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

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**Abstract.** *Mycoplasma cynos* is recognized as an emerging causative pathogen of canine infectious respiratory disease (CIRD) worldwide. We developed a new open-source real-time PCR (rtPCR) assay for *M. cynos* that performs well under standard rtPCR conditions. Primers and probes were designed to target the *M. cynos tuf* gene. Reaction efficiencies for the *M. cynos tuf* gene assay on 2 platforms were based on amplification of standard curves spanning 8 orders of magnitude: ABI 7500 platform, 94.3–97.9% ($r^2 \geq 0.9935$); QuantStudio OpenArray platform, 119.1–122.5% ($r^2 = 0.9784$). The assay performed very well over a range of template input, from $10^9$ copies to the lower limit of quantification at 4 copies of the *M. cynos* genome on the ABI 7500 platform. Diagnostic performance was estimated by comparison with an in-house legacy assay on clinical specimens as well as testing isolates that were characterized previously by intergenic spacer region (ISR) sequencing. Exclusivity was established by testing 12 other *Mycoplasma* species. To substantiate the high specificity of the *M. cynos tuf* gene assay, sequence confirmation was performed on ISR PCR amplicons obtained from clinical specimens. One ISR amplicon sequence revealed *M. mucosicanis* rather than *M. cynos*. The complete protocol of the newly developed *M. cynos tuf* assay is provided to facilitate assay harmonization.

**Key words:** canine infectious respiratory disease; *Mycoplasma cynos*; *tuf* gene.

## 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 influenza virus, canine parainfluenza virus, canine pneumovirus, and canine respiratory coronavirus.[7,17,18] Previous studies and case reports have established an association between the presence of *Mycoplasma cynos* and canine infectious respiratory disease worldwide.[6,7,12,18,27] It is notable that no association was found between CIRD and 9 other *Mycoplasma* species found in the canine respiratory tract.[5]

At least 17 *Mycoplasma* species have been detected in dogs, although at least half of these are also found in other mammalian hosts.[3,5] *M. cynos* was identified as a new *Mycoplasma* species of dogs in 1973.[20] The type strain, originally known as Rosendal$^T$ and subsequently as H 831$^T$, was isolated from the lung of a dog with pneumonia,[19] and additional *M. cynos* strains were isolated from respiratory and genital tracts of both healthy dogs and dogs with respiratory disease.[20] *M. cynos* has been detected in samples from canine conjunctiva, nasal cavity, oral cavity, lower respiratory tract, genital tract, and urine.[3,13,19,20] Remarkably, *M. cynos* was also the only *Mycoplasma* species isolated from the air of a kennel.[5] Experimental infection of dogs with *M. cynos* can induce pneumonia.[19,21]

Detection tests with high specificity are imperative given that dogs can be infected simultaneously with several *Mycoplasma* species.[3,4] *Mycoplasma* speciation can be accomplished by culture followed by sequencing of the 16S rRNA gene or intergenic spacer region (ISR).[4] However, this approach is not rapid or simple, given that mycoplasmas are fastidious organisms requiring complex media.[3] Although sequences obtained from *Mycoplasma* cultures can result in species identification, the high frequency of mixed infections can obscure the ability to distinguish multiple species of *Mycoplasma*.[3,4] Although the genome sequence of *M. cynos* strain C142 has been published,[26] little sequence information is available for type strain Rosendal/H 831$^T$ or other *Mycoplasma* species likely to be present in the same clinical specimen. There is a

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need for a highly specific *M. cynos* real-time PCR (rtPCR) assay; only one such assay has been published, as part of a recent epidemiologic study on CIRD.[17] Our focus was to provide a comprehensive evaluation of a *M. cynos* assay that met established criteria for assay validation so that it could be adopted easily by other laboratories.

We used an in-house assay to detect the *M. cynos* 16S-23S rRNA intergenic (internal transcribed spacer region [ITS]) sequence 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* minor groove binder (MGB) probe–based assay that would perform well under standard PCR and cycling conditions on both the Applied Biosystems (ABI) 7500 and OA platforms (Thermo Fisher Scientific, Waltham, MA), while providing high specificity on canine respiratory swab and tissue specimens. The OA platform was included in our test validation because it enables detection of respiratory pathogens in a high-throughput manner.[11]

## Materials and methods

### Clinical specimens and reference strains

We used clinical specimens submitted for routine testing to the Cornell Animal Health Diagnostic Center (Ithaca, NY), with the need for *M. cynos* PCR testing being determined by the referring veterinarian. The starting amount of sample used for purification was one nasal swab, pooled nasal swabs, or 100–200 mg of respiratory tissue. Tissue samples were minced and combined with 1 mL of Dulbecco modified eagle medium (DMEM; Thermo Fisher Scientific) and then homogenized (TissueLyser; Qiagen, Germantown, MD) for 2 min at 18 Hz. Following centrifugation at $825 \times g$ for 3 min, the supernatant was used for nucleic acid extraction.

For estimation of diagnostic specificity and sensitivity, 71 canine respiratory specimens submitted for routine canine respiratory panel testing in 2016 were examined with the in-house assay that targeted a different region of the *M. cynos* genome (ITS) and the new *M. cynos tuf* assay. Additionally, 67 bacterial isolates previously characterized to species level by culture and ISR sequencing were tested with the new *M. cynos tuf* assay. This panel of *Mycoplasma* bacterial isolates was comprised of *M. canis, M. cynos, M. edwardii, M. felis, M. maculosum*, and *M. spumans*.

Thirteen *Mycoplasma* reference strains were tested to evaluate the analytical specificity of the assay, including *M. alkalescens* ATCC 29103$^T$, *M. arginini* ATCC 23838$^T$, *M. bovigenitalium* D0108 721, *M. bovigenitalium* ATCC 19852$^T$, *M. bovirhinis* ATCC 27748$^T$, *M. bovis* D0200 473, *M. bovis* ATCC 25523$^T$, *M. californicum* ST-6 (ATCC 33461$^T$), *M. canadense* ATCC 29418$^T$, *M. canis* ATCC 19525$^T$, *M. cynos* ATCC 27544$^T$, *M. edwardii* ATCC 23462$^T$, and *M. 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; Thermo Fisher Scientific; Supplementary Table 1). 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 *B. bronchiseptica, M. cynos*, canine adenovirus 1 (CAdV; *Canine mastadenovirus A*), canine distemper virus (CDV; *Canine morbillivirus*), canine parainfluenza virus 5 (PIV5; *Mammalian orthorubulavirus 5*), canine pneumovirus (CPnV; *Murine orthopneumovirus*), canine respiratory coronavirus (CRCoV; *Betacoronavirus 1*), influenza A virus (IAV; *Influenza A virus*), and MS2 phage RNA.

### 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; Thermo Fisher Scientific). The purified nucleic acid was eluted in 90 µL of buffer.

### Exogenous internal control

We used the MS2 phage as an exogenous internal control (XIC) for the complete extraction process by combining it with the MagMAX lysis buffer followed by quantification in sample eluates.[8] An acceptable range was established based on 2 standard deviations (SDs) from the average cycle threshold (Ct) level in respiratory matrix, inclusive of tissues. PCR inhibition as a result of the sample matrix is indicated by the MS2 XIC signal being out of the acceptable range or undetected.

### Assay design

Primer Express software (Thermo Fisher Scientific) was used to design primers (forward: 5'-TCTTCGTATTTAGCATCA CCTTCAAGT-3'; reverse: 5'-TGATGGAGATAATGCGC-CAAT-3'), and an MGB probe (5'-FAM-CTTTTAAAGCT-GAACCACG-MGB-3') using an alignment of all available *Mycoplasma tuf* gene sequences including *M. cynos* strains H 831$^T$ and C142 (genome NC_019949).[26] The amplicon length is 72 bp.

For the ABI 7500 Fast real-time PCR platform (Thermo Fisher Scientific), forward and reverse primers at 400 nM final concentration and probe at 120 nM final concentration were added to the master mix (ABI Path-ID multiplex one-step RT-PCR; Thermo Fisher Scientific) in a 21-µL mix and combined with 4 µL of template. Cycling conditions were 48°C for 10 min, 95°C for 10 min, followed by 40 cycles of

95°C for 15 sec and then 60°C for 45 sec, using the standard cycling mode. Sequence detection software version 1.4 (ABI; Thermo Fisher Scientific) was used with automatic analysis settings. No template controls were included with each assay, and no amplification was detected.

For high-throughput testing, the new *M. cynos tuf* assay was also validated on the QuantStudio 12K Flex system with an OpenArray (OA) block for nanoscale PCR (ABI; Thermo Fisher Scientific). This nanoscale PCR workflow has been described previously.[11] Custom nanoscale PCR amplification plates arranged in an 18×3 gene expression format were ordered with the *M. cynos tuf* oligonucleotides listed above, in addition to oligonucleotides for other pathogen and control tests.[11] 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, 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 (ABI; Thermo Fisher Scientific), and 7 μL of template in a 10-μL total volume. Pre-amplification cycling conditions were 50°C for 15 min, 95°C for 1 min, 20 cycles of 95°C for 15 sec and then 60°C for 2 min, followed by 99.9°C for 5 min in a conventional thermal cycler (ABI GeneAmp; Thermo Fisher Scientific). This pre-amplification product was then diluted in 40 μL of 1× TE buffer. The pre-amplified and diluted samples were then combined with an equal volume of TaqMan OA real-time PCR master mix (ABI; Thermo Fisher Scientific), from which 33 nL was transferred to the nanoscale PCR amplification plate by the AccuFill system (ABI; Thermo Fisher Scientific). Note that the OA platform uses an 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.

When a sample was assayed on the OA platform, it was simultaneously tested for these canine and equine respiratory pathogens: *B. bronchiseptica, M. cynos, M. felis, Streptococcus equi, S. zooepidemicus*, CAdV, CDV, PIV5, CPnV, CRCoV, equine adenoviruses 1 and 2 (EAdV-1, –2; *Equine mastadenovirus A* and *B*), equine herpesvirus 1 and 4 (EHV-1, –4; *Equid alphaherpesvirus 1* and *4*), equine rhinitis viruses A and B (ERAV, *Equine rhinitis A virus*; ERBV, *Erbovirus A*), and IAV. Routine controls were run in parallel to diagnostic samples, including a negative extraction control, negative amplification control, XIC (MS2 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 3 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. Inter-assay 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 wherein 100% of the replicates were positive plus 2 SDs. This was based on 12 replicates on the 7500 platform and 36 replicates on the OA platform.

Analyses and description of the *M. cynos tuf* assay performance comply with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[1]

## Diagnostic performance

A proprietary, in-house legacy *M. cynos* ITS real-time PCR assay performed on the 7500 platform, which targeted a region of the *M. cynos* genome distinct from the *tuf* gene, was chosen as the comparison method. Results of the ITS assay were compared to results of the novel *tuf* assay using 71 canine respiratory specimens submitted for routine canine respiratory panel testing in 2016. Respiratory disease signs listed on sample accession forms by the referring veterinarian were recorded.

Sequencing of the 16S-23S rRNA ISR was utilized to confirm that the *tuf* PCR-positive specimens tested in 2017 and 2018 were *M. cynos* positive. The PCR primers used to amplify the ISR of *Mycoplasma* species were described previously.[10] PCR products were visualized by gel electrophoresis. Initially, 5 μL of PCR product were loaded on 1.5% agarose gel and electrophoresed at 100 V to observe the size and 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 (QIAquick gel extraction kit; Qiagen). The concentration of purified PCR products was determined (Qubit 3.0 fluorometer; Thermo Fisher Scientific). Direct sequencing of amplicons with the PCR primers was performed by the Institute of Biotechnology at Cornell University (Ithaca, NY). Chromatograms were visualized and edited manually (Geneious 8.1.7; https://www.geneious.com). The ISR 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, and the tree was inferred using the neighbor-joining method.[14,22] The bootstrap test with 1,000 replicates was used to determine the percentage of replicate trees in which the associated taxa clustered.[9] ISR sequences obtained from GenBank included *Acholeplasma laidlawii* (CP000896) and the

following *Mycoplasma* species: *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).

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 article submission date (October 7, 2016 to February 14, 2019). These data were analyzed with Access (Microsoft, Redmond, WA) to quantify and characterize coinfection rates and pathogen prevalence. GraphPad Prism (GraphPad Software, San Diego, CA) was used to generate plots of the data.

Sequences determined in our study are associated with NCBI BioProject PRJNA525414. PCR product ISR sequence GenBank accessions are MK675548–MK675633.

### Statistical analyses

GraphPad Prism was used to analyze standard curve data by linear regression, CV, and also for 2×2 test agreement analyses.

## Results

### Assay design and performance

Standard curves with 8 orders of magnitude were run on 3 different days and analyzed by linear regression using GraphPad Prism (Supplementary Table 2, Supplementary Fig. 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 of 94.3–97.9%. For the OA platform, amplification was also linear ($p = 0.22$ for deviation from linearity; Supplementary Fig. 1); efficiency was 119.1–122.5%. Dilutions of 0.1 and 0.01 copies/µL were consistently not detected on either platform.

### Repeatability

In intra-assay variation, for the 7500 platform (Supplementary Table 3), all SDs were < 1.0, and none of the CVs exceeded the maximum desired value of 3% (untransformed) or 50% (linearized using $2^{-Ct}$). For the OA platform (Supplementary Table 4), 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%).

To assess inter-assay variation, runs were performed on 3 different days, all SDs were < 1.0, and none of the CVs exceeded the maximum desired value of 3% (untransformed) or 50% (linearized using $2^{-Ct}$; Supplementary Table 5). The slopes of the standard curves were not significantly different on the 3 different days for each platform ($p = 0.46$ for 7500, $p = 0.83$ for OA).

### Analytical sensitivity and specificity

The analytical limit of quantification was < 10 bacterial genome copy equivalents 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 through exclusivity, the *Mycoplasma* reference strains listed previously were tested (see Materials and methods) and were not detected with the *M. cynos tuf* assay.

The positive amplification control tested individually on the OA platform was uniquely detected by the new *M. cynos tuf* assay, and no other targets from a panel of canine and equine respiratory assays were detected (see Materials and methods for the full list of pathogens). However, many of the clinical samples that were negative by the new *tuf* assay were positive for other pathogens including *B. bronchiseptica*, CDV, PIV-5, CPnV, CRCoV, and IAV.

### Diagnostic sensitivity and specificity estimation

No rtPCR assay has been recognized as a gold standard for *M. cynos* detection to date. In lieu of a gold standard, we considered an in-house assay that targeted a different region of the *M. cynos* genome (ITS) to be the reference test to estimate diagnostic sensitivity and specificity. Seventy-one canine respiratory specimens submitted for routine testing for canine respiratory disease were tested directly (without bacterial enrichment) with the ITS assay and the new *tuf* assay. The new *tuf* assay demonstrated 95.8% agreement with the ITS assay (Table 1). Thirty-three specimens were positive by both assays, and 35 specimens were negative by both assays. The one false-negative was most likely the result of another *Mycoplasma* species being detected by the ITS assay; ISR sequencing revealed the presence of *M. spumans* in the sample. The other 2 discrepant samples were detected by the OA but not the 7500 platform. The kappa coefficient for detection of canine respiratory specimens between the *M. cynos tuf* and ITS assays was 0.915 (95% confidence interval: 0.822–1.000).

The new *tuf* assay was further scrutinized using 67 isolates that were characterized by *Mycoplasma* 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

**Table 1.** Comparison of *Mycoplasma cynos* quantitative PCR assays using 71 canine respiratory samples.

| | ITS PCR positive | ITS PCR negative |
|---|---|---|
| *tuf* PCR positive | 33 | 2 |
| *tuf* PCR negative | 1 | 35 |

ITS = in-house *M. cynos* 16S-23S rRNA intergenic sequence qPCR assay; *tuf* = newly developed *M. cynos* qPCR assay.

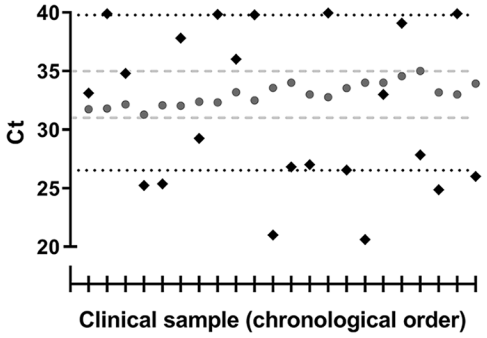


**Figure 1.** Detection of *Mycoplasma cynos* target and exogenous internal control in clinical specimens on the ABI 7500 platform (Thermo Fisher Scientific). The cycle threshold (Ct) for each specimen is plotted on the y-axis; no Ct values were < 20. *M. cynos* was detected over a range of high-, moderate-, and low-positive values (black diamonds; moderate-positive range bracketed by black dotted line) in 22 clinical specimens. Five low-positive values were detected and are plotted above the black dotted line. The MS2 exogenous internal control was detected in the same specimens, and MS2 Ct values remained within a span of 2 standard deviations (gray dots; gray dashed line = 2 SDs).

had *M. canis* and *M. edwardii* as the 2 top BLAST hits, but included *M. cynos* in the list of significant hits. Strains of *M. canis* (ATCC 19525$^T$) and *M. edwardii* (ATCC 23462$^T$) were tested and both yielded negative results with the *tuf* assay. The sixth discrepant isolate was typed 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, for 22 clinical specimens tested on 15 independent 7500 platform runs (Fig. 1). In these specimens, *M. cynos* Ct values were 20.6–40.0; the XIC Ct values were 31.3–35.0. The OA assay detected a range of *M. cynos* Ct values (2.3–18.4) from 23 clinical specimens obtained from 15 independent runs (Fig. 2). The XIC was detected in the same specimens and with corresponding Ct values that remained within a span of 2 SDs (Ct range: 10.7–15.8; 2 SD range: 10.7–16.1; Fig. 2).

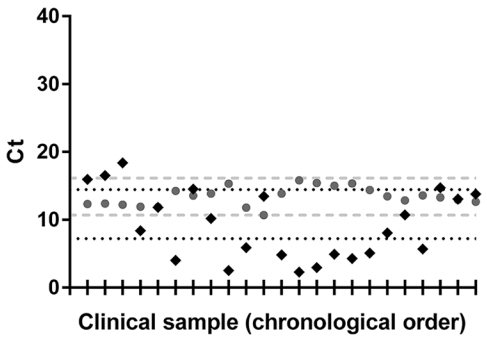


**Figure 2.** Detection of *Mycoplasma cynos* target and exogenous internal control (XIC) in clinical specimens on the QuantStudio OpenArray platform (Thermo Fisher Scientific). The cycle threshold (Ct) for each specimen is plotted on the y-axis. A range of high, moderate, and low *M. cynos* positive values was detected in 23 clinical specimens (black diamonds; moderate-positive range bracketed by black dotted line). The MS2 XIC was detected in the same specimens (gray dots; gray dashed line = 2 SDs).

## Sequence confirmation

To confirm that *tuf* PCR-positive specimens contained *M. cynos*, the ISR was amplified and sequenced. Amplification of the ISR was performed on 107 *tuf* PCR-positive specimens tested in 2017 and 2018, subsequent to the initial set of 71 specimens used to estimate diagnostic performance in 2016. Visualization of the ISR PCR products revealed that 89 *tuf* PCR-positive specimens yielded a single ISR band at the 457-bp 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 (Fig. 3). 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, 1 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 that each had an ambiguous (N) base that could not be resolved manually. 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

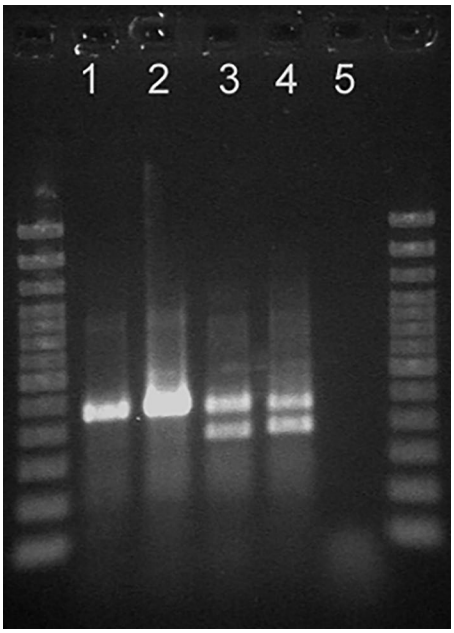


**Figure 3.** Gel electrophoresis of *Mycoplasma* intergenic spacer region (ISR) PCR products. Most *tuf* PCR-positive clinical specimens yielded a single *Mycoplasma* ISR band at the 457-bp size expected (lanes 1 and 2) for the *M. cynos* genome. A subset of *tuf* PCR-positive clinical specimens yielded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

of 169 nucleotide identities; these 2 PCR product sequences were identical); these represent specimens with coinfection of at least 2 *Mycoplasma* species.

To visualize the evolutionary relationship of the ISR sequences obtained from clinical specimens in the context of previously characterized *Mycoplasma* species, phylogenetic analysis was performed.[4,24] Phylogeny of *Mycoplasma* species has been established based on 16S rRNA sequences, and phylogenetic clusters are named after a representative genus or species (https://www.patricbrc.org/view/Taxonomy/2093). *M. cynos* ISR sequences were placed within the *M. synoviae* cluster, including those obtained from sequence confirmation of *tuf* PCR-positive specimens (all 82 represented by sequence "Cornell ISR 1"), the *M. cynos* strain Rosendal/H 831$^T$ whole genome sequence, and strain C142 (Fig. 4). *M. mucosicanis* ISR sequences from PCR and *M. mucosicanis* 1642$^T$ GenBank accessions (whole genome sequence SMDN00000000 and intergenic spacer sequence FM180556) were all located in the *M. bovis* cluster. The remaining ISR sequences determined in our study, representing *M. spumans* and *M. canadense*, were present within the *M. hominis* cluster.

To further explore whether the *M. cynos tuf* assay was likely to cross-react with *M. mucosicanis* DNA, the whole genome sequences of *M. cynos* strain Rosendal/H 831$^T$ (GenBank accession SMDO00000000) and *M. mucosicanis* strain 1642$^T$ (GenBank accession SMDN00000000) were determined by our laboratory. The sequences of the *tuf* gene, primers, and probe were compared between *M. cynos* Rosendal/H 831$^T$, *M. cynos* strain C142, and *M. mucosicanis* 1642$^T$ genome sequences using the BLASTN tool.[2] The *M. cynos* Rosendal/H 831$^T$ sequence shared 99.8% nucleotide identity (1,186 of 1,188 bases) with the C142 *tuf* sequence; *M. mucosicanis* shared 83.6% identity (999 of 1,195 bases, including 11 gaps). The *M. cynos* Rosendal/H 831$^T$ sequence had exact matches to the *tuf* primer and probe sequences. The corresponding regions in the *M. mucosicanis* 1642$^T$ sequence shared 23 of 27 nucleotide identities with the forward primer, 16 of 21 with the reverse primer, and 16 of 19 with the probe. Over the 72-bp region spanning the forward and reverse primers, the *M. cynos* Rosendal/H 831$^T$ sequence was identical to the C142 genome, and the *M. mucosicanis* 1642$^T$ sequence had 13 variable sites.

### *M. cynos* prevalence and coinfections

We have performed 1,982 *M. cynos tuf* tests on canine respiratory specimens since initiating use of the new assay in late 2016, and of those, 634 (32%) were positive. In the same time frame, canine respiratory panel results were analyzed to investigate *M. cynos* coinfections. The canine respiratory panel includes tests for *B. bronchiseptica*, CAdV, CDV, PIV-5, CPnV, CRCoV, and IAV, in addition to *M. cynos*. Of 1,368 canine respiratory panel tests, 358 (26.2%) results identified coinfections that included *M. cynos*. These coinfections consisted of *M. cynos* plus another 1 (214 specimens, 59.8%), 2 (103 specimens, 28.87%), 3 (28 specimens, 7.8%), 4 (10 specimens, 2.8%), or 5 (3 specimens, 0.8%) pathogens (Fig. 5A). The prevalence of pathogens present simultaneously with *M. cynos* in specimens was assessed, and PIV-5 was found most frequently (157 specimens, 28.1%), followed by *B. bronchiseptica* (119 specimens, 21.3%), and CRCoV (111 specimens, 19.9%). Present less frequently in *M. cynos* coinfections were CPV (84, 15.0%), CDV (41, 7.3%), IAV (29, 5.2%), and CAdV (18, 3.2%; Fig. 5B).

## Discussion

To design a novel assay for *M. cynos* with high specificity, we targeted the gene encoding elongation factor Tu (*tuf*) because it contains *Mycoplasma* species–specific variable regions.[16,23,25] The *M. cynos tuf* assay performed well on the ABI 7500 platform, even at very high template concentrations of up to $10^9$ copies/μL. On the OA platform, reaction efficiencies were high compared to typical benchmarks for the 7500 platform given the fact that samples were preamplified and visualized using a white LED excitation source. The *tuf* PCR assay exhibited excellent performance and specificity on the OA platform, an attribute that enables high-throughput testing and facilitates coinfection monitoring. The sequence confirmation data, supported by phylogenetic

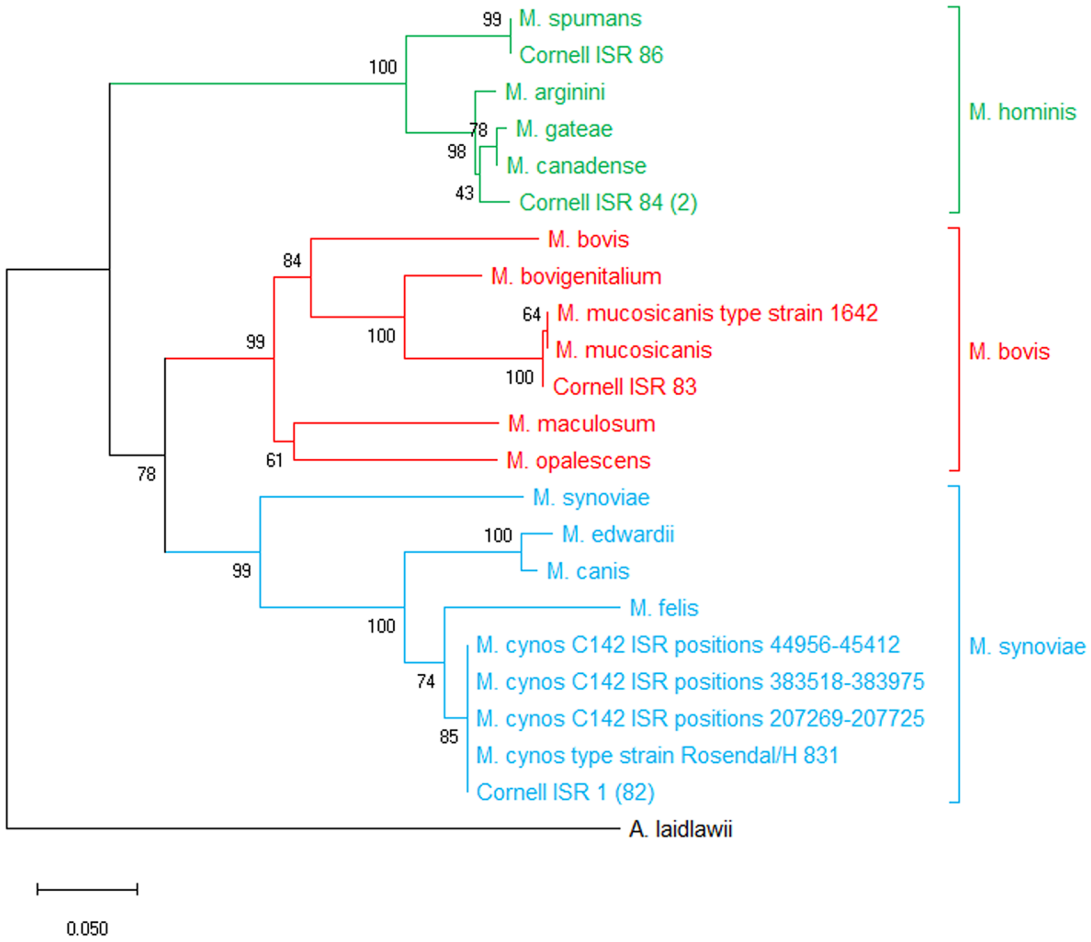


**Figure 4.** Phylogenetic tree of *Mycoplasma* intergenic spacer region sequences. Evolutionary analyses were conducted in MEGA X, and the tree was inferred using the neighbor-joining method.[14,22] The bootstrap test with 1,000 replicates was used to determine the percentage of replicate trees in which the associated taxa clustered, and these values are shown next to the branches.[9] Phylogenetic clustering of *Mycoplasma* strains is distinguished by color; sequences determined in our work by PCR amplicon sequencing 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. Sequences determined by whole genome sequencing of ATCC type strains in our laboratory are indicated by the text "type strain." The ISR sequence of *Acholeplasma laidlawii* was included as an outgroup. Sequences obtained from GenBank include *A. laidlawii* (CP000896) and the following *Mycoplasma* species: *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).

analysis, provided 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 16.8% of *tuf* PCR-positive specimens producing 2 distinct ISR amplicons. Reference strains of the other *Mycoplasma* species present in the sample, identified by ISR sequencing, were tested with the *tuf* assay and did not amplify.

Phylogenetic analysis was performed to view how the ISR sequences obtained from clinical specimens fit into the established evolutionary relationships between *Mycoplasma* species.[4,24] Placement of the 82 *M. cynos* ISR sequences and the *M. cynos* genome sequences in the *M. synoviae* cluster was expected from previous reports of *Mycoplasma* phylogenetic analysis.[4] The identity of the other 4 ISR sequences based on BLAST results was confirmed in the phylogenetic tree: the single *M. mucosicanis* ISR sequence was located with *M. mucosicanis* genome sequences in the *M. bovis* cluster, and the 3 sequences derived from the shorter ISR PCR products were placed in the *M. hominis* cluster.

The high prevalence of *M. cynos*, close to one-third of clinical specimens were positive over 2 y of running the assay routinely, is consistent with previous observations,

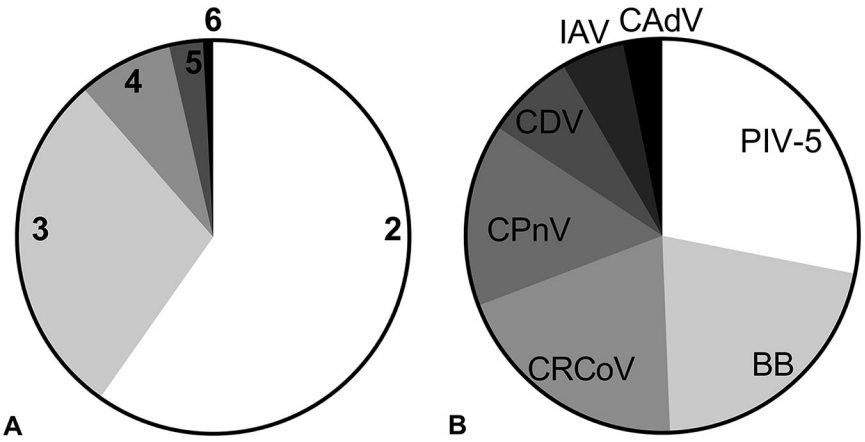


**Figure 5.** Number and prevalence of pathogens present in *Mycoplasma cynos* coinfections. **A.** Since the *tuf* assay went into production, 358 *M. cynos* coinfections have been identified, consisting of 2 (59.8%, white), 3 (28.8%, light gray), 4 (7.8%, gray), 5 (2.8%, dark gray), or 6 (0.8%, black) pathogens. **B.** Coinfections included *M. cynos* and one or more of the following pathogens: canine parainfluenza virus (PIV-5, 28.1%), *Bordetella bronchiseptica* (BB, 21.3%), canine respiratory coronavirus (CRCoV, 19.9%), canine pneumovirus (CPnV, 15.0%), canine distemper virus (CDV, 7.3%), influenza A virus (IAV, 5.2%), or canine adenovirus (CAdV, 3.2%).

which have largely focused on bacterial coinfections. The high proportion of viral coinfecting agents stresses the importance of comprehensive testing for the management of CIRD, particularly in the context of American Veterinary Medical Association 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, coinfection of *M. cynos* with up to 4 other pathogens have been described. Pathogens identified in coinfections with *M. cynos* include *B. bronchiseptica*, CAdV, CDV, and PIV–5.[6,7,15] A review of our canine respiratory panel results since the *M. cynos tuf* assay went into use revealed similar findings. Specifically, *M. cynos* coinfections identified by us were comprised of up to 6 pathogens, including 1 or more of the following (listed in decreasing order of prevalence): PIV-5, *B. bronchiseptica*, CRCoV, CPnV, CDV, IAV, and CAdV.

A study[17] published subsequent to completion of our work described a multiplex assay that targets the *M. cynos* and *M. canis* 16S rRNA genes rather than the *tuf* gene. Three findings were consistent between that publication and our work: 1) the *M. cynos* limit of detection is < 10 genome copies with both assays; 2) *M. cynos* was one of the most commonly detected pathogens (24.5% prevalence in their report and 32% in our study); and 3) the most common coinfection pair in both studies was *M. cynos* and PIV–5. Future work of interest to the veterinary diagnostic community may involve comparisons of these assays on the same sample sets in order to further promote harmonization between laboratories.

The *tuf* assay described herein is intended for the detection 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 diagnostic laboratories. The validation data presented encompass both the 7500 and OA real-time PCR platforms. The *tuf* assay is compatible with the MS2 phage RNA XIC to facilitate PCR inhibition monitoring according to best practice guidelines of the AAVLD Laboratory Technology Committee. Additionally, use of this assay in our laboratory under ISO/IEC:17025 Biological scope was approved by the American Association for Laboratory Accreditation (A2LA, certificate 2880.01), indicating that it is compatible with a quality management system adhering to these requirements. Through publication, we hope to promote assay harmonization in veterinary testing in order to facilitate surveillance and the use of validated assays.

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## Supplementary material

Supplementary material for this article is available online.

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