Characterization of a novel *Mycoplasma cynos* real-time PCR assay

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Running head: Novel *M. cynos* real-time PCR assay

Abstract

Mycoplasma cynos is recognized as an emerging causative pathogen of canine infectious respiratory disease (CIRD) world-wide. The goal of this work was to develop a new open-source real-time PCR assay for *M. cynos* that performs well under standard real-time PCR conditions. Primers and probes were designed to target the *M. cynos tuf* gene, and the assay was evaluated on the ABI 7500 and QuantStudio OpenArray platforms. The *M. cynos tuf* gene assay reaction efficiencies on 7500 and OpenArray platforms ranged from 94.3 to 97.9% and 119.1 to 122.5% ($R^2 \geq 0.9935$ and 0.9784) respectively, based on amplification of standard curves spanning 8 orders of magnitude. The assay performed very well over a range of template input, from 10^9 copies to the lower limit of quantitation at 4 copies of the *M. cynos* genome. Diagnostic performance was assessed by comparison of results with a legacy assay on clinical specimens as well as testing isolates that were previously characterized by intergenic spacer region sequencing. Exclusivity was established by testing 12 ~~other veterinary~~ *Mycoplasma* strains isolated from different animals. To substantiate the high specificity of the *M. cynos tuf* gene assay, sequence confirmation was performed on intergenic spacer region PCR amplicons. Further, whole genome sequencing of *M. ycoplasma cynos* and *M. ycoplasma mucosicanis* type strains are reported here for the first time. The complete sequences, protocol, and ~~excellent~~ performance of the newly developed *M. cynos tuf* assay is provided to facilitate assay harmonization. Additionally, the *M. cynos tuf* assay also performs very well on the OpenArray platform, enabling high-throughput testing capacity.

Keywords: *Mycoplasma cynos*, *Mycoplasma mucosicanis*, canine infectious respiratory disease

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Commentato [PM2]: Same thing

Introduction

Canine infectious respiratory disease (CIRD), also referred to as kennel cough, is a complex disease that can be caused by bacterial and/or viral pathogens such as *Bordetella bronchiseptica*, canine respiratory coronavirus (CRCoV), canine influenza virus (CIV), canine parainfluenza virus (CPIV), and canine pneumovirus (CnPnV).[1,5,17] More recent studies and case reports have established an association between the presence of *M. cynos* and canine infectious respiratory disease world-wide.[1,4,5,11,27] It is notable that no association was found between CIRD and 9 other *Mycoplasma* species found in the canine respiratory tract.[1]

At least 17 *Mycoplasma* species have been detected in dogs, although at least half of these are also found in other mammalian hosts.[1,2] *Mycoplasma cynos* was identified as a new *Mycoplasma* species of dogs by Rosendal in 1973.[17] The type strain, deposited as Rosendal strain and subsequently designated H 831, was isolated from the lung of a dog with pneumonia,[16] and additional *M. cynos* strains were isolated from respiratory and genital tracts of both healthy dogs and dogs with respiratory disease.[17] *M. cynos* has been detected in samples from canine conjunctiva, nasal cavity, oral cavity, lower respiratory tract, genital tract, and urine.[2,11,16,17] Remarkably, *M. cynos* was also the only *Mycoplasma* species isolated from the air of a kennel.[1] Experimental infection of dogs with *M. cynos* can induce pneumonia.[16,18]

Diagnostic testing for *M. cynos* can be performed with culture and/or molecular methods. *M. cynos* is a fastidious organism requiring complex growth media, and culture methods still require sequencing for species identification. Sequencing approaches used to date have been useful, but one cannot assume that all *Mycoplasma* species present are always detected.[2,3] In an effort to improve molecular characterization of *M. cynos*, the genome sequence of strain C142 has been published,[24] however little sequence information is available for type strain Rosendal/H 831

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or other *Mycoplasma* species likely to be present in the same clinical specimen. There is need for a highly-specific and validated *M. cynos* real-time PCR assay to be published, including oligonucleotide sequences in order to allow for rapid results and assay harmonization. High specificity is imperative because it is not unusual for dogs to be infected with several *Mycoplasma* species simultaneously.[1,2]

A legacy assay detecting the *M. cynos* 16S-23S rRNA intergenic (ITS) sequence was used in our laboratory prior to this work. However, that assay exhibited high variability in analytic performance on the QuantStudio 12K Flex OpenArray (OA) platform and this compelled us to develop a novel *M. cynos* MGB probe-based assay that would perform well under standard PCR and cycling conditions on both ABI 7500 and OA platforms, while providing high specificity on canine respiratory swab and tissue specimens. The OA platform was included in this test validation because it enables detection of respiratory pathogens in a high-throughput manner.[9]

Materials and Methods

Clinical specimens and reference strains

For routine diagnostic testing, the starting amount used for purification is one nasal swab or 100-200 mg of tissue. Tissue samples are minced and combined with 1 mL DMEM (ThermoFisher Scientific, Waltham, MA) and then homogenized with a TissueLyser (Qiagen, Germantown, MD) for 2 minutes at 18 Hz. Following centrifugation at 3,000 rpm for 3 minutes, this supernatant is used for nucleic acid extraction.

For determination of diagnostic specificity and sensitivity, 71 canine respiratory specimens submitted for routine canine respiratory panel diagnosis were tested with a legacy *M. cynos* assay (ITS) and the new *M. cynos tuf* assay. Additionally, 67 bacterial isolates previously characterized by *Mycoplasma ss. spp.* culture and intergenic spacer region (ISR) sequencing for species

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determination were tested with the new *M. cynos tuf* assay. This panel of bacterial isolates was comprised of *M. canis*, *M. cynos*, *M. edwardii*, *M. felis*, *M. maculosum*, and *M. spumans* strains.

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Thirteen reference strains were tested to evaluate the analytical specificity of the assay, including *Mycoplasma alkalesc~~e~~ens* ATCC 29103, *Mycoplasma arginini* ATCC 23838, *Mycoplasma bovigenitalium* D0108 721, *Mycoplasma bovigenitalium* ATCC 19852, *Mycoplasma bovirhinis~~aris~~* ATCC 27748, *Mycoplasma bovis* D0200 473, *Mycoplasma bovis* ATCC 25523, *Mycoplasma californicum~~ason~~* ST-6T or 6J Dellinger, *Mycoplasma canadense* ATCC 29418, *Mycoplasma canis* ATCC 19525, *Mycoplasma cynos* ATCC 27544, *Mycoplasma edwardii* ATCC 23462, and *Mycoplasma mucosicanis* ATCC BAA-1895.

Positive amplification control

A positive amplification control (PAC) based on the *tuf* gene target region was synthesized and cloned by an ISO 9001:2008 certified facility (GenScript, Piscataway, NJ) and quantified using an intercalating dye (PicoGreen, ThermoFisher Scientific). The confirmed sequence is as follows:

AGTGTTCCACGTTCAACACGTCCAGTAGCAACTGTTCCACGTCCTGTAATTGTAAAT
ACGTCCTCAACAGCCATTAAGAATGGTTTGTCATATTCTTTAACAGGTGTTTCAATAT
ATGAATCAACTGCGTCCATTAATTCTAATATTTTTTCTTCGTATTTAGCATCACCTTC
AAGTGCTTTTAAAGCTGAACCACGAATAATTGGCGCATTATCTCCATCAAATCCATA
TTCTGATAAAAGGCTACGAATTTCAACTTCAACTAATTCAATCATTTCTTCTTCACCT
TCTAACATATCAACTTTGTTTAAGAAAACAACGATACGCGGAACACCAACTTGTTTA
GATAAAAGAATGTGTTCACGTGTTTGAGGCATAGGCCCATCTGTTGCAGCAACAA.

For high-throughput testing as part of a canine respiratory panel on the OA platform, a pool consisting of all positive amplification controls was created, including beta-coronavirus,

B. ~~ordetella~~ bronchiseptica, canine adenovirus, canine distemper virus, canine parainfluenza virus, canine pneumovirus, influenza A, *M. cynos*, and MS2 RNA phage.

Nucleic acid extraction

Total nucleic acid (RNA and DNA) was purified with an automated magnetic bead-based extraction kit (MagMAX™ Total Nucleic Acid Isolation Kit AM1840, ThermoFisher Scientific). The purified nucleic acid was eluted in 90 µl.

Exogenous internal control

We used the MS2 RNA phage as an exogenous internal control (XIC) by combining it with the MagMAX lysis buffer and detection in sample eluates.[6] An acceptable range was established based on two standard deviations from the average cycle threshold (Ct) level in respiratory matrix, inclusive of tissues. PCR inhibition due to the sample matrix is indicated by the MS2 XIC signal being out of acceptable range or undetected.

Assay design and amplification platforms

Primer Express software (Thermo Fisher Scientific) was used to design primers (forward 5' TCTTCGTATTTAGCATCACCTTCAAGT 3'; reverse 5' TGATGGAGATAATGCGCCAAT 3'), and an MGB probe (5' FAM-CTTTTAAAGCTGAACCACG-MGB 3') using an alignment of all available *Mycoplasma* species *tuf* gene sequences including *M. cynos* genome NC_019949.[23]

For the Applied Biosystems™ 7500 Real-Time PCR platform (Applied Biosystems, Foster City, CA), forward and reverse primers at 400 nM final concentration and probe at 120 nM final concentration were added to the Path-ID Multiplex One-Step RT-PCR master mix (Applied Biosystems) in 21µl and combined with 4 µl of template. Cycling conditions were 48°C for 10 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds then 60°C for 45 seconds.

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For high-throughput testing, the new *M. cynos tuf* assay was also validated on the Applied Biosystems™ QuantStudio™ 12K Flex system with an OpenArray block (Thermo Fisher Scientific). This nanoscale diagnostic PCR workflow is optimized for use at Animal Health Diagnostic Center (AHDC) ~~in our laboratory~~ and has been described previously.[9] Custom nanoscale PCR amplification plates arranged in an 18x3 gene expression format were ordered with the *M. cynos tuf* oligonucleotides listed above, in addition to oligonucleotides for other pathogen and control tests.[9] The forward and reverse primers were also included in a pre-amplification/reverse-transcription pool in combination with the other respiratory panel primers at 9 µM (Integrated DNA Technologies, Inc., Coralville, IA). This pool was combined with random primers at 300 nM final concentration (New England Biolabs, Ipswich, MA), TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems), and 7 µL template in a 10 uL total volume. Pre-amplification cycling conditions were 50°C for 15 minutes, 95°C for 1 minute, 20 cycles of (95°C for 15 seconds and then 60°C for 2 minutes), followed by 99.9°C for 5 minutes in a conventional PCR machine. This pre-amplification product was then diluted in 40 µL of 1X TE buffer. The pre-amplified and diluted samples are then combined with an equal volume of TaqMan™ OpenArray™ Real-Time PCR Master Mix (Applied Biosystems), from which 33 nL is transferred to the nanoscale PCR amplification plate by the AccuFill system (Applied Biosystems). Note that the OA platform uses a different algorithm for calculating results called "C_RT" (relative cycle threshold). Values are calculated based on the shape of individual amplification curves rather than the cycle of crossing a particular fluorescence threshold. For simplicity, herein we refer to C_RT as Ct (cycle threshold), which is the value reported by the 7500 platform.

Routine controls were run in parallel to diagnostic samples, including a negative extraction control, negative amplification control, XIC (MS2 RNA phage), and PAC at 10^3 copies/µL.

Analytic Performance

A standard curve spanning 8 orders of magnitude was made from serial dilutions of the PAC. These dilutions were tested in triplicate on three different days. Amplification efficiency was calculated from the slope of the standard curve. Intra-assay variation was determined by calculating the percent coefficient of variation (%CV) from the replicates within each run. Interassay variation was determined by calculating the %CV from the replicates on different days.

The assay limit of detection (LOD) was calculated based on the mean of the Ct value at the lowest copy number where 100% of the replicates are positive plus 2 standard deviations. This was based on 12 replicates on the 7500 platform and 36 replicates on the OA platform. Platform-specific interpretation ranges were established according to internal guidelines. For the 7500 platform, Ct>LOD = "low positive", Ct between LOD and the value of (LOD divided by 1.5) = "moderate positive", Ct<(LOD divided by 1.5) = "high positive". For the OA platform, Ct>LOD = "low positive", Ct between LOD and the value of (LOD divided by 2) = "moderate positive", Ct<(LOD divided by 2) = "high positive".

Diagnostic performance

A proprietary and internally approved legacy *M. cynos* ITS real-time PCR performed on the 7500 platform was chosen as the comparison method. Results of the legacy ITS assay were compared to results of the novel *tuf* assay using 71 canine respiratory specimens submitted for routine canine respiratory panel diagnosis.

Sequencing of the 16S-23S rRNA ISR was chosen as the gold standard. PCR primers to amplify the ISR of *Mycoplasma* species were described previously.[8] PCR products were visualized by gel electrophoresis. Initially, 5 μL of PCR product were loaded on a 1.5% agarose gel and electrophoresed at 100 volts to observe the number of discrete bands present. GelRed nucleic acid

gel stain (Biotium, Fremont, CA) was used to stain DNA for visualization on a ChemiDoc Imaging system (Bio-Rad, Hercules, CA) with the accompanying Quantity One software. Amplicons were purified directly (QIAquick PCR Purification kit, Qiagen) or excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen). The concentration of purified PCR products was determined by a Qubit 3.0 fluorometer (Thermo Fisher Scientific). Direct sequencing of amplicons was performed by the Institute of Biotechnology at Cornell University, Ithaca, NY. Chromatograms were visualized and manually edited with Geneious 8.1.7 (https://www.geneious.com). Intergenic spacer region sequences obtained from clinical specimens were compared to *Mycoplasma* ISR sequences in the NCBI nucleotide collection database with BLAST. Phylogenetic analysis was performed with MEGA version X.[12]

Results of canine respiratory specimens tested with the *M. cynos tuf* assay individually or as one of the canine respiratory panel assays were obtained from our laboratory information management system, from the time the *tuf* assay went into production to the present (October 7, 2016 to February 14, 2019). This data was analyzed with Microsoft Access to quantify and characterize co-infection rates and pathogen prevalence. GraphPad Prism (GraphPad Software, San Diego, CA) was used to generate plots of the data.

Whole Genome Sequencing

The MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems) was used to extract DNA from *M. cynos* (ATCC 27544) and *M. mucosicanis* (ATCC BAA-1895) type strains after enrichment. DNA was quantified with a Qubit 3.0 fluorometer (Thermo Fisher Scientific). The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) was used to prepare fragment libraries of the DNA for whole genome sequencing; this was performed according to the manufacturer's instructions. Tagmented libraries were purified using AMPure XP Beads

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(Beckman Coulter Life Sciences, Indianapolis, IN) at a concentration of 0.5x. Barcoded *M. cynos* and *M. mucosicanis* whole genome libraries were then pooled and run on Illumina MiSeq Reagent Kit (V3) cartridge at 2x250 cycles using a MiSeq next-generation sequencer.

Sequence reads were trimmed using the sliding window program in Trimmomatic with a window size of 4 and a minimum quality score of 20 [10.1093/bioinformatics/btu170]. Draft genomes were assembled using SPAdes 3.10.1 [10.1089/cmb.2012.0021]. Read depth and assembly statistics were calculated using BBMap 38.26 (https://jgi.doe.gov/data-and-tools/bbtools/) and Quast 4.0 [10.1093/bioinformatics/btt086].

The *tuf* gene was identified in each of the assemblies by running a BLASTN search against them using the *tuf* nucleotide sequence from the *M. cynos* C142 reference genome (HF559394.1:c930548-929361) [10.1186/1471-2105-10-421]. The *tuf* assay primer and probe sequences were also compared to our *M. cynos* and *M. mucosicanis* assemblies and to the published C142 genome.

All sequences determined in this study have been submitted to NCBI BioProject XX (in process).

Statistical analyses

GraphPad Prism (GraphPad Software) was used to analyze standard curve data by linear regression, coefficient of variation, and 2x2 test agreement analyses.

Results

Assay design and performance

Primers and probes for the *tuf* gene were designed based on alignment of *M. cynos* strains H 831 and C142 with 18 other *Mycoplasma tuf* gene sequences available on NCBI.

Standard curves with 8 orders of magnitude were run on three different days and analyzed by linear regression using GraphPad Prism (Table 1, Figure 1). The assay performance on the 7500 platform using the master mix manufacturer's recommended concentrations was linear ($p = 0.21$ for deviation from linearity) with $r^2 \geq 0.9935$, and efficiency values of 94.3% to 97.9%. Dilutions of 0.1 and 0.01 copies per microliter were consistently not detected. For the OA platform, amplification was also linear ($p = 0.22$ for deviation from linearity, Figure 1). The efficiency values ranged from 119.1% to 122.5%.

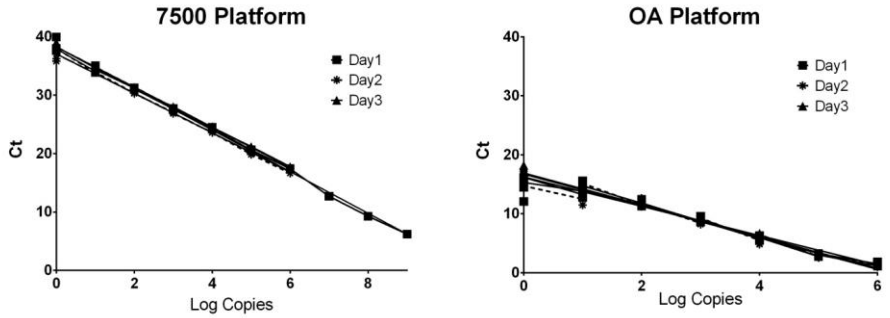

Figure 1. *Mycoplasma cynos* standard curve dilution series. The *Mycoplasma cynos* positive amplification control was serially diluted and then amplified with the *tuf* assay on the ABI 7500 and OpenArray platforms. Dilutions of 0.1 and 0.01 copies per uL were consistently not detected (not shown).

Table 1. Standard curves demonstrating *M. cynos tuf* assay performance.

Platform	Day	Slope	R^2	Efficiency
7500	1	-3.467 ± 0.062	0.993	94.3%
7500	2	-3.374 ± 0.056	0.994	97.9%
7500	3	-3.437 ± 0.036	0.997	95.4%
OA	1	-2.721 ± 0.050	0.986	122.5%
OA	2	-2.762 ± 0.069	0.978	119.1%
OA	3	-2.763 ± 0.049	0.989	119.7%

Repeatability

Intra-assay variation: On the 7500 platform (Table 2), all SDs were <1.0, and none of the CVs exceeded the maximum desired value of 3% (untransformed) or 50% (linearized using 2^{-Ct}).

Table 2. Intra-assay variation of Cycle Threshold (Ct) values on the 7500 platform.

Day	Copies per µl	Mean	SD	%CV (Ct)	%CV (2^{-Ct})
1	Low = 10^2	31.2	0.08	0.24	5.30
1	Med = 10^3	27.7	0.05	0.16	3.14
1	High = 10^4	24.4	0.10	0.39	6.53
2	Low = 10^2	30.4	0.05	0.17	3.57
2	Med = 10^3	27.0	0.07	0.27	5.21
2	High = 10^4	23.6	0.04	0.19	3.06
3	Low = 10^2	31.2	0.08	0.27	5.86
3	Med = 10^3	27.9	0.10	0.38	7.25
3	High = 10^4	24.5	0.06	0.24	4.06

On the OA platform (Table 3), all SDs were <1.0. There were a number of failed CVs (>3%) using the untransformed values, but none of the CVs failed by the more appropriate linearized method (<50%).

Table 3. Intra-assay variation of Cycle Threshold (Ct) values on the OA platform.

Day	Copies per µl	Mean	SD	%CV (Ct)	%CV (2^{-Ct})
1	Low = 10^2	11.8	0.32	2.72	20.88
1	Med = 10^3	9.0	0.27	2.98	16.75
1	High = 10^4	5.8	0.27	4.70	17.32
2	Low = 10^2	11.8	0.51	4.35	32.24
2	Med = 10^3	8.7	0.32	3.69	20.72
2	High = 10^4	5.7	0.47	8.30	34.19
3	Low = 10^2	11.9	0.27	2.24	16.65
3	Med = 10^3	8.8	0.28	3.20	16.98
3	High = 10^4	5.9	0.29	4.91	17.22

Inter-assay variation: Variation between runs on 3 different days is shown in Table 4. All SDs were <1.0, and none of the CVs exceeded the maximum desired value of 3% (untransformed) or 50% (linearized using 2-Ct). The slopes of the standard curves were not

significantly different on the three different days for each platform (p = 0.46 for 7500, p = 0.83 for OA).

Table 4. Inter-assay variation of Ct values.

Platform	Template concentration (per µl)	Mean	SD	%CV (Ct)	%CV (2^{-Ct})
7500	Low = 10^2	30.9	0.49	1.60	36.7
7500	Med = 10^3	27.5	0.49	1.79	35.9
7500	High = 10^4	24.2	0.49	2.03	36.1
OA	Low = 10^2	11.9	0.05	0.44	5.3
OA	Med = 10^3	8.8	0.14	1.55	9.9
OA	High = 10^4	5.8	0.12	2.10	10.3

Analytical sensitivity and specificity

The analytical limit of quantitation was less than 10 copies of the bacterial genome on both platforms. This was based on 12 replicates on the 7500 platform and 36 replicates on the OA platform.

To define the analytical specificity, the following *Mycoplasma* reference strains were tested and not detected with the *M. cynos tuf* assay: *M. alkalescens~~alkalescans~~*, *M. arginini*, *M. bovigenitalium* (D0108 721 and ATCC 19852), *M. bovirhinis~~bovirharis,~~ M. bovis* (D0200 473 and ATCC 25523), *M. californicum~~ason~~*, *M. canadense*, *M. canis*, *M. edwardii*, and *M. mucosicanis*.

The positive amplification control run individually on the OA platform uniquely detected the new *M. cynos tuf* target, and no other targets from a panel of canine and equine respiratory assays; the latter was comprised of: beta-coronavirus, *B.~~ordetella~~ bronchiseptica*, canine adenovirus, canine distemper virus, canine parainfluenza virus, canine pneumovirus, equine adenoviruses 1 and 2, equine herpesvirus 1 and 4, equine rhinitis viruses A and B, influenza A, *Mycoplasma felis*, *Streptococcus equi*, and *Streptococcus zooepidemicus*. Many of the clinical samples that were negative by the new *tuf* assay were positive for other pathogens including

beta-coronavirus, *B. ~~ordetella~~ bronchiseptica*, canine distemper virus, canine parainfluenza virus, canine pneumovirus, and influenza A virus.

Diagnostic sensitivity and specificity

To assess the diagnostic sensitivity and specificity, 71 canine respiratory specimens submitted for routine diagnosis of canine respiratory disease were previously tested directly (without bacterial enrichment) with the legacy ITS assay and the new *tuf* assay. The new *tuf* assay demonstrated 95.77% agreement with legacy ITS assay (Table 5). The one false negative was most likely due to another *Mycoplasma* species being detected by the legacy assay as ISR sequencing revealed the presence of *M. spumans* in the sample. The other 2 discrepant samples were detected by the OpenArray but not the 7500 platform. The Kappa correlation for detection of canine respiratory specimens was 0.915 (95% confidence interval 0.822-1.000).

Table 5. Diagnostic specificity assessment on canine respiratory samples.

	ITS PCR +	ITS PCR -
tuf PCR +	33	2
tuf PCR -	1	35

The diagnostic specificity of the new *tuf* assay was also assessed using 67 isolates that were characterized by *Mycoplasma* ~~S~~spp. culture and sequencing. The results identified 5 true positive isolates and 56 true negative isolates. The 56 negative samples were comprised of 22 *M. canis* isolates, 12 *M. felis* isolates, 12 *M. spumans* isolates, 7 *M. edwardii* isolates, and 3 *M. maculosum* isolates. No false negatives were found, although 6 isolates were determined to be false positives because they were positive with the *tuf* PCR assay but negative by ISR sequencing. Five of the 6 discrepant ISR sequencing results had *M. canis* and *M. edwardii* as the two top BLAST hits but included *M. cynos* in the list of significant hits. ATCC isolates for *M. canis* and *M. edwardii* were tested and both yielded negative results with the *tuf* assay. The sixth discrepant isolate was typed

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as *M. maculosum* or *M. leopharyngis* but also included many other significant hits including several that were excluded by testing the ATCC isolates listed above, thus we chose not to pursue this isolate further.

Inhibition monitoring

Continuous monitoring of the XIC did not reveal any systematic indications of inhibition in respiratory matrix, inclusive of tissues. Results from 22 clinical specimens tested on15 independent ABI 7500 runs are shown in Figure 2. In these specimens, *M. cynos* Ct values ranged from 20.609 – 39.958 while the XIC Ct values ranged from 31.283 – 35.000. The OA assay detected a range of *M. cynos* Ct values (2.282 – 18.369) from 23 clinical specimens obtained from 15 independent runs (Figure 3). The XIC was detected in the same specimens and with corresponding Ct values that remained within a span of 2 standard deviations (Ct range 10.694 – 15.814; 2 standard deviation range10.682 – 16.164; Figure 3).

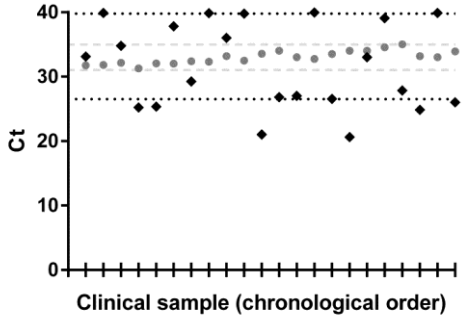

Figure 2. Detection of *Mycoplasma cynos* target and exogenous internal control in clinical specimens on the ABI 7500 platform. *Mycoplasma cynos* was detected over a range of high, moderate, and low positive values (black diamonds; moderate positive range bracketed by black dotted line) in 22 clinical specimens. The MS2 exogenous internal control was detected in the

same specimens and MS2 Ct values remained within a span of 2 standard deviations (gray circles; 2 standard deviations represented by gray broken line).

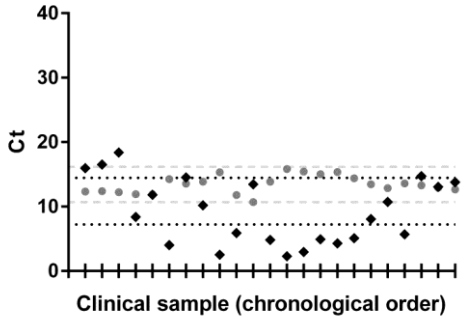

Figure 3. Detection of *Mycoplasma cynos* target and exogenous internal control in clinical specimens on the OpenArray platform. A range of high, moderate, and low *M. ~~ycoplasma~~ cynos* positive values was detected in 23 clinical specimens (black diamonds; moderate positive range bracketed by black dotted line). The MS2 exogenous internal control was detected in the same specimens (gray circles; 2 standard deviations represented by gray broken line).

Sequence confirmation

To confirm that *tuf* PCR positive specimens contained *M. cynos*, the intergenic spacer region (ISR) was amplified and sequenced. Amplification of the ISR was performed on 107 *tuf* PCR positive-specimens. Visualization of the ISR PCR products revealed that 89 *tuf* PCR positive-specimens yielded a single ISR band at the 457 base pair size expected for the *M. cynos* genome. The remaining 18 *tuf* PCR positive-specimens yielded 2 bands; one at the size expected for the *M. cynos* genome and a second smaller band (Figure 4). Ninety-four PCR products were purified and were directly sequenced (91 of the amplicon size expected for *M. cynos* and 3 smaller). Of the 91 amplicon sequences expected to be *M. cynos*, NCBI BLAST revealed that 82 were ≥99.6% identical to the *M. cynos* ISR sequence, one sequence shared high identity with *M. mucosicanis*

ISR sequence, and the remaining 8 had poor sequence quality that prevented definitive identification of the *Mycoplasma* species present in the specimen. The *M. cynos* 1642 strain genome sequence (HF559394) includes 3 discrete ISR, 2 of which are 257 bases in length and share 100% identity; the third ISR contains a 1 base insertion. Of the 82 sequences that matched *M. cynos* ISR sequence, 78 of these were 100% identical to the *M. cynos* 1642 strain ISR of 257 bases (HF559394), 2 were 100% identical to the *M. cynos* 1642 strain ISR of 258 bases (HF559394), and 2 were 100% identical to the *M. cynos* 1642 strain ISR of 257 bases (HF559394) except each had an ambiguous (N) base that could not be manually resolved. The PCR sequence that shared high identity with *M. mucosicanis* strain 1642 sequence (FM180556.1) was identical at 307 of 309 ISR positions. Sequence analysis of the 3 smaller ISR PCR products identified the presence of *M. spumans* (100% identical to AF538684) and *M. canadense* (the most significant BLAST result was to AP014631 with 163 out of169 nucleotide identities; these 2 PCR product sequences were identical to each other); these represent specimens with co-infection of at least 2 *Mycoplasma* species.

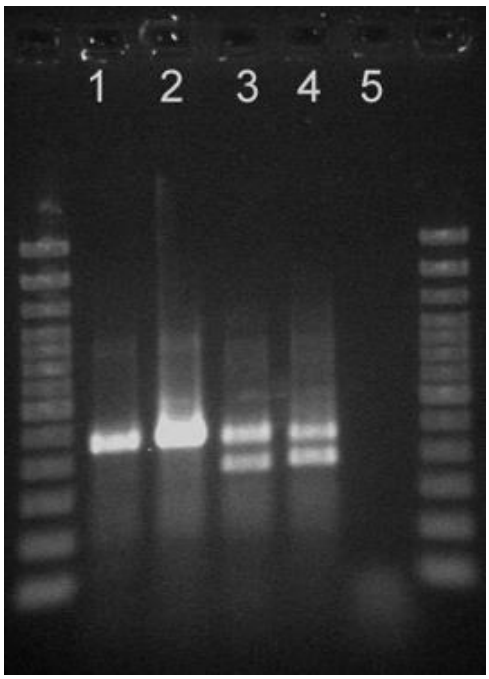

Figure 4. Gel electrophoresis of *Mycoplasma* ISR PCR products. Most *tuf* PCR positive-clinical specimens yielded a single *Mycoplasma* ISR band at the 457 base pair size expected (lanes 1 and 2) for the *M. cynos* genome. A subset of *tuf* PCR positive-clinical specimens yielded 2 bands; one at the size expected for the *M. cynos* genome and a second smaller band (lanes 3 and 4). Lane 5 contained the negative amplification control. The outermost lanes were loaded with 100 bp DNA ladder.

Phylogenetic analysis of the ISR sequences obtained in this work and from reference *Mycoplasma* strain sequences was performed. *M. cynos* ISR sequences from strain C142 and from sequence confirmation of *tuf*-PCR positive specimens (all 82 represented by sequence "Cornell ISR 1") were placed within the *M. synoviae* cluster (Figure 5), consistent with previous reports of

Mycoplasma phylogenetic analysis.[3] Similarly, the ISR sequences from *M. mucosicanis* strain 1642 and PCR sequencing herein were found in the *M. bovis* cluster. The remaining ISR sequences determined in this study, representing *M. spumans* and *M. canadense*, were present within the *M. hominis* cluster.

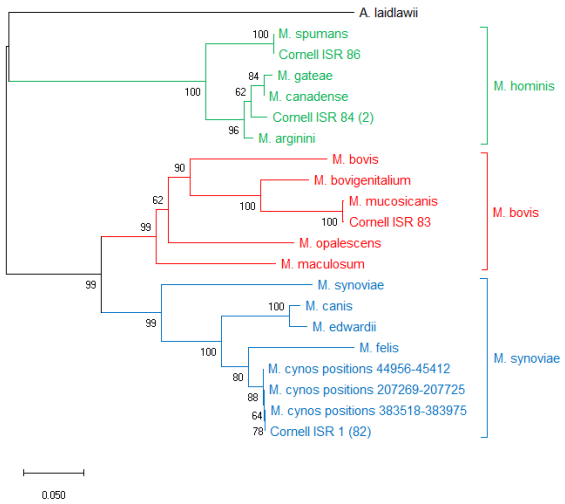

Figure 5. Phylogenetic tree of *Mycoplasma* intergenic spacer region sequences. Evolutionary analyses were conducted in MEGA X and the tree was inferred using the Neighbor-Joining method.[12,19] The bootstrap test with 1,000 replicates was used to determine the percentage of replicate trees in which the associated taxa clustered together and these values are shown next to the branches.[7] Phylogenetic clustering of *Mycoplasma* strains are distinguished by color; sequences determined in this work are denoted by "Cornell ISR" prefix. Sequences identified multiple times are indicated by (#) following the name, with '#' reflecting the number of times that sequence was obtained. *Mycoplasma* sequences obtained from GenBank include: *A. laidlawii* (CP000896), *M. arginini* (AY737013), *M. bovigenitalium* (AP017902), *M. bovis*

(CP002513), *M. canadense* (AY800341), *M. canis* (AF443605), *M. cynos* (NC_019949), *M. edwardii* (AF443607), *M. felis* (AF443608), *M. gateae* (AF443609), *M. maculosum* (AF443610), *M. mucosicanis* (FM180556), *M. opalescens* (AF443612), *M. spumans* (AF538684), and *M. synoviae* (AJ781002). The ISR sequence of *Acholeplasma laidlawi* was included as an outgroup.

Whole genome sequencing

Our assembled *M. cynos* type strain Rosendal/H 831 (ATCC 27544) genome was 853,159 bp with an N50 of 5,333 and a GC content of 26.35% (NCBI accession in process). Our *M. mucosicanis* type strain 1642 (ATCC BAA-1895) assembly consisted of 870,218 bp with an N50 of 64,609 and GC content of 29.55% (NCBI accession in process). Average read depths were 15.4 for *M. cynos* and 77.6 for *M. mucosicanis*. The *M. cynos* assembly shared 99.8% nucleotide identity (1186/1188 bases) with the C142 *tuf* sequence, while the *M. mucosicanis* shared 83.6% identity (999/1195 bases including 11 gaps). Our *M. cynos* assembly had exact matches to the *tuf* primer and probe sequences. The corresponding regions in our *M. mucosicanis* assembly shared 23/27 nucleotide identities with the forward primer, 16/21 with the reverse primer, and 16/19 with the probe. Over the 72 bp region spanning the forward and reverse primers, our *M. cynos* assembly was identical to the C142 genome and our *M. mucosicanis* assembly had 13 variable sites.

The ABI 7500 *tuf* gene assay was performed on the DNA extracted from the *M. cynos* and *M. mucosicanis* type strains obtained from ATCC and *M. cynos* was positive while *M. mucosicanis* was not detected.

***M. cynos* prevalence and co-infections**

Our laboratory has performed 1,982 *M. cynos tuf* tests on diagnostic canine respiratory specimens since initiating production of the new assay in late 2016, and of those, 634 (31.99%) were positive. In the same time frame, canine respiratory panel results were analyzed to investigate

M. cynos co-infections. The canine respiratory panel includes tests for beta-coronavirus, *Bordetella bronchiseptica*, canine adenovirus, canine distemper virus, canine parainfluenza virus, canine pneumovirus, and influenza A virus in addition to *M. cynos*. Of 1,368 canine respiratory panel tests, 358 (26.17%) results identified co-infections that included *M. cynos*. These co-infections consisted of *M. cynos* plus another 1 (214 specimens, 59.78%), 2 (103 specimens, 28.77%), 3 (28 specimens, 7.82%), 4 (10 specimens, 2.79%), or 5 (3 specimens, 0.84%%) pathogens (Figure 6A). The prevalence of pathogens present simultaneously with *M. cynos* in specimens was assessed, and canine parainfluenza virus was found most frequently (157 specimens, 28.09%), followed by *Bordetella bronchiseptica* (119 specimens, 21.29%), and beta-coronavirus (111 specimens, 19.86%). Also present less frequently in *M. cynos* co-infections were canine pneumovirus (84, 15.03%), canine distemper virus (41, 7.33%), influenza A virus (29, 5.19%), and canine adenovirus (18, 3.22%) (Figure 6B).

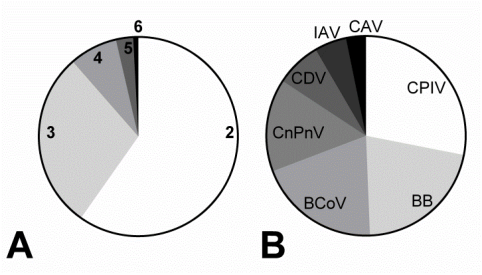

Figure 6. Number and prevalence of pathogens present in *M. cynos* co-infections. Panel **A**) Instances of 358 *M. cynos* co-infections since the *tuf* assay went into production consisted of 2 (59.78%, white), 3 (28.77%, light gray), 4 (7.82%, gray), 5 (2.79%, dark gray), or 6 (0.84%, black) pathogens. Panel **B**) Co-infections included *M. cynos* and one or more of the following pathogens: canine parainfluenza virus (CPIV), 28.09%), *Bordetella bronchiseptica* (BB,

21.29%), beta-coronavirus (BCoV, 19.86%), canine pneumovirus (CnPnV, 15.03%), or canine distemper (CDV, 7.33%), influenza A (IAV, 5.19%), or canine adenovirus (CAV, 3.22%).

Discussion

The *tuf* assay described herein is intended for the diagnosis of *M. cynos* in canine respiratory samples with high specificity in a rapid and economical format that conforms to reagents and conditions commonly used in AAVLD laboratories. The validation data presented encompasses both the ABI 7500 and OA real-time PCR platforms. The *tuf* assay is compatible with the MS2 RNA phage XIC to facilitate PCR inhibition monitoring according to best practice guidelines of the AAVLD Technology Committee. Collectively, the performance of this assay meets the AAVLD guidelines and the authors' internal validation standards. Additionally, use of this assay in our laboratory under ISO/IEC:17025 scope was approved by the American Association for Laboratory Accreditation (A2LA, certificate number 2880.01).

To design a novel assay for *M. cynos* with high specificity, the gene encoding elongation factor Tu (*tuf*) was targeted because it contains *Mycoplasma* species-specific variable regions.[14,20,22] The *M. cynos tuf* assay performed well on the ABI 7500 platform, even at very high template concentrations of up to 10^9 copies per microliter. On the OA platform, reaction efficiencies were high compared to typical benchmarks for the 7500 platform due to the fact that samples were pre-amplified and visualized using a white LED excitation source. Otherwise, the *tuf* PCR assay exhibited excellent performance and specificity on the OA platform; this attribute enables high-throughput testing and facilitates co-infection monitoring. The sequence confirmation data and phylogenetic analysis provide robust support for the specificity of the *tuf* PCR assay. The discrepant results identified between ISR sequencing and the new *tuf* assay, including the identification of *M. mucosicanis* in a *tuf* PCR positive-specimen, suggest instances

of mixed *Mycoplasma* infections. This was supported by the visualization of multiple bands upon ISR amplification, with 16.82% of *tuf* PCR positive-specimens producing 2 distinct ISR amplicons. Reference isolates of the other *Mycoplasma* species present in the sample, identified by ISR sequencing, were tested with the *tuf* assay and did not amplify.

The ISR identity match of *M. mucosicanis* in a *tuf* PCR positive-specimen was of concern, and little is known about *M. mucosicanis* as it was recently identified.[21] No full genome sequence was previously available for this species or for *M. cynos* type strain Rosendal/H 831. To address this, type strains of both *M. mucosicanis* and *M. cynos* were obtained from ATCC, and whole genome sequencing was performed in our laboratory. The whole genome and ISR sequence data determined here is publicly available to support efforts to better understand the clinical significance of *Mycoplasma* infection in canine health and to facilitate the identification of novel species. As *Mycoplasma* strains are able to cause disease directly as well as part of a co-infection, it is difficult to delineate their precise role. The association of this agent with morbidity in CIRD was previously characterized and is outside the scope of the present study. The high prevalence of close to one-third of clinical specimens being positive over two years of running the assay routinely is consistent with previous observations, which have largely focused on bacterial coinfections. The high proportion of viral co-infecting agents stresses the importance of comprehensive diagnostic testing for the management of CIRD, particularly in the context of AVMA guidelines for judicious use of antimicrobials (https://www.avma.org/KB/Policies/Pages/Judicious-Therapeutic-Use-of-Antimicrobials.aspx).

In some instances of dogs manifesting CIRD, co-infection of *M. cynos* with up to four other pathogens have been described. Pathogens identified in co-infections with *M. cynos* include *Bordetella bronchiseptica*, canine adenovirus type 2, canine distemper virus, and canine

parainfluenza virus.[4,5,13] A review of our canine respiratory panel results since the *M. cynos tuf* assay went into production revealed similar findings. Specifically, *M. cynos* co-infections identified by us were comprised of up to 6 pathogens, including one or more of the following: canine parainfluenza virus, *Bordetella bronchiseptica*, beta-coronavirus, canine pneumovirus, canine distemper virus, influenza A virus, and/or canine adenovirus.

In conclusion, this report describes the first comprehensive validation of an open-source assay for *M. cynos*. With its publication, the authors hope to promote assay harmonization in veterinary diagnostics in order to facilitate surveillance and the use of validated assays.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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