

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

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

17 **Abstract**

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

36

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

Commentato [PM1]: Not clear to me why we speak about *M. mucosicanis*

Commentato [PM2]: Same thing

38 **Introduction**

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

46 At least 17 *Mycoplasma* species have been detected in dogs, although at least half of these
47 are also found in other mammalian hosts.^{1,2} *Mycoplasma cynos* was identified as a new
48 *Mycoplasma* species of dogs by Rosendal in 1973.¹⁷ The type strain, deposited as Rosendal strain
49 and subsequently designated H 831, was isolated from the lung of a dog with pneumonia,¹⁶ and
50 additional *M. cynos* strains were isolated from respiratory and genital tracts of both healthy dogs
51 and dogs with respiratory disease.¹⁷ *M. cynos* has been detected in samples from canine
52 conjunctiva, nasal cavity, oral cavity, lower respiratory tract, genital tract, and urine.^{2,11,16,17}
53 Remarkably, *M. cynos* was also the only *Mycoplasma* species isolated from the air of a kennel.¹
54 Experimental infection of dogs with *M. cynos* can induce pneumonia.^{16,18}

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

Commentato [PM3]: I THINK WE NEED TO ADD ALSO THE TIME
TAKE TO GROW 7-8 DAYS

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

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

73 **Materials and Methods**

74 **Clinical specimens and reference strains**

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

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

Commentato [PM4]: Do we need to add the period?

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

Commentato [PM5]: Do we need to add the number for each Myco?

86 Thirteen reference strains were tested to evaluate the analytical specificity of the assay,
87 including *Mycoplasma alkalescens* ATCC 29103, *Mycoplasma arginini* ATCC 23838,
88 *Mycoplasma bovis* D0108 721, *Mycoplasma bovis* ATCC 19852,
89 *Mycoplasma bovis* ATCC 27748, *Mycoplasma bovis* D0200 473, *Mycoplasma bovis*
90 ATCC 25523, *Mycoplasma californicum* ST-6T or 6J Dellinger, *Mycoplasma canadense*
91 ATCC 29418, *Mycoplasma canis* ATCC 19525, *Mycoplasma cynos* ATCC 27544, *Mycoplasma*
92 *edwardii* ATCC 23462, and *Mycoplasma mucosicanis* ATCC BAA-1895.

93 Positive amplification control

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

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98 AGTGTTCACGTTCAACACGTCCAGTAGCAACTGTTCCACGTCCTGTAATTGTAAAT  
99 ACGTCCTCAACAGCCATTAAGAATGGTTTGTTCATATTCTTTAACAGGTGTTTCAATAT  
100 ATGAATCAACTGCGTCCATTAATTCTAATATTTTTTCTTCGTATTTAGCATCACCTTC  
101 AAGTGCTTTTAAAGCTGAACCACGAATAATTGGCGCATTATCTCCATCAAATCCATA  
102 TTCTGATAAAAGGCTACGAATTTCAACTTCAACTAATTCAATCATTCTTCTTCACCT  
103 TCTAACATATCAACTTTGTTTAAAGAAAACAACGATACGCGGAACACCAACTTGTTTA  
104 GATAAAAGAATGTGTTACGTGTTTGAGGCATAGGCCCATCTGTTGCAGCAACAA.
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105 For high-throughput testing as part of a canine respiratory panel on the OA platform, a pool
106 consisting of all positive amplification controls was created, including beta-coronavirus,

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

Commentato [PM6]: Check is MS2 RNA phage or MS2 phage RNA

109 **Nucleic acid extraction**

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

112 The purified nucleic acid was eluted in 90 µl.

113 **Exogenous internal control**

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

Commentato [PM7]: same

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119 **Assay design and amplification platforms**

120 Primer Express software (Thermo Fisher Scientific) was used to design primers (forward
121 5' TCTTCGTATTAGCATCACCTTCAAGT 3'; reverse 5' TGATGGAGATAATGCGCCAAT
122 3'), and an MGB probe (5' FAM-CTTTTAAAGCTGAACCACG-MGB 3') using an alignment of
123 all available *Mycoplasma* species *tuf* gene sequences including *M. cynos* genome NC_019949.²³

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

130 For high-throughput testing, the new *M. cynos tuf* assay was also validated on the Applied
131 Biosystems™ QuantStudio™ 12K Flex system with an OpenArray block (Thermo Fisher
132 Scientific). This nanoscale diagnostic PCR workflow is optimized for use [at Animal Health](#)
133 [Diagnostic Center \(AHDC\) in our laboratory](#) and has been described previously.⁹ Custom
134 nanoscale PCR amplification plates arranged in an 18x3 gene expression format were ordered with
135 the *M. cynos tuf* oligonucleotides listed above, in addition to oligonucleotides for other pathogen
136 and control tests.⁹ The forward and reverse primers were also included in a pre-
137 amplification/reverse-transcription pool in combination with the other respiratory panel primers at
138 9 μM (Integrated DNA Technologies, Inc., Coralville, IA). This pool was combined with random
139 primers at 300 nM final concentration (New England Biolabs, Ipswich, MA), TaqMan™ Fast
140 Virus 1-Step Master Mix (Applied Biosystems), and 7 μL template in a 10 uL total volume. Pre-
141 amplification cycling conditions were 50°C for 15 minutes, 95°C for 1 minute, 20 cycles of (95°C
142 for 15 seconds and then 60°C for 2 minutes), followed by 99.9°C for 5 minutes in a conventional
143 PCR machine. This pre-amplification product was then diluted in 40 μL of 1X TE buffer. The pre-
144 amplified and diluted samples are then combined with an equal volume of TaqMan™
145 OpenArray™ Real-Time PCR Master Mix (Applied Biosystems), from which 33 nL is transferred
146 to the nanoscale PCR amplification plate by the AccuFill system (Applied Biosystems). Note that
147 the OA platform uses a different algorithm for calculating results called “C_RT” (relative cycle
148 threshold). Values are calculated based on the shape of individual amplification curves rather than
149 the cycle of crossing a particular fluorescence threshold. For simplicity, herein we refer to C_RT as
150 Ct (cycle threshold), which is the value reported by the 7500 platform.

151 Routine controls were run in parallel to diagnostic samples, including a negative extraction
152 control, negative amplification control, XIC (MS2 RNA phage), and PAC at 10³ copies/μL.

153 **Analytic Performance**

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

159 The assay limit of detection (LOD) was calculated based on the mean of the Ct value at the
160 lowest copy number where 100% of the replicates are positive plus 2 standard deviations. This
161 was based on 12 replicates on the 7500 platform and 36 replicates on the OA platform. Platform-
162 specific interpretation ranges were established according to internal guidelines. For the 7500
163 platform, $C_t > LOD$ = “low positive”, C_t between LOD and the value of (LOD divided by 1.5) =
164 “moderate positive”, $C_t < (LOD \text{ divided by } 1.5)$ = “high positive”. For the OA platform, $C_t > LOD$
165 = “low positive”, C_t between LOD and the value of (LOD divided by 2) = “moderate positive”,
166 $C_t < (LOD \text{ divided by } 2)$ = “high positive”.

167 **Diagnostic performance**

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

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

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

186 Results of canine respiratory specimens tested with the *M. cynos tuf* assay individually or
187 as one of the canine respiratory panel assays were obtained from our laboratory information
188 management system, from the time the *tuf* assay went into production to the present (October 7,
189 2016 to February 14, 2019). This data was analyzed with Microsoft Access to quantify and
190 characterize co-infection rates and pathogen prevalence. GraphPad Prism (GraphPad Software,
191 San Diego, CA) was used to generate plots of the data.

192 **Whole Genome Sequencing**

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

Commento [PM8]: not clear to me why we add here also mucosicanis

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

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

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

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

214 **Statistical analyses**

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

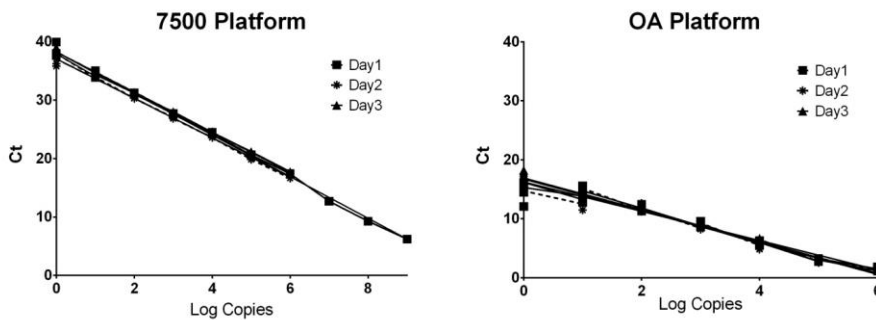
217

218 **Results**

219 **Assay design and performance**

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

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



229
 230 **Figure 1. *Mycoplasma cynos* standard curve dilution series.** The *Mycoplasma cynos* positive
 231 amplification control was serially diluted and then amplified with the *tuf* assay on the ABI 7500
 232 and OpenArray platforms. Dilutions of 0.1 and 0.01 copies per uL were consistently not detected
 233 (not shown).

234
 235 **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%

236

237 **Repeatability**

238 **Intra-assay variation:** On the 7500 platform (Table 2), all SDs were <1.0, and none of
 239 the CVs exceeded the maximum desired value of 3% (untransformed) or 50% (linearized using
 240 2^{-Ct}).

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

Day	Copies per μ l	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

242

243 On the OA platform (Table 3), all SDs were <1.0. There were a number of failed CVs
 244 (>3%) using the untransformed values, but none of the CVs failed by the more appropriate
 245 linearized method (<50%).

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

Day	Copies per μ l	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

247

248 **Inter-assay variation:** Variation between runs on 3 different days is shown in Table 4.

249 All SDs were <1.0, and none of the CVs exceeded the maximum desired value of 3%
 250 (untransformed) or 50% (linearized using 2^{-Ct}). The slopes of the standard curves were not

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

253 **Table 4.** Inter-assay variation of Ct values.

Platform	Template concentration (per μ l)	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

254

255 Analytical sensitivity and specificity

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

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

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

271 beta-coronavirus, *Bordetella bronchiseptica*, canine distemper virus, canine parainfluenza virus,
272 canine pneumovirus, and influenza A virus.

273 **Diagnostic sensitivity and specificity**

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

282 **Table 5.** Diagnostic specificity assessment on canine respiratory samples.

	ITS PCR +	ITS PCR -
<i>tuf</i> PCR +	33	2
<i>tuf</i> PCR -	1	35

283

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

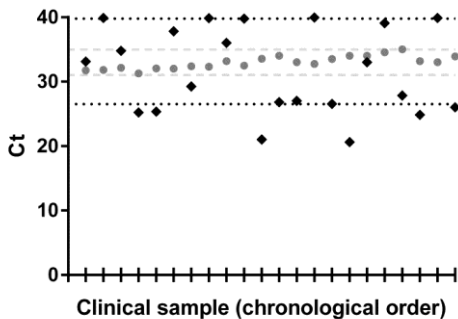
Commentato [PM9]: do we need this sentence?

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

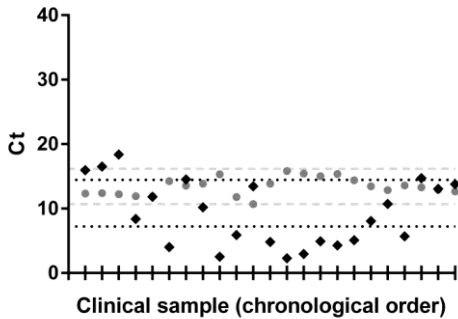
296 **Inhibition monitoring**

297 Continuous monitoring of the XIC did not reveal any systematic indications of inhibition
298 in respiratory matrix, inclusive of tissues. Results from 22 clinical specimens tested on 15
299 independent ABI 7500 runs are shown in Figure 2. In these specimens, *M. cynos* Ct values ranged
300 from 20.609 – 39.958 while the XIC Ct values ranged from 31.283 – 35.000. The OA assay
301 detected a range of *M. cynos* Ct values (2.282 – 18.369) from 23 clinical specimens obtained from
302 15 independent runs (Figure 3). The XIC was detected in the same specimens and with
303 corresponding Ct values that remained within a span of 2 standard deviations (Ct range 10.694 –
304 15.814; 2 standard deviation range 10.682 – 16.164; Figure 3).



305
306 **Figure 2. Detection of *Mycoplasma cynos* target and exogenous internal control in clinical**
307 **specimens on the ABI 7500 platform. *Mycoplasma cynos* was detected over a range of high,**
308 **moderate, and low positive values (black diamonds; moderate positive range bracketed by black**
309 **dotted line) in 22 clinical specimens. The MS2 exogenous internal control was detected in the**

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



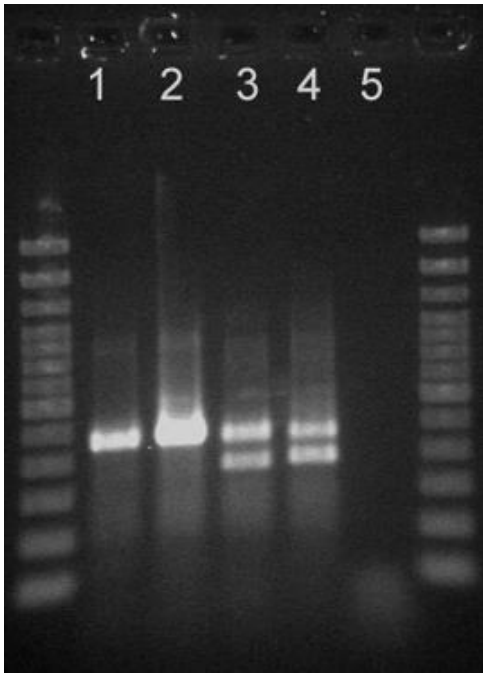
312
313 **Figure 3. Detection of *Mycoplasma cynos* target and exogenous internal control in clinical**

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

318 **Sequence confirmation**

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

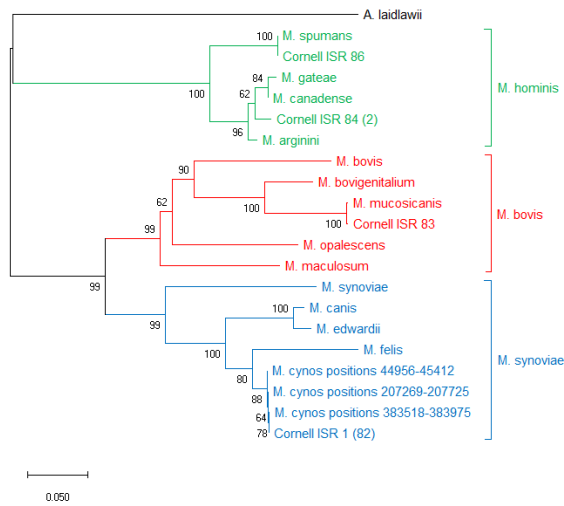
328 ISR sequence, and the remaining 8 had poor sequence quality that prevented definitive
329 identification of the *Mycoplasma* species present in the specimen. The *M. cynos* 1642 strain
330 genome sequence (HF559394) includes 3 discrete ISR, 2 of which are 257 bases in length and
331 share 100% identity; the third ISR contains a 1 base insertion. Of the 82 sequences that matched
332 *M. cynos* ISR sequence, 78 of these were 100% identical to the *M. cynos* 1642 strain ISR of 257
333 bases (HF559394), 2 were 100% identical to the *M. cynos* 1642 strain ISR of 258 bases
334 (HF559394), and 2 were 100% identical to the *M. cynos* 1642 strain ISR of 257 bases (HF559394)
335 except each had an ambiguous (N) base that could not be manually resolved. The PCR sequence
336 that shared high identity with *M. mucosicanis* strain 1642 sequence (FM180556.1) was identical
337 at 307 of 309 ISR positions. Sequence analysis of the 3 smaller ISR PCR products identified the
338 presence of *M. spumans* (100% identical to AF538684) and *M. canadense* (the most significant
339 BLAST result was to AP014631 with 163 out of 169 nucleotide identities; these 2 PCR product
340 sequences were identical to each other); these represent specimens with co-infection of at least 2
341 *Mycoplasma* species.



342
343 **Figure 4. Gel electrophoresis of *Mycoplasma* ISR PCR products.** Most *tuf* PCR positive-
344 clinical specimens yielded a single *Mycoplasma* ISR band at the 457 base pair size expected
345 (lanes 1 and 2) for the *M. cynos* genome. A subset of *tuf* PCR positive-clinical specimens yielded
346 2 bands; one at the size expected for the *M. cynos* genome and a second smaller band (lanes 3
347 and 4). Lane 5 contained the negative amplification control. The outermost lanes were loaded
348 with 100 bp DNA ladder.

349
350 Phylogenetic analysis of the ISR sequences obtained in this work and from reference
351 *Mycoplasma* strain sequences was performed. *M. cynos* ISR sequences from strain C142 and from
352 sequence confirmation of *tuf*-PCR positive specimens (all 82 represented by sequence “Cornell
353 ISR 1”) were placed within the *M. synoviae* cluster (Figure 5), consistent with previous reports of

354 *Mycoplasma* phylogenetic analysis.³ Similarly, the ISR sequences from *M. mucosicanis* strain
 355 1642 and PCR sequencing herein were found in the *M. bovis* cluster. The remaining ISR sequences
 356 determined in this study, representing *M. spumans* and *M. canadense*, were present within the *M.*
 357 *hominis* cluster.



358
 359 **Figure 5. Phylogenetic tree of *Mycoplasma* intergenic spacer region sequences.** Evolutionary
 360 analyses were conducted in MEGA X and the tree was inferred using the Neighbor-Joining
 361 method.^{12,19} The bootstrap test with 1,000 replicates was used to determine the percentage of
 362 replicate trees in which the associated taxa clustered together and these values are shown next to
 363 the branches.⁷ Phylogenetic clustering of *Mycoplasma* strains are distinguished by color;
 364 sequences determined in this work are denoted by “Cornell ISR” prefix. Sequences identified
 365 multiple times are indicated by (#) following the name, with ‘#’ reflecting the number of times
 366 that sequence was obtained. *Mycoplasma* sequences obtained from GenBank include: *A.*
 367 *laidlawii* (CP000896), *M. arginini* (AY737013), *M. bovisgenitalium* (AP017902), *M. bovis*

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

372 **Whole genome sequencing**

373 Our assembled *M. cynos* type strain Rosendal/H 831 (ATCC 27544) genome was 853,159
374 bp with an N50 of 5,333 and a GC content of 26.35% (NCBI accession in process). Our *M.*
375 *mucosicanis* type strain 1642 (ATCC BAA-1895) assembly consisted of 870,218 bp with an N50
376 of 64,609 and GC content of 29.55% (NCBI accession in process). Average read depths were 15.4
377 for *M. cynos* and 77.6 for *M. mucosicanis*. The *M. cynos* assembly shared 99.8% nucleotide
378 identity (1186/1188 bases) with the C142 *tuf* sequence, while the *M. mucosicanis* shared 83.6%
379 identity (999/1195 bases including 11 gaps). Our *M. cynos* assembly had exact matches to the *tuf*
380 primer and probe sequences. The corresponding regions in our *M. mucosicanis* assembly shared
381 23/27 nucleotide identities with the forward primer, 16/21 with the reverse primer, and 16/19 with
382 the probe. Over the 72 bp region spanning the forward and reverse primers, our *M. cynos* assembly
383 was identical to the C142 genome and our *M. mucosicanis* assembly had 13 variable sites.

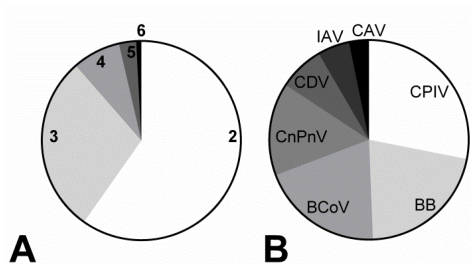
384 The ABI 7500 *tuf* gene assay was performed on the DNA extracted from the *M. cynos* and
385 *M. mucosicanis* type strains obtained from ATCC and *M. cynos* was positive while *M. mucosicanis*
386 was not detected.

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

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

391 *M. cynos* co-infections. The canine respiratory panel includes tests for beta-coronavirus,
 392 *Bordetella bronchiseptica*, canine adenovirus, canine distemper virus, canine parainfluenza virus,
 393 canine pneumovirus, and influenza A virus in addition to *M. cynos*. Of 1,368 canine respiratory
 394 panel tests, 358 (26.17%) results identified co-infections that included *M. cynos*. These co-
 395 infections consisted of *M. cynos* plus another 1 (214 specimens, 59.78%), 2 (103 specimens,
 396 28.77%), 3 (28 specimens, 7.82%), 4 (10 specimens, 2.79%), or 5 (3 specimens, 0.84%
 397 pathogens (Figure 6A). The prevalence of pathogens present simultaneously with *M. cynos* in
 398 specimens was assessed, and canine parainfluenza virus was found most frequently (157
 399 specimens, 28.09%), followed by *Bordetella bronchiseptica* (119 specimens, 21.29%), and beta-
 400 coronavirus (111 specimens, 19.86%). Also present less frequently in *M. cynos* co-infections were
 401 canine pneumovirus (84, 15.03%), canine distemper virus (41, 7.33%), influenza A virus (29,
 402 5.19%), and canine adenovirus (18, 3.22%) (Figure 6B).

403



404

405 **Figure 6. Number and prevalence of pathogens present in *M. cynos* co-infections. Panel A)**

406 Instances of 358 *M. cynos* co-infections since the *tuf* assay went into production consisted of 2
 407 (59.78%, white), 3 (28.77%, light gray), 4 (7.82%, gray), 5 (2.79%, dark gray), or 6 (0.84%,
 408 black) pathogens. Panel B) Co-infections included *M. cynos* and one or more of the following

409 pathogens: canine parainfluenza virus (CPIV), 28.09%), *Bordetella bronchiseptica* (BB,

410 21.29%), beta-coronavirus (BCoV, 19.86%), canine pneumovirus (CnPnV, 15.03%), or canine
411 distemper (CDV, 7.33%), influenza A (IAV, 5.19%), or canine adenovirus (CAV, 3.22%).

412 **Discussion**

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

422 To design a novel assay for *M. cynos* with high specificity, the gene encoding elongation
423 factor Tu (*tuf*) was targeted because it contains *Mycoplasma* species-specific variable
424 regions.^{14,20,22} The *M. cynos tuf* assay performed well on the ABI 7500 platform, even at very high
425 template concentrations of up to 10⁹ copies per microliter. On the OA platform, reaction
426 efficiencies were high compared to typical benchmarks for the 7500 platform due to the fact that
427 samples were pre-amplified and visualized using a white LED excitation source. Otherwise, the
428 *tuf* PCR assay exhibited excellent performance and specificity on the OA platform; this attribute
429 enables high-throughput testing and facilitates co-infection monitoring. The sequence
430 confirmation data and phylogenetic analysis provide robust support for the specificity of the *tuf*
431 PCR assay. The discrepant results identified between ISR sequencing and the new *tuf* assay,
432 including the identification of *M. mucosicanis* in a *tuf* PCR positive-specimen, suggest instances

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

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

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

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

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

464

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477

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