DEVELOPMENT OF A BEAD-BASED MULTIPLEXED PCR ASSAY FOR THE SIMULTANEOUS DETECTION OF MULTIPLE *MYCOPLASMA* SPECIES

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Veterinary Science

WASHINGTON STATE UNIVERSITY
Department of Veterinary Microbiology & Pathology

DECEMBER 2010

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ACKNOWLEDGMENT

I would to thank Terry McElwain for the tremendous amount of guidance throughout all phases of this project, Fred Rurangirwa for additional project oversight and techniques required for cultivating and extracting DNA from mycoplasma, Doug Call for making his laboratory available at all times and for his assistance with the manuscript, Tim Baszler for serving on the committee, Jonathon Anderson for conducting preliminary assay development experiments, Lisa Orfe for technical assistance, especially as it pertains to primer and probe design, Dan Bradway for culturing *Mycoplasma mycoides* subsp. *capri*, and additional technical assistance, Niles Reichardt for providing bi-monthly lists of field samples to be collected by WADDL's Central Processing staff and Jenn Baisley for saving field samples.

The funding for this project was provided by USDA, NIFA, NAHLN Cooperative Agreement, the Washington Animal Disease Diagnostic Laboratory, and the WSU CVM Department of Veterinary Microbiology and Pathology.

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Abstract

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December 2010

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We describe the development and analytical validation of a specific and sensitive 7-plex polymerase chain reaction assay coupled to a bead-based liquid suspension array for high throughput detection of multiple *Mycoplasma* spp. of ruminants. The assay employs a combination of newly designed and previously validated primer-probe sets that target genetic loci specific for *Mycoplasma bovis*, *Mycoplasma mycoides* cluster, *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC) and *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp). PCR products were hybridized to specific oligonucleotide probes bound to spectrally unique, 5.6 µm diameter, carboxylated beads, and subsequently analyzed by flow cytometry. Analytical sensitivity for the targeted *Mycoplasma* species ranged from 10 fg to 1 pg of purified gDNA extracted from broth cultures (approximately 8-800 MmmSC genome equivalents). *In silico* comparison of primers and probes, and analytical assessment with a range of near-neighbor *Mycoplasma* species and multiple bacterial respiratory pathogens of ruminants demonstrated 100% analytical specificity of the assay. To assess assay performance and diagnostic specificity, 192 bovine respiratory samples were analyzed by incorporating a high throughput DNA extraction platform. The assay correctly classified all 192 field samples as negative for MmmSC

or Mccp with an apparent 100% diagnostic specificity for these pathogens. 33/192 field samples were positive for *M. bovis* and all were confirmed by PCR or sequence verification of the *uvrC* gene. The results from this study indicate that the bead-based liquid suspension array will provide a reliable, analytically sensitive and specific platform to simultaneously interrogate ruminant respiratory samples for multiple *Mycoplasma* species, including *Mycoplasma mycoides* cluster organisms exotic to the United States. Sequential addition of primer-probe sets to the assay did not significantly impact analytical sensitivity of individual primer-probe combinations, suggesting that expanding the array to include more *Mycoplasma* species will not compromise overall performance.

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Dedication

This thesis is dedicated to my wife Jamie Lee for her unwavering emotional support and sacrifice over the last three years.

Introduction

Mycoplasma bovis and members of the *Mycoplasma mycoides* cluster are significant ruminant respiratory pathogens of economic importance (10, 11, 12, 16, 17, 18). *M. bovis* is associated with pneumonia in young calves and feedlot cattle (17, 19, 20). While all five taxa of the recently reclassified *M. mycoides* cluster are pathogenic, four are associated with three disease syndromes that involve the respiratory tract of small ruminants or cattle, and are listed as notifiable agents by the OIE (World Organization for Animal Health): (i) contagious agalactiae of sheep and goats (*Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma mycoides* subsp. *capri* (including the recently reclassified serovar *Mycoplasma mycoides* subsp. *mycoides* biotype Large Colony) (ii) contagious bovine pleuropneumonia of cattle (*Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony), and (iii) contagious caprine pleuropneumonia of goats (*Mycoplasma capricolum* subsp. *capripneumoniae*) (21). The fifth, recently reclassified *M. mycoides* cluster member, *Mycoplasma leachii* sp. nov. (21), (formerly *Mycoplasma* sp. bovine group 7 of Leach) has been isolated from calves with pneumonia (23). There are also reports of members of the *M. mycoides* cluster being isolated from non-traditional hosts. For instance, *M. mycoides* subsp. *capri* has been isolated from cattle, *M. leachii* and *M. mycoides* subsp. *mycoides* biotype Small Colony from goats, and *M. capricolum* subsp. *capripneumoniae* has been isolated from sheep (18, 22, 23).

Identification of specific *Mycoplasma* spp. from the ruminant respiratory tract typically involves cultivation followed by immunologic-based assays or PCR. Members of the *M. mycoides* cluster are particularly difficult to differentiate because of phenotypic and genotypic features that lead to serological cross-reactivity and challenges with sequence verification (9). Cross-reactions by growth inhibition assays are frequently seen among *M. mycoides* subsp.

mycoides biotype Small Colony and *M. mycoides* subsp. *capri*, as well as between *M. leachii* and *M. capricolum* subps. *capripneumoniae* (9). Further, in the case of small ruminants, *M. mycoides* subsp. c*apri* grows relatively well, which could result in concurrent infections with the more fastidious *M. capricolum* subsp. *capripneumoniae* or *M. capricolum* subsp. *capricolum* not being detected by preliminary cultivation techniques (23) without specifically investigating their presence by PCR.

Because of the unique challenges for efficiently identifying *M. bovis,* accurate identification in the case of the *M. mycoides* cluster, and the importance of early detection of the causative agents of contagious bovine and caprine pleuropneumonia, the aim of the present study was to develop a single assay capable of resolving these targets from ruminant respiratory samples. A 7-plex PCR coupled to a liquid bead array assay was employed that targets two genetic loci each for *M. bovis*, *M. capricolum* subsp. *capripneumoniae*, and *M. mycoides* subsp. *mycoides* biotype Small Colony, and one primer-probe set targeting the *M. mycoides* cluster as a whole. This assay was chosen for its diagnostic efficiency, capacity to overcome the limited number of target organisms detectable using multiplex rt-PCR assays, its ability to identify multiple respiratory pathogens in a single sample, and for the opportunity to include in the future primer-probe sets targeting other *Mycoplasma* spp. and non-mycoplasma microbial pathogens.

TABLE 1. Bacteria used for determining analytical specificity

Strain	Source	Target(s) for strain verification
M. agalactiae	ATCC 35890	16s rRNA/16s-23s intergenic spacer region
M. alkalescens	Cornell; 2-1-1-46 strain	16s rRNA/16s-23s intergenic spacer region
M. arginini	ATCC 23243	16s rRNA/16s-23s intergenic spacer region
M. bovigenitalium	ATCC 14173	16s rRNA
M. bovirhinis	IOM Mollicutes Collection; PG43 strain	16s-23s intergenic spacer region
M. bovis	ATCC 25025	16s rRNA/16s-23s intergenic spacer region
M. bovoculi	ATCC 29104	16s rRNA
M. californicum	ATCC 33461	16s rRNA/16s-23s intergenic spacer region
M. canadense	ATCC 29418	16s rRNA
M. canis	ATCC 19525	16s rRNA
M. dispar	ATCC 27140	16s rRNA
M. ovipneumoniae	WADDL[1]	16s rRNA[2]
M. putrefaciens	IOM Mollicutes Collection; KS-1 strain	16s rRNA
M. mycoides cluster		
M. capricolum subsp. *capricolum*	IOM Mollicutes Collection; goat 14 strain	16s rRNA/*fusA*/putative integral membrane protein H2 gene
M. capricolum subsp. *capripneumoniae*	FADDL[3]	16s rRNA/*fusA*/*arcD*
M. leachii sp. nov.	IOM Mollicutes Collection; N29 Leach strain	16s rRNA/*fusA*/*glk*
M. mycoides subsp. *capri*	Bradway	16s rRNA/*fusA*/*glk*
M. mycoides subsp. *capri* (serovar Large Colony)	IOM Mollicutes Collection; 4933 strain	16s rRNA/fusA/glk
M. mycoides subsp. *mycoides* Small Colony	FADDL, Gladysdale strain	16s rRNA/*fusA*/*glk*
Non-mycoplasma bacteria		
Arcanobacterium pyogenes	WADDL	N/A[4]
Escherichia coli	WADDL	N/A
Histophilus somni	WADDL	N/A
Mannheimia hemolytica	WADDL	N/A
Pasteurella multocida	WADDL	N/A

[1]Washington Animal Disease Diagnostic Laboratory

[2]WADDL rt-PCR (25, 26)

[3]United States Foreign Animal Disease Diagnostic Laboratory

[4] Classified by biochemical assays

Materials and methods

Bacterial strains and growth conditions: The bacteria used in this study are listed in Table 1. Mycoplasma strains were cultured at 37°C under 5% CO_2 in PPLO or general Mycoplasma Broth (Hardy Diagnostics, USA). *Mycoplasma dispar* was grown at 37°C under 5% CO_2 in modified Friis broth (UC Davis Biological Media Services, CA, USA). Agar plates streaked for isolation of *Arcanobacterium pyogenes*, *Escherichia coli*, *Histophilus somni*, *Mannheimia hemolytica* and *Pasteurella multocida* were provided by the Washington Animal Disease Diagnostic Laboratory (WADDL, Pullman, WA) for DNA extraction.

DNA extraction and sequence analysis: *Mycoplasma* strains were pelleted by centrifuging broth cultures at 10,000 X *g* for 10 min. Pellets of the mycoplasma strains and non-mycoplasma bacterial colonies on agar were re-suspended in 200 µl of 1X PBS, and DNA was extracted using the DNeasy tissue kit (Qiagen, USA). DNA from *M. mycoides* subsp. *mycoides* biotype Small Colony and *M. capricolum* subsp. *capripneumoniae* was extracted and safety treated (see below) at the United States Foreign Animal Disease Diagnostic Laboratory (FADDL) (Plum Island, NY) prior to being shipped. Invitrogen's (Carlsbad, CA) Easy-DNA™ kit was used, per manufacturer's instructions (*E. coli* specific protocol), to extract 5 to 12 ml of the mycoplasma cultures. The DNA was suspended in Tris buffer (pH 8.0, Ambion, Austin, TX) and submitted for safety treatment for removal from BSL3 facilities. The extracted DNA was safety treated by adding 10 µl of 3 M NaCl (1/10 volume), 3.5 µl 3 M Acetate (1/30 volume), and 5 µl ribonuclease A (0.5 Kunitz units/100 µl, USB, Cat. 70194Z) followed by boiling the sample for 10 min, slowly cooled, and frozen at 70°C prior to being shipped.

The identity of *Mycoplasma* spp. was confirmed by amplification and sequence analysis of DNA targets listed in Table 1. Modifications of previously described PCR assay conditions were used to amplify 16s rRNA, *fusA*, *glk*, *arcD*, and putative integral membrane protein H2 gene sequences (7, 8, 9, 10, 11, 12). For sequence analysis of the 16s-23s rRNA intergenic spacer region, an alignment of the partial 16s rRNA sequence, complete 16s-23s intergenic spacer sequence, and partial 23s rRNA gene sequence of two *M. bovis* strains available in GenBank (accession nos. AY729934, AY765211), three *M. bovigenitalium* strains (AY780797, AY973571, AY974058), and one strain each of *M. agalactiae* (AY770623), *M. alkalescens* (AY816348), *M. californicum* (AY736031), and *M. canadense* (AY800341) was created using Vector NTI software (Invitrogen,USA). Primers L-ITS and R-ITS were developed (L-ITS: AACCATGGGAGCTGGTAATG; R-ITS: GTTCGCTATCGGTGTCTGGT) using primer3 (http://frodo.wi.mit.edu/primer3/) based on conserved regions of this alignment that would lead to an expected 939 bp amplicon. The PCR conditions for amplifying the 16-23s rRNA spacer region for sequencing consisted of those optimized for the multiplex PCR assay (see "development and optimization of multiplex PCR" section below). Bands of the expected size in a 1.5% agarose gel were visualized under ultraviolet light, excised, and purified using Freeze 'N Squeeze DNA gel extraction spin column kit (Bio-Rad Laboratories, USA). Purified PCR products (10 µl) were combined with 5 µl of the forward or reverse primer (5 µM) and submitted for sequence analysis (Genewiz Inc., USA).

TABLE 2. Primer-probe sets used for multiplex assay

targeted mycoplasma	primer/probe	sequence 5'-3'	gene target	size (bp)	reference
M. mycoides cluster	MYC1	TTCTAAATTAGTTACTCGTGCA	ribosomal protein S7	457-466	1
	MYC2	AATAAACTGTATTCTCTAGCCA			
	MYC probe	ATTGGAGGAGCTAACTATCAAGTTC			this study
M. mycoides subsp. *mycoides* SC	SG1352	GTTTTTTAGGAATTTTGTAAATAGCTCAATT	hypothetical prolipoprotein	90	2
	SG1353	AGTGAAGTTTCTAAATCAATCCAATCAG			
	SGAB010 probe	TCAATTAGACTGTAATGAAATAT			
M. mycoides subsp. *mycoides* SC	MSC-382F	ATGCAAGAAGTTATTAATGTTTATCATTC	52 amino acid hypothetical protein	106	3
	MSC-382R	CGTAATATATTTGTTTAACATATGGAATAA			
	MSC-382 probe	CTTTTATTAAGCTCATCTCTTGG			this study
M. capricolum subsp. *capripneumoniae*	MccpF	CGCTCACATAGCCAATCATC	*lppa* (lipoprotein A)	152	4
	MccpR	TCGTTTTTAAGAGAAAATCAAGCA			
	Mccp probe	CAAGCTGATGAACATAAAAATGATG			this study
M. capricolum subsp. *capripneumoniae*	CPN1F	ATGTTTGAAGGGGCAGAGAA	putative integral membrane protein (membrane protein H2)	216	this study
	CPN1R	TTTGGGCTTAACCAAAGGTG			
	CPN1 probe	ATGACCCTAACAAAAGACCGGAT			
M. bovis	Mb-F	GCTTCAGTATTTTGACGG	*uvrC*	303	5
	Mb-R	GGTTTAGCTCCATAACCAGA			
	PMyb probe	CATATATAAGTGAGACTAACTTATT			
M. bovis	mb-mp1F	TATTGGATCAACTGCTGGAT	*mb-mp81* (membrane lipoprotein P81 gene)	447	6
	mb-mp1R	AGATGCTCCACTTATCTTAG			
	mb-mp1 probe	GCAGCTAAAGGAAAAGGCGC			this study

Development and optimization of multiplex PCR: The primers and probes used in the multiplex assay are listed in Table 2 (1, 2, 3, 4, 5, 6). Primers CPN1F and CPN1R were designed from a highly conserved region of the putative integral membrane protein H2 gene (13) present in each of ten *M. capricolum* subsp. *capripneumoniae* strain sequences available in Genbank (accession numbers: AF378153, AF378154, AF378155, AF378156, AF378157, AF378158, AF378159, AF162991, EU827517, and GU992420) using CLC DNA Workbench 5.6 software (Cambridge, MA, USA) and primer3. Probes mb-mp1, CPN1, Mccp and MSC-382 were designed using a combination of CLC DNA workbench 5.6 software and Oligo Calculator version 3.5 (http://www.basic.northwestern.edu/biotools/oligocalc.html) from sequences available in Genbank (accession numbers: AY627040 (mb-mp1 probe), AF111424 (Mccp probe), AF162991 (CPN1 probe) and, BX293980 (MSC-382 probe)).

The PCR assay was optimized using the PCR Optimization Kit (Roche, USA), for assessment of optimal pH, $MgCl_2$ concentration, and affects of several additives (DMSO, glycerol, $(NH_4)_2SO_4$ and gelatin). The annealing temperature was optimized using a temperature gradient setting on the iCycler thermocycler ((Bio-Rad Laboratories, USA). The cycling parameters consisted of an initial denaturation step of 95°C for 5 min followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 1 min with a final extension step of 72°C for 7 min. The amount of DNA was standardized at 10 ng (1 ng/µl) of genomic DNA for each of the targeted mycoplasmas using the NanoDrop ND1000 spectrophotometer (Thermo Scientific, USA). Reactions with greatest band intensities in agarose gels were observed with the following constituents (50 µl final reaction volume): 5 µl 1X FastStart reaction buffer with $MgCl_2$ (FastStart High Fidelity PCR System, Roche, USA), 1 µl dNTP mix (10 mM) (Roche, USA), 1.2

µl FastStart high fidelity enzyme blend (5 U/ µl Taq) (Roche, USA), 7 µl $MgCl_2$ (25 mM) (Roche, USA), 11.8 µl water, and 1 µl of each forward and reverse primer (20 µM) (IDT, USA).

Bead preparation: A modification of Luminex Corporation's *Protocol for Carbodiimide Coupling of Amine-modified Oligonucleotides Probes to Carboxylated Microspheres* was used to bind oligonucleotide probes modified with a 5'amine C12 spacer (IDT, USA) to seven spectrally unique fluorescent beads (14). Aliquots of each bead set (5.0 X 10^6 beads; 400 µl) were centrifuged at 8000 X g for 2 min. The bead pellet was resuspended in 50 µl of 0.1 M 2-[N-morpholino] ethanesulfonic acid (MES) buffer, pH 4.5 (Sigma) by vortex and sonication. Oligonucleotide probe suspension (2 µl of 0.1 nmole/µl) and 2.5 µl of 10 mg/ml 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) (Pierce) was added to the bead solution and incubated at room temperature in the dark for 30 min. Another 2.5 µl of 10 mg/ml EDC was added and similarly incubated for an additional 30 min. The beads were washed by resuspending in 1.0 ml of 0.02% TWEEN-20 (Sigma), and centrifuging at 8000 X g for 2 min. The supernatant was removed and a second wash was performed by resuspension in 1ml of 0.1% SDS followed by the previous centrifugation step. The supernatant was removed and beads were resuspended in 100 µl TE buffer pH 8.0. The final bead concentration was estimated by two replicate hemacytometer counts.

Probe hybridization: A modification of Luminex Corporation's *Sample Protocol for Oligonucleotide Hybridization* (15) was used for hybridizing biotinylated PCR products to coupled beads. First, a bead mixture was prepared that contained 100 beads/µl/bead set suspended in 1.5 X tetramethylammonium chloride (TMAC) (Sigma) hybridization buffer. Bead mixture (33 µl) was combined with 10 µl of PCR product, and 7µl of TE, pH 8.0 buffer to the

wells of a 96-well PCR plate. The hybridization reaction was carried out in an iCycler Thermocycler set with a single denaturation step of 95°C for 5 minutes followed by a 30 minute hybridization step at 55°C. The hybridization products were transferred to a 96-well 1.2 µm filter plate (Millipore) and washed 3 times using 100 µl 1 X TMAC buffer. Beads were resuspended in 100 µl 0.75 X TMAC reporter buffer containing 4 µg/ml recombinant Phycolink streptavidin-R-phycoerythrin (Prozyme, USA). Reporter mix was incubated in the dark on the Bio-Plex 100 Suspension Array System platform (Bio-Rad Laboratories, USA) at 45°C for 5 min, and each well was subsequently analyzed by the instrument at a 45°C holding temperature.

Assessment of diagnostic specificity: 192 bovine lung samples submitted to WADDL from Washington, Idaho, Utah, Oregon, Montana, Colorado, Nevada, and New York during 2008-2009 were stored at -70°C prior to analysis in 2010. The majority of samples were suspect bacterial or viral pneumonia cases; however, other diseases such as enteritis, encephalitis, sepsis, metritis, salmonellosis, clostridial myositis, malignant catarrhal fever, bovine viral diarrhea, toxicity, renal failure, and abortion were also represented. Cattle breeds included Angus, Hereford, Holstein, Charlois, Limousin, Brown Swiss, Shorthorn, Jersey, and variety of crosses. The age of cattle ranged from fetuses to aged-adults (11 years-old); however, the majority of lung samples were from calves less than 1 year-old. Heifers, steers and adult cows and bulls were all represented.

The samples were processed by swabbing a freshly cut surface of each lung with a flocked swab (Hardy Diagnostics, USA). Each swab was rinsed in a 96 well storage plate containing 400 µl 1X PBS, pH 7.2. DNA was extracted using the BioSprint 96 One-For-All Vet Kit (Qiagen, USA) on the BioSprint 96 robotic workstation.

Results

Analytical specificity: The seven primer-probe sets (Table 2) were initially evaluated using 1 ng of purified genomic DNA (gDNA) from a representative strain of each target *Mycoplasma* species. The suspension array detected three probes for *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony and *Mycoplasma capricolum* subsp. *capripneumoniae* samples (each sample positive for the *M. mycoides* cluster probe (MYC), and two probes specific for each subspecies (SGAB010; MSC-382 and Mccp; CPN1, respectively)), one probe for the remaining members of the *M. mycoides* cluster samples (MYC probe), and two species specific probes for the *M. bovis* sample (PMyb and mb-mp1). No probe cross-reactivity was observed (Fig.1), and the signal to noise ratio of the mean fluorescence intensities comparing signal among target and non-target strains ranged from 20.8 (Mccp probe) to 88.3 (PMyb probe). Members of this group of seven target *Mycoplasma* spp. served as both positive and negative internal controls for the remaining analytical specificity experiments. No probe cross-reactivity was seen among the remaining mycoplasma strains or common bacterial respiratory pathogens of cattle (Fig. 2).

From these data, we established a threshold for classifying positive and negative results. A sample was considered positive if the mean fluorescence intensity of a probe was greater than each of two calculated values: (i) mean fluorescence intensity of the negative internal control samples plus three times the standard deviation and (ii) two times the mean fluorescence intensity of the negative internal control samples.

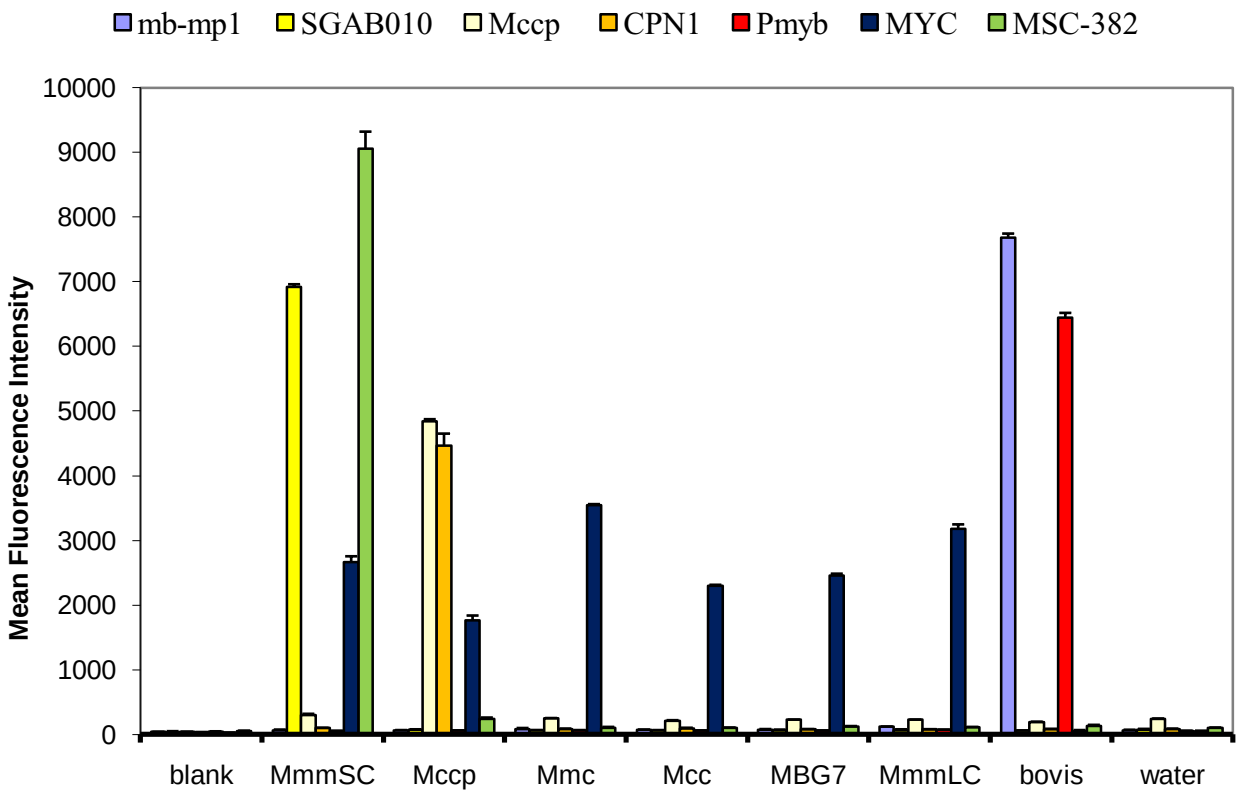

Figure 1. Preliminary analytical specificity assessing 1 ng gDNA of the *M. mycoides* cluster members and *M. bovis.* Error bars represent standard error of the mean. Two probes are specific for *M. bovis* (mb-mp1; PMyb), *M. mycoides* subsp. *mycoides* Small Colony (SGAB010; MSC-382), *M. capricolum* subsp. *capripneumoniae* (Mccp; CPN1), and one probe (MYC) is specific for the *M. mycoides* cluster members. MmmSC=M. *mycoides* subsp. *mycoides* Small Colony, Mccp=*M. capricolum* subsp. *capripneumoniae*, Mmc=*M. mycoides* subsp. *capri*, Mcc=*M. capricolum* subsp. *capricolum*, MBG7=*M. leachii*, MmmLC=*M. mycoides* subsp. *mycoides* biotype Large Colony, bovis=*M. bovis.*

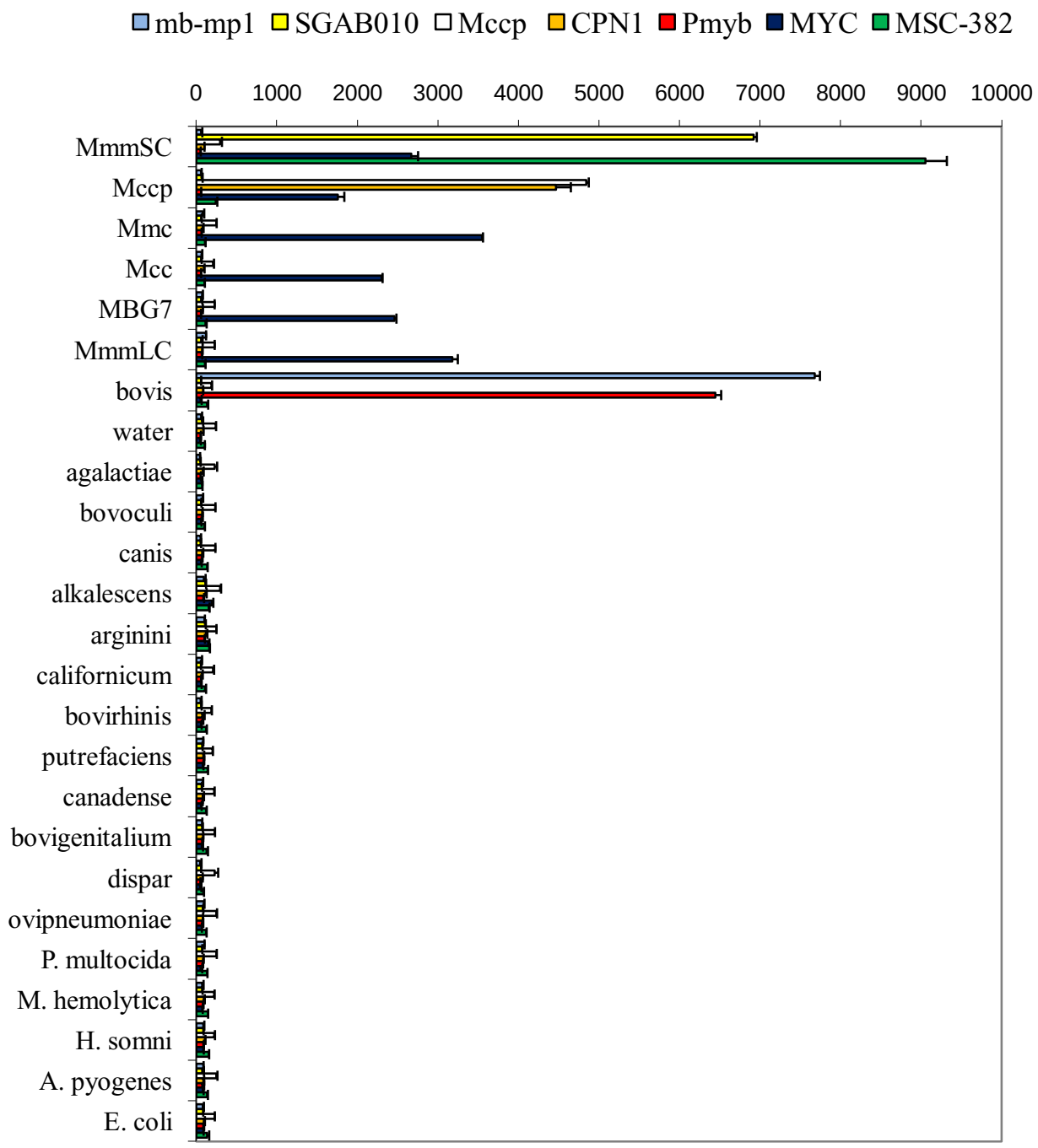

Figure 2. Analytical specificity run assessing *Mycoplasma species* and non-mycoplasma bacteria commonly isolated from the ruminant respiratory tract. Error bars represent standard error of the mean. Two probes are specific for *M. bovis* (mb-mp1; PMyb), *M. mycoides* subsp. *mycoides* Small Colony (SGAB010; MSC-382), *M. capricolum* subsp. *capripneumoniae* (Mccp; CPN1), and one probe (MYC) is specific for the *M. mycoides* cluster members.MmmSC=M. *mycoides* subsp. *mycoides* Small Colony, Mccp=*M. capricolum* subsp. *capripneumoniae*, Mmc=*M. mycoides* subsp. *capri*, Mcc=*M. capricolum* subsp. *capricolum*, MBG7=*M. leachii*, MmmLC=*M. mycoides* subsp. *mycoides* biotype Large Colony, bovis=*M. bovis*.

Analytical sensitivity and intra-assay variability: The limit of detection (LOD) of all seven probes for each target *Mycoplasma* spp. was determined by preparing ten-fold serial dilutions that ranged from 1 ng to 1 fg of purified genomic DNA (gDNA). Both multiplex amplification and detection steps of the assay were performed in triplicate for each dilution series, and the limit of detection was defined as the highest dilution at which all three replicates were above the negative cut-off value. The cut-off value for classifying a sample as positive was calculated similar to previously described except a PCR control sample containing water in place of non-target gDNA served as the negative control. The limit of detection of probes and corresponding target *Mycoplasma* spp. are listed in Table 3, and mean fluorescence intensities with standard error of the means for determining intra-assay variability are illustrated in Figure 3. The LOD for *M. bovis* was 10 fg gDNA, and 100 fg for *M. capricolum* subsp. *capricolum*, *M. leachii*, *M. mycoides* subsp. *capri*, 1 pg for *M. mycoides* subsp. *mycoides* biotype Small Colony, and 10 pg for *M. capricolum* subsp. *capripneumoniae*. Based on complete genome sequence data available in GenBank, 1 pg gDNA of *M. mycoides* subsp. *mycoides* biotype Small Colony, *M. mycoides* subsp. *capri*, and *M. capricolum* subsp. *capricolum* strains represents approximately 10^3 genome copies.

TABLE 3. Analytical sensitivity of the multiplex assay

Mycoplasma	Probes						
	MYC	SGAB010	MSC-382	Mccp	CPN1	PMyb	mb-mp1
M. mycoides subsp. mycoides SC	10 pg[1]	1 pg	1 pg				
M. capricolum subsp. capripneumoniae	100 pg			10 pg	10 pg		
M. mycoides subsp. capri (serovar MmmLC)	100 fg						
M. mycoides subsp. capri	100 fg						
M. capricolum subsp. capricolum	100 fg						
M. leachii	100 fg						
M. bovis						10 fg	10 fg

[1]Lowest quantity of target DNA at which all 3 replicates were positive

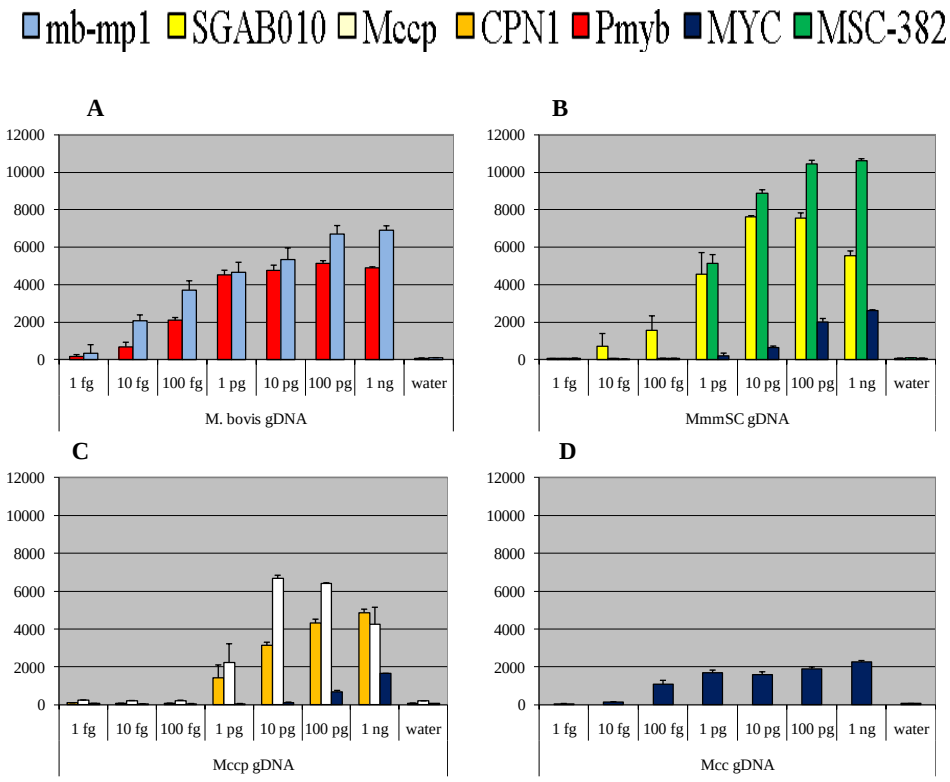

Figure 3. Analytical sensitivity of the multiplex assay. The y-axis represents mean fluorescence intensity. Panel A=serial dilutions *of M. bovis* gDNA. Panel B= *M. mycoides* subsp. *mycoides* biotype Small Colony gDNA. Panel C=*M. capricolum* subsp. *capripneumoniae*. Panel D=*M. capricolum* subsp. *capricolum*. Error bars represent standard error of the mean. Each probe is designated a specific color (see Figs. 1 & 2).

Interassay variability: To assess repeatability of the multiplex assay, amplification and detection steps were performed in triplicate using high, medium, and low concentrations based on LOD of target gDNA on 3 separate days. Aliquoted samples of gDNA at the specified concentrations were prepared on a single day and frozen at -20°C. Aliquots were thawed and a fresh PCR master mix was prepared on each of the three days. Table 4 summarizes the 3-day interassay coefficient of variation for each probe at varying concentrations of target gDNA. The coefficient of variation percentage was less than 30% for the majority of the cases. Not surprisingly, in some cases lower template concentrations yielded higher variance estimates.

Assay performance on field samples: To initiate stage two validation (OIE) of the assay, lung samples from 192 bovine cases submitted to WADDL during 2008-2009 were analyzed. Ten microliters of gDNA extracted from each bovine lung sample (N=192) was combined with 40 µl of master mix for a single multiplex PCR step. The probe hybridization and detections steps were performed in duplicate using 10 µl of PCR product. Each 96-well plate had five wells reserved for the following control samples: 1 ng MmmSC gDNA, 1ng Mccp gDNA, 1 ng *M. bovis* gDNA, water PCR control, and a blank (mixture containing the probe hybridization and SAPE reporter buffers).

Table 4. Interassay variability (CV%)

Mycoplasma		Probes						
		MYC	**SGAB010**	**MSC-382**	**CPN1**	**Mccp**	**PMyb**	**mb-mp1**
M. mycoides subsp. *mycoides SC*	1 ng	2.9 [1]	9.5	17.6				
	100 pg	4.0	1.5	14.4				
	1 pg	ND[2]	32.8	17.2				
M. capricolum subsp. *capripneumoniae*	1 ng	21.0			10.3	16.7		
	100 pg	29.7			30.2	1.4		
	10 pg	ND			4.5	6.5		
M. capricolum subsp. *capricolum*	100 pg	4.9						
	1 pg	8.8						
	100 fg	12.1						
M. mycoides subsp. *capri (serovar MmmLC)*	100 pg	7.2						
	1 pg	26.6						
	100 fg	37.7						
M. leachii	100 pg	3.0						
	1 pg	6.1						
	100 fg	38.2						
M. bovis	100 pg						9.8	8.5
	1 pg						9.8	8.6
	100 fg						5.4	9.7

[1] Percentage coefficient of variation
[2] Not done

Mycoplasma culture was performed by WADDL on 43/192 field samples, and 14/43 were positive for mycoplasma growth. Each of these 14 samples was identified as *M. bovis* by the bead-based assay using the cut-off value algorithm for determining analytical specificity. A total of 33 samples were positive in the bead-based assay for *M. bovis*. Of these 33 samples, 32 were positive based on the PMyb probe and 13 based on the mb-mp1 probe. One sample was positive for probe mb-mp1 and negative for PMyb, while 20 samples were positive for probe PMyb and negative for mb-mp1. All 33 samples positive for *M. bovis* were either positive for mycoplasma growth by WADDL (n=14), or confirmed by sequence verification (n=19) of the *uvrC* gene using the primer set in Table 2. Two culture negative samples were positive for *M.*

bovis in the multiplex assay and were confirmed as *M. bovis* by *uvrC* gene sequence verification. All 192 field samples were negative for *M. mycoides* subsp. *mycoides* biotype Small Colony, *M. capricolum* subsp. *capripneumoniae* and *M. mycoides* cluster group (probes SGAB010, MSC-382, CPN1, Mccp, and MYC).

Discussion

Diagnosis and differentiation of *Mycoplasma* spp. is challenging. The OIE recognizes multiple tests for identifying the causative agents of contagious bovine and caprine pleuropneumonia including culture and biochemical testing, and serological and PCR assays (27, 28). Multiple PCR assays have been described that specifically identify *Mycoplasma* species and members of the *M. mycoides* cluster. To our knowledge, there is no single assay that can be used to accomplish this task. Multiplex PCR combined with suspension bead array offers the opportunity to interrogate a single sample for multiple bacterial species. We utilized this platform to determine the potential for a single assay to accurately accomplish this. As an initial assessment, the assay was designed for the purpose of utilizing the routine diagnostic stream of ruminant respiratory cases in the United States for surveillance of mycoplasma causing bovine and caprine pleuropneumonia. Primers and probes for which analytical data had previously been published were adapted to the multiplex suspension array format. In addition, five new probe sequences and one new primer-probe set were designed so that two probes were available for each of the three specific strains targeted.

The limit of detection varied by probe. In general, there was no improvement of analytical sensitivity when comparing primer-probe sets in a singleplex or multiplex format for *M. mycoides* subsp. *mycoides* biotype Small Colony and *M. capricolum* subsp. *capripneumoniae*

with the exception of MYC probe where a 10-fold increase in the LOD was observed for *M. capricolum* subsp. *capripneumoniae*. The decreased sensitivity observed with *M. mycoides* subsp. *mycoides* biotype Small Colony and *M. capricolum* subsp. *capripneumoniae* probes in the multiplex assay could be related in part to safety treatment and/or shipment of DNA prepared for these pathogens (DNA from *M. mycoides* subsp. *mycoides* biotype Small Colony and *M. capricolum* subsp. *capripneumoniae* was generously provided by the United States Foreign Animal Disease Diagnostic Laboratory, Plum Island, NY). This assumption is based on the relative drop in the MYC probe sensitivity for these two strains when compared to the other *M. mycoides* cluster members in a singleplex format, and by a comparison of the limit of detection of gDNA prepared from two different *M. leachii* strains. *M. leachii* genomic DNA prepared with the DNeasy tissue kit had a 1000-fold increase in analytical sensitivity over another *M. leachii* strain whose DNA had been safety treated and shipped by FADDL (data not shown). Moreover, when the MYC primer-probe set was tested on *M. mycoides* subsp. *mycoides* biotype Small Colony and *M. capricolum* subsp. *capripneumoniae* in a singleplex format, the limit of detection remained 10-100-fold less sensitive than the remaining *M. mycoides* cluster members.

Additional target and non-target *Mycoplasma* species also need to be evaluated for a more complete assessment of analytical and diagnostic sensitivity and specificity. Nevertheless, six of seven primer sets were previously evaluated using a more broad range of strains within the same taxa and other closely related *Mycoplasma* species, suggesting conservation of the targeted loci. The one novel primer-probe set designed in this study (CPN1) amplifies a sequence encoding a putative integral membrane protein that is highly conserved among all ten strains available in GenBank. Notably, 5/7 probes were developed specifically for this assay, and their performance with other field isolates needs to be determined.

Preliminary evaluation of the assay's performance on field samples is encouraging. The diagnostic specificity is 100% (95% CI 98-100%) for *M. mycoides* subsp. *mycoides* biotype Small Colony and *M. capricolum* subsp. *capripneumonia* with an assumption that these organisms are not present in the United States (US declared free by the OIE; 29). Mycoplasma culture results were available for 43 of the field samples tested. Of these 43 samples, the multiplex assay correctly identified *M. bovis* as present in all 14 that were culture positive. Of the 29 culture negative samples, 2 were positive in the multiplex assay and were confirmed as positive *M. bovis* samples by *uvrC* amplification and sequencing. This suggests that the multiplex assay may offer increased diagnostic sensitivity for *M. bovis* over culture, while requiring much less time. Clearly, however, diagnostic sensitivity of the assay must be thoroughly assessed using a larger number of known positive samples, not only for *M. bovis*, but for *M. mycoides* subsp. *mycoides* biotype Small Colony and *M. capricolum* subsp. *capripneumoniae*, the latter two of which will require studies outside the United States in endemic regions. Specificity of the assay analyzed *in silico* and using type strains of multiple mycoplasma and common prokaryotic respiratory pathogens of ruminants was 100%.

The likelihood of a false negative result arising from a mutated field strain is expected to decrease by targeting two loci. The utility of 2 primer-probe sets was demonstrated for *M. bovis* in this study, as one known *M. bovis* positive lung sample from the field set was negative for PMyb probe, while nearly two-thirds of the known *M. bovis* positive samples were negative with the mb-mp1 probe. It is unclear whether the decreased diagnostic sensitivity associated with the mb-mp1 probe is due to a mutation in *M. bovis* field isolates or if it is the result of competing amplification reactions with the PMyb primer set.

Previous studies involving development and validation of a multiplex PCR-coupled liquid suspension array indicate that this form of microbial detection can be highly sensitive and specific, and is an efficient method for screening multiple pathogens in a single assay, exceeding the capacity of real-time PCR (24). By combining multiplex amplification steps to a liquid suspension array, up to 100 gene targets can be resolved using the flow cytometer platform. Clearly, this number is unattainable from a single multiplex PCR assay; however, the quantity of pathogens screened in a single well of a 96-well plate can be increased by combining products from several multiplex PCR assays. During the development phase of this assay, no decrease in analytical sensitivity was observed by increasing the number of primer-probe sets from three to seven (data not shown). This finding is encouraging for further development aimed at discriminating additional members of the *M. mycoides* cluster and other *Mycoplasma* species. Moreover, additional multiplex PCR assays targeting non-mycoplasma bacteria or viral pathogens could be developed to create a syndrome-specific assay capable of screening a more complete set of pathogens from a single ruminant respiratory sample. This would encourage use of the assay in the routine diagnostic workflow for simultaneous diagnosis of respiratory pathogens and surveillance for foreign mycoplasma strains, facilitating rapid identification of accidental or deliberate introduction of these pathogens into non-endemic countries.

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