however, the assay under these conditions cannot identify *M. bovis* by *uvrC* amplification (sequencing of the *Mycoplasma* ITS is necessary).

5.4 Limit of Quantitation (LOQ)

N/A

5.5 Analytical Selectivity/Specificity or Inclusivity/Exclusivity

Analytical specificity (ASp) data collected included selectivity (effect of mixed cultures or sample reagents on performance of method), exclusivity (lack of interference from a relevant range of nontarget strains which are potentially cross-reactive), and inclusivity (ability of the method to detect target from a wide range of strains).

It is anticipated that most samples submitted for analysis by this method will be pure cultures. However, in some cases where a pure culture is not available, surrounding sample DNA may interfere with analysis. In other cases, where samples are not culture plates but biological fluids or swabs, the possibility exists that other DNA will be present. In both situations, it is important to test coamplification effects on the performance of the assay. It was found for one swab sample that there was diverse DNA and coamplification of 16S and *Mycoplasma* ITS that could be visually identified on a gel. In 4 other cases, mixed DNA taken from a sparse growth of mycoplasma on a milk sample plate resulted in

coamplification not visible on a gel. During sequencing and sequence analysis, the coamplification could be seen as mixed sequence traces, and could not be distinguished from each other. These were only a few cases amongst several hundred, and so this situation is not considered routine. In practice, samples are typically well isolated colonies, pure cultures, or monoculture in biological fluids or swab samples. Where DNA was extracted directly from Amies swab storage fluid, from tracheal aspirate, or from joint fluid, it was found that in all cases (4 samples) DNA was successfully amplified and sequenced cleanly.

To test exclusivity, 30 isolates of bacteria were selected that might be found in bovine milk and might be cross-reactive. These bacteria had been previously sequenced to confirm identity, are among the QMPS Molecular Lab strain collection, and are listed below:

ISOLATE #	INTERNAL ID	STRAIN DESCRIPTION
1	FSL Z3-001	*Streptococcus agalactiae* ATCC 13813
2	FSL Z3-002	*Streptococcus agalactiae* ATCC 27956
3	FSL Z3-003	*Streptococcus dysgalactiae* ATCC 27957
4	QMP B4-273	*Streptococcus oralis*
5	FSL Z3-004	*Streptococcus uberis* ATCC 27958
6	FSL K2-017	*Listeria monocytogenes*
7	QMP W1-037	*Lactococcus lactis subsp. lactis* ATCC 11454
8	QMP B4-277	*Enterococcus gallinarum*
9	QMP W1-051	*Pediococcus pentosis*
10	QMP S1-197	*Staphylococcus epidermidis* ATCC 12228
11	QMP W1-127	*Bacillus subtilis*
12	QMP S1-027	*Staphylococcus aureus*
13	QMP S1-194	*Staphylococcus xylosus* ATCC 29971
14	QMP S1-195	*Staphylococcus simulans* ATCC 11631
15	QMP W1-126	*Bacillus cereus*
16	QMP AY1-066	*Streptococcus infantarius* subsp. *infantarius*
17	QMP S1-003	*Shigella sonnei*
18	QMP AY1-061	*Enterococcus faecalis*

19	QMP AY1-064	*Enterococcus casseliflavus*
20	QMP W1-182	*Pseudomonas aeruginosa*
21	FSL S3-908	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
22	QMP W1-089	*Enterobacter cloacae* ATCC 23355
23	FSL Z3-206	*Klebsiella pneumoniae* ATCC 13883
24	QMP Z4-137	*Serratia marcescens*
25	QMP S1-212	*Escherichia coli* ATCC 25922
26	QMP W1-183	*Pasteurella multocida*
27	QMP M2-741	*Raoultella terrigena*
28	QMP M2-351	*Raoultella planticola*
29	QMP M2-348	*Klebsiella variicola*
30	QMP Z3-591	*Clostridium perfringens*

The DNA used was normalized to 40 ng/rxn for all three assays. For the *uvrC/16S* assay, it was found that *Klebsiella pneumoniae, Staphylococcus aureus,* and *Streptococcus oralis* produced 269 bp *16S* amplicons. *Streptococcus uberis, S. agalactiae, S. dysgalactiae,* and *Lactococcus lactis* did show amplification, but the amplicon was of a distinctly different size than what is required to indicate a mollicute. *Str. 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 amplified when tested with the *Mycoplasma* ITS or *Acholeplasma* ITS assay. Due to this possibility of cross-reactivity, all three assays must be run together for an isolate in order to be exclusive.

To test inclusivity, 50 different *M. bovis, Mycoplasma* species*,* and *Acholeplasma* species were tested with our assay. For *M. bovis* these isolates were obtained from 50 different farms or veterinary clinics and originated from sample specimen types of milk, environmental swabs and samples, synovial membrane, joint fluid, and lung tissue. For *Mycoplasma*, those isolates tested represented the target bovine species, where *M. bovigenitalium, M. bovirhinis, M. canadense, M. californicum, M. alkalescens,* and *M. argnini* from 50 different farms and originating from sample specimen types including milk, environmental swabs and samples, synovial membrane, joint fluid, and lung tissue. For *Acholeplasma* species, the 50 isolates tested represented 4 different species (*A. laidlawii, A. oculi, A. granularum,* and *A. pleceiae*). These isolates were obtained from 7 different farms, representing both milk and environmental samples. In all cases, isolates selected for testing were obtained between 2012 and 2013. For all *M. bovis* tested, *uvrC, 16S,* and *Mycoplasma* ITS amplified but not *Acholeplasma* ITS. For all *Mycoplasma* species tested,

only *16S* and *Mycoplasma* ITS amplified. For all *Acholeplasma* species tested, only *16S* and *Acholeplasma* ITS amplified. Results were confirmed by sequence analysis of *Mycoplasma* ITS amplicon for all *Mycoplasma* (100 isolates total) and by sequence analysis of *Acholeplasma* ITS for all *Acholeplasma* (50 isolates).

Analytical sensitivity (ASe) data collected included determining the lower limit of detection (LOD) for the smallest and largest genome *Mycoplasma* species of interest and for *Acholeplasma* species. This is discussed in a previous section (5.3 Limit of Detection). *Mycoplasma arginini* was representative of the smallest genome size *Mycoplasma* species tested (610 kbp). For this organism, the lower limit of detection for the uvrC/16S and *Mycoplasma* ITS assays combined was found to be 40 ng DNA/reaction or 6.06×10^6 copies/rxn. *Mycoplasma bovis* was representative of the largest genome *Mycoplasma* species tested (1003 kbp). For this organism, the lower limit of detection for the uvrC/16S and *Mycoplasma* ITS assays combined was found to be 40 pg DNA/rxn or 3.75×10^3 copies/rxn. *Acholeplasma laidlawii* was selected as the representative *Acholeplasma* species, with a genome size of 1497 kbp. The lower limit of detection for the *uvrC/16S* and *Acholeplasma* ITS assays combined was found to be 4 pg DNA/rxn or 2.46×10^3 copies/rxn.

5.6 Range and Linearity

N/A

5.7 Robustness

This assay has been tested with two different models of BioRad thermocyclers, with good consistency between the two models.

To prevent well-to-well contamination during PCR amplification, either PCR reaction tube strips with individual domed caps (VWR 732-0545) or 96-well plates (Greiner BioOne 82050-648) with microseal B adhesive sealing tape (BioRad MSB1001) must be used. This amplification plasticware performance was tested to be optimal for the BioRad thermocycler models tested, and would need reassessment if additional models or brands of thermocyclers were being tested for use with these assays.

It was found that lot-to-lot variations in the *uvrC* primer sets and in the 5X TBE electrophoresis running buffer concentrate produced some variability in *uvrC* amplicon

visualization. Any new primer lots or 5X TBE lots should be tested first by performing the complete assays with *M. bovis* ATCC control DNA. Lot acceptability can thus be determined prior to testing diagnostic samples.

It was found that different brands of sterile, filter-tip, molecular biology grade pipette tips performed differently in terms of pipetting accuracy and in the effectiveness of the filter in preventing pipettor contamination (and thus sample-to-sample contamination). As a result of testing several different brands of each size range of tips, it was found that this assay requires use of the following tips: Axygen TXLF 10LRS (1-10 µl); VWR 53510-106 (10-100 µl); and VWR 16466-008 or Fisher 02-707-431 (100-1000 µl).

To prevent sample-to-sample contamination of DNA samples during DNA extraction heating and centrifugation steps, 1.5 ml molecular biology grade microtubes with locking lids are used (Eppendorf 0030108051).

5.8 Sensitivity/specificity/ROC Analysis or other appropriate statistical analysis

The sampling design created to assess diagnostic sensitivity (DSe) and specificity (DSp) was based on commonly used statistical formulas (Lesaffre et al., 2007), where tables and online calculators are readily available for easily determining the number of samples required to estimate different levels of DSe and DSp.

For each of the assay pairs (*uvrC/16S* and *Mycoplasma* ITS; *uvrC/16S*; and *Acholeplasma* ITS*)* for each of the target groups (*M. bovis, Mycoplasma,* and *Acholeplasma*) estimates of DSe and DSp were first established as performance indicators. Given the past proven use of some components of the assay in commercial kits, and our prior experience, a 99% confidence interval was selected for DSe and DSp parameters as a reference. Since 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 (World Organization for Animal Health, 2010), a minimum sample size of 95 known positives and 95 known negatives was established. The study design must consider that three different groups are discriminated: *M. bovis*, *Mycoplasma* species, and *Acholeplasma* species. The 95 bovine *Mycoplasma* species included 20 isolates of *M. bovis* and 75 isolates representing other bovine *Mycoplasma* species.

Between January 2012 and November 2013, 424 presumptive positive bovine mycoplasma field isolates were tested by our assay. These isolates originated as submissions to the

QMPS Molecular Lab by both internal (QMPS Bacteriology Labs Ithaca/Geneseo, AHDC Bacteriology Lab) and external labs (NYS and other US veterinary labs). Submitted samples were either pure subcultures or original isolates presumptively identified on a variety of media streaked with samples of bovine milk, environmental swabs, nasal swabs, tracheal washes, lung aspirate, or lung tissue washes. Presumptively identified mycoplasmas were found in relatively pure culture, as a single isolated colony, or as one to a few colonies amongst non-mycoplasmal growth. The AHDC Bacteriology Lab submitted an additional 4 DNA extracts that were taken directedly from clinical diagnostic samples including tissue washes and joint aspirate where myoplasma was suspect but could not be cultured. During 2013 an additional 86 mycoplasmas were tested which were from canine, caprine, ovine, feline, avian, and reptilian clinical diagnostic sample sources, and submitted by the AHDC Bacteriology Lab.

Among the 424 bovine isolates and 4 diagnostic specimens, 282 isolates and all 4 diagnostic specimens were identified by the assay as *M. bovis* (286 total). Fifty three were identified as *Acholeplasma* species. Eighty five were identified to species level as bovine *Mycoplasma* species other than *M. bovis.* Fifty five of these were sequenced further, and the remaining 30 isolates have not yet been further speciated. This and an additional 10 isolates are needed to complete our analysis for bovine *Mycoplasma* species (not *M. bovis*), and this testing is ongoing.

The 286 *M. bovis* showed positive amplification of *uvrC, 16S,* and *Mycoplasma* ITS. Ninety five were sequenced and NCBI BLAST analyzed to confirm identity as *M. bovis*. No false positives were found; all were identified as *M. bovis*.

The 53 *Acholeplasma* showed positive amplification of *16S* and *Acholeplasma* ITS (negative amplification of *uvrC* or *Mycoplasma* ITS*)*. These 53 were sequenced and NCBI BLAST analyzed to confirm identities as *Acholeplasma* sp.; all were further identified to species level by sequencing and BLAST analysis. No false positive were found. An additional 42 isolates are needed to complete the selectivity analysis, bringing the number of total true positives to 95 and the confidence level in estimating DSe and DSP for the *uvrC/16S* and *Acholeplasma* ITS from 85% to 95% (2% allowable error). Testing is ongoing to complete this established required number of samples.

The 55 *Mycoplasma* species showed positive amplification of *Mycoplasma* ITS and *16S* (with negative amplification of *uvrC* and *Acholeplasma* ITS). These were further analysed to species level after amplicon sequencing and NCBI BLAST analysis. All were confirmed as *Mycoplasma* species with no false positives. The 55 isolates were identified as follows:

22 *M. alkalescens*

2	*M. arginini*
7	*M. bovigenitalium*
2	*M. bovirhinis*
10	*M. californicum*
12	*M. canadense*

Additionally, the 86 *Mycoplasma* species obtained from other non-bovine host sources (3 avian; 1 reptilian; 64 canine; 1 caprine; and 16 feline) and submitted by AHDC Bacteriology were tested by our assay. A majority of these isolates could not be subcultured onto secondary media for confirmation, and/or were sparsely growing on the primary plate, often with considerable mixed growth. Several of the isolates submitted originated from the same plate, but were of distinctly different colony morphologies. All showed positive amplification of *Mycoplasma* ITS and *16S* (negative amplification of *uvrC* and *Acholeplasma* ITS) and were further analysed to species level after amplicon sequencing and NCBI BLAST analysis. All were confirmed as *Mycoplasma* species with no false positives. Only two could not be confirmed to species level. For one isolate, there was one colony to work with and a very mixed plate contaminated with fungus; not enough clean DNA could be amplified for sequencing purposes. For a second isolate, obtained from a goat, the speciation results were not discriminatory; this isolate was of the Mycoplasma mycoides cluster, which is not within the taxonomic grouping of our target Mycoplasma. The remaining 84 isolates were identified as follows:

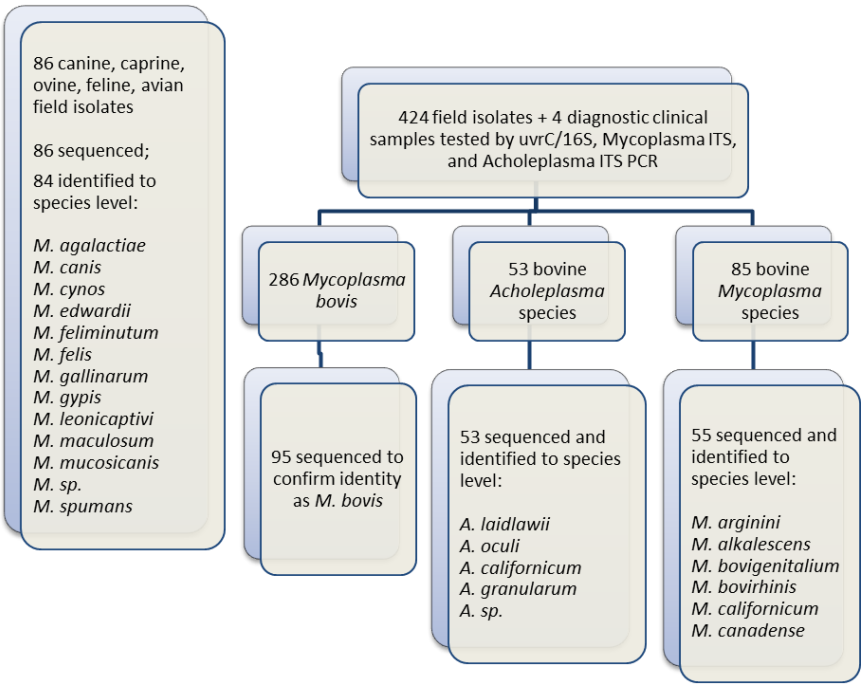
1	*M. agalactiae*
27	*M. canis*
5	*M. cynos*
10	*M. edwardii*
1	*M. falconis*
2	*M. feliminutum*
14	*M. felis*
1	*M. gallinarum*
1	*M. gypis*
3	*M. leonicaptivi*
4	*M. maculosum*

1	*M. mucosicanis*
12	*M. spumans*
2	*Mycoplasma sp.*

Interestingly, in 4 of the 5 cases where colonies of two different morphologies were tested from the same sample culture plate, the two colonies resulted in two different species identities.

These data show there is a broader scope utility of this assay for sequencing identification, specifically for species classified within the phylogenetic taxonomic mycoplasma clusters Bovis-lipophilum, Synoviae, and Hominis.

The chart below shows a simplified breakdown of the results.



To test DSp for *uvrC/16S* (know negative *M. bovis)*, 95 negative samples were selected among isolates of non-*M. bovis Mycoplasma* species (45, representing the 19 different species listed above) and isolates of *Acholeplasma* species (50). To test DSp for *Mycoplasma* ITS (known negative *Mycoplasma*) negative samples were selected among isolates of *Acholeplasma* (50) and non-mollicutes (45). To test DSp for *Acholeplasma* ITS, true negative samples were selected among isolates of *Mycoplasma* (50) and non-mollicutes (45). For all cases described these true negatives failed to amplify in PCR.

In summary, for *uvrC/16S* and *Mycoplasma* ITS (where *M. bovis* is the known positive):

		Number of reference samples required			
		Known positive (95)		known negative (95)	
	Positive				
Test results		95	TP true positives	FP false positives	0
	Negative		FN false negatives	TN true negatives	
		0			95
		Diagnostic Sensitivity		Diagnostic Specificity	
		TP/(TP+FN)		TN/(TN+FP)	
		100% (96.1%-100%)		100% (96.1%-100%)	

In summary, for *uvrC/16S* and *Mycoplasma* ITS (where bovine *Mycoplasma* species NOT *M. bovis* are the known positive):

		Number of reference samples required			
		Known positive (85)		known negative (95)	
	Positive				
Test results		85	TP true positives	FP false positives	0
	Negative		FN false negatives	TN true negatives	
		0			95

Diagnostic sensitivity*	Diagnostic specificity
TP/(TP+FN)	TN/(TN+FP)
100% (96.1%-100%)	100% (96.1%-100%)

In summary, for *uvrC/16S* and *Acholeplasma* ITS (where *Acholeplasma* species are the known positive):

		Number of reference samples required			
		Known positive (53)		known negative (95)	
Test results	Positive	53	TP true positives	FP false positives	0
	Negative	0	FN false negatives	TN true negatives	95
		Diagnostic sensitivity**		Diagnostic specificity	
		TP/(TP+FN)		TN/(TN+FP)	
		100% (96.9-100%)		100% (96.1%-100%)	

*DSe confidence limit reduced to 93% with reduced sample size

**DSe confidence limit reduced to 85% with reduced sample size

5.9 Matrix Effects

No matrix inhibitory effects have been identified with swab samples. In one case a heterogeneous DNA sample was found having multiple organism DNA present; in this situation, amplification products were atypical and could not be used for confirmation of *Mycoplasma* or *Acholeplasma* or for further sequencing and analysis.

DISCUSSION

CONCLUSION

All of the data collected indicate this is a reliable assay with excellent specificity, sensitivity, and repeatability. Used alone the 3-component assay is capable of identifying a presumptive positive mycoplasma colony isolated from a bovine host diagnostic sample as *M. bovis*, as another *Mycoplasma* species, or as an *Acholeplasma* species. Based on testing of a panel of eubacteria typically found in bovine milk, the 3-component assay can indicate a true negative isolate. This assay can be completed within 4-6 hours, unless further speciation is required. When the assay is followed by sequencing and sequence analysis, mycoplasmas or acholeplasmas can be further identified to the species level with a high level of discrimination. In this case, an additional 2 working days are needed to complete these additional steps.

Ongoing work is planned. Additional field samples will be needed to add to the specificity data for *Acholeplasma* and *Mycoplasma*. Initial work with other mycoplasma species isolated from hosts other than bovine indicates that there is a broader scope utility of this assay, specifically for species classified within the phylogenetic taxonomic mycoplasma clusters Bovis-lipophilum, Synoviae, Hominis, and Acholeplasma (as described by Volokhov et al., 2012). Thus, further verification of this assay for additional mycoplasma species is recommended.

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