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ORIGINAL ARTICLE

Development of a real-time PCR assay for detection and differentiation of *Mycoplasma ovipneumoniae* and a novel respiratory-associated *Mycoplasma* species in domestic sheep and goats

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Abstract

A novel respiratory-associated *Mycoplasma* species (*M.* sp. nov.) of unknown clinical significance was recently identified that causes false positive results with multiple published PCR methods reported to specifically detect *Mycoplasma ovipneumonaie*, a well-known respiratory pathogen in small ruminants. This necessitates our objective to develop a real-time PCR (qPCR) assay for improved specificity and sensitivity, and more rapid detection and differentiation of *M. ovipneumoniae* and the *M.* sp. nov. in domestic sheep (DS) and domestic goat (DG) samples, as compared to a conventional PCR and sequencing (cPCR-seq) assay. Primers and probes were designed based on available *M. ovipneumoniae* 16S rRNA gene sequences in the GenBank database, and partial 16S rRNA gene sequences provided by the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) for *M. ovipneumoniae* and *M.* sp. nov. USDA-ARS provided DS ($n = 153$) and DG ($n = 194$) nasal swab nucleic acid that previously tested positive for either *M. ovipneumoniae* ($n = 117$) or *M.* sp. nov. ($n = 138$), or negative for both targets ($n = 92$) by cPCR-seq. A host 18S rRNA gene was included as an internal control to monitor for the failure of nucleic acid extraction and possible PCR inhibition. For samples positive by cPCR-seq, qPCR agreement was 88.0% (103/117; $\kappa = 0.81$) and 89.9% (124/138; $\kappa = 0.84$) for *M. ovipneumoniae* and *M.* sp. nov., respectively; 12 of 255 (4.7%) cPCR-seq positive samples were qPCR positive for both targets. Of samples negative by cPCR for both mycoplasmas, qPCR detected *M. ovipneumoniae* and *M.* sp. nov. in 6.5% (6/92) and 4.3% (4/92), respectively. Samples with discordant results between the cPCR and sequencing assay and the new qPCR were analyzed by target sequencing; successfully sequenced samples had identity

matches that confirmed the qPCR result. The increased target specificity of this qPCR is predicted to increase testing accuracy as compared to other published assays.

KEYWORDS
goats, mycoplasma, *Mycoplasma ovipneumoniae*, real-time PCR, polymerase chain reaction, respiratory system, sheep

1 | INTRODUCTION

Mycoplasma ovipneumoniae, first identified in a sheep flock in Queensland, Australia in 1972 (Carmichael et al., 1972), is now recognized as a globally distributed respiratory pathogen of domestic sheep and goats (Alley et al., 1999; Cheng et al., 2015; Manlove et al., 2019). The organism has also been detected in domestic cattle and a variety of non-domestic animals, including bighorn sheep, Dall sheep, mountain goats, moose, Beira antelope, caribou, mule deer, white-tailed deer, and muskoxen (Besser et al., 2008; Gull et al., 2014; Handeland et al., 2014; Highland et al., 2018; Wolfe et al., 2010; Wolff et al., 2019). Infection in small ruminants can be subclinical or range from mild symptoms including lethargy and coughing to severe bronchopneumonia (Ayling & Nicholas, 2007; Nicholas et al., 2008). *M. ovipneumoniae* infection can interfere with normal ciliary activity, predisposing the host to pulmonary infections by other respiratory pathogens (Ayling & Nicholas, 2007). Although domestic sheep are also susceptible to other respiratory disease-causing mycoplasmas, *M. ovipneumoniae* is the most commonly reported (Ayling & Nicholas, 2007). The United States Department of Agriculture, Animal and Plant Health Inspection Service Veterinary Services' (USDA-APHIS-VS) National Animal Health Monitoring System (NAHMS) Sheep 2011 study reported the presence of *M. ovipneumoniae* in 88.5% (401/453) of sheep operations in 22 major sheep-producing states in the United States (USDA-APHIS, 2015).

Goats are also susceptible to respiratory infections with *Mycoplasma* spp., including *M. ovipneumoniae*. Data regarding the prevalence of *M. ovipneumoniae* in goats in the United States are regionally limited to the Western United States and Alaska, and results vary, with 12% (4/32), 17% (14/83), and 44% (7/16) of premises tested having at least one animal positive for *M. ovipneumoniae* (Heinse et al., 2016; USDA-APHIS, 2020). A USDA-APHIS-VS NAHMS Goat 2019 study examining the prevalence of *M. ovipneumoniae* in domestic goats across the United States is currently underway (USDA-APHIS, 2020). Prevalence studies, like this one and others, will need to rely on fast and accurate *M. ovipneumoniae* testing methods. Considering the potential disease impact in domestic sheep and goat populations (Ayling & Nicholas, 2007; Gonçalves et al., 2010; Rifatbegovic et al., 2011), as well as recently implemented regulations (Alaska Department of Environmental Conservation, 2021) requiring *M. ovipneumoniae* testing on sheep and goats prior to movement into Alaska, accurate and rapid testing methods are of clinical and regional regulatory importance.

Traditional diagnostic procedures for the detection of *M. ovipneumoniae* are culture based (Ruffin et al., 2001), which are still considered by some to be the gold standard for diagnosis (Wang et al., 2020; Weiser et al., 2012). Due to the fastidious nature of *M. ovipneumoniae*, culture-based methods may take weeks and can be labour-intensive (Ackerman et al., 2019; Jennings-Gaines et al., 2016; Ongor et al., 2011; Weiser et al., 2012). The long turnaround time for results renders these methods impractical for routine testing applications. If successfully cultured, the small centreless *M. ovipneumoniae* colonies may appear indistinctive from other bacterial growth, and thereby require further serological or PCR-based confirmatory testing (Ayling & Nicholas, 2007; Ackerman et al., 2019). Finally, culture-based methods for *M. ovipneumoniae* detection have been shown to be less sensitive compared to PCR-based methods (Weiser et al., 2012; Jennings-Gaines et al., 2016).

Several conventional PCR (cPCR) assays have been described for detection of *M. ovipneumoniae* (Highland et al., 2018; Lauerman, 1998; McAuliffe et al., 2003); however, cPCR testing is generally less sensitive (Biassoni & Raso, 2014; Noll et al., 2015) and requires time-consuming post-PCR analysis steps that can delay results. Analysis of genotypic profiles of *M. ovipneumoniae* from caprine and ovine species demonstrates bacterial genetic heterogeneity between species (Maksimovic et al., 2017), which may impact the sensitivity of molecular diagnostics. Recombinase polymerase amplification (RPA) assays have been recently developed (Gupta et al., 2021; Wang et al., 2020) that provide rapid nucleic acid-based detection of *M. ovipneumoniae* in domestic sheep. Due to the isothermal (37–42°C) running conditions and recombinase-facilitated primer hybridization, RPA primer binding has a low tolerance of binding site mismatches that can occur among closely related bacteria, which may impact specificity (Daher et al., 2015; Deng & Gao, 2015). In a thorough study on the influence of sequence mismatches on RPA specificity, Daher et al. (2015) report that RPA primers with >1 mismatch at their 3′ end can reduce or even prevent amplification, which may impact sensitivity. Compared to PCR, RPA is less amenable to multiplexing and has a higher cost of reagents (Lobato et al., 2018).

Real-time quantitative PCR (qPCR) is still considered the "gold standard" technology for nucleic acid-based detection and is reported to offer a 10- to 100-fold increase in sensitivity, in general, compared to cPCR (Biassoni & Raso, 2014; Noll et al., 2015). There is an inherent increase in specificity associated with the probe-based detection that qPCR offers (Noll et al., 2020), which is important when considering multiple related *Mycoplasma* species can be present in the respiratory tract of sheep and goats (Ayling & Nicholas, 2007; Rifatbegovic et al., 2011). In fact, Herndon et al. (2021) recently described the draft genome sequence (Accession #JADDYD000000000) of a novel *Mycoplasma* species (*Mycoplasma* sp. nov.) isolated from a female moose calf, which authors have also detected in domestic sheep and goats

and non-domestic ungulate species. *M.* sp. nov. was first detected by Herndon et al. (2021) while using cPCR and qPCR methods published as being specific for *M. ovipneumoniae* (McAuliffe et al., 2003; Ziegler et al., 2014). McAuliffe et al. (2003) developed a cPCR to improve the speed and accuracy of *M. ovipneumoniae* detection; however, the forward and reverse primers share 55% (11/20 nucleotides) and 80% (16/20 nucleotides) continuous 5′ to 3′ identity, respectively, to *M.* nov. sp. (Accession #JADDYD000000000). Lawrence et al. (2010) developed a qPCR for detection of *M. ovipneumoniae*; however, 100% of the forward and reverse primers, and a majority of the probe (17/22 continuous nucleotides at 5′ end), share a sequence identity match to *M.* sp. nov. (Accession #JADDYD000000000). In a study examining the presence of *M. ovipneumoniae* on U.S. domestic sheep operations to infer associated risk factors for infection and productivity losses, Manlove et al. (2019) used the qPCR assay published by Ziegler et al. (2014), which utilizes the same forward primer and probe described by Lawrence et al. (2010), in combination with the same reverse primer from McAuliffe et al. (2003). Authors report "false positive reactions associated with the presence of an unidentified Mycoplasma-like organism", a probable reference to the then undescribed *M.* sp. nov. To combat the issue, Manlove et al. (2019) re-designed the forward primer by adding eight nucleotides to the 3′ end, yet it still shares a high sequence identity match (26/28 continuous nucleotides at 5′ end) to *M.* sp. nov. (Accession #JADDYD000000000). Highland et al. (2018) describe in detail a 40-cycle partial 16S rRNA cPCR using the McAuliffe et al. (2003) primers followed by Sanger sequencing of amplicon visible by gel electrophoresis and GenBank alignment (cPCR-seq) to differentiate amplified *M. ovipneumoniae* and *M.* sp. nov.

The described, previously published, methods for *M. ovipneumoniae* detection are either labour-intensive, potentially insensitive, and/or non-specific. This necessitates our objective to develop a real-time PCR (qPCR) assay for improved specificity and sensitivity, and more rapid detection and differentiation of *M. ovipneumoniae* and the *M.* sp. nov. in domestic sheep (DS) and domestic goat (DG) samples, as compared to a conventional PCR and sequencing (cPCR-seq) assay.

2 | MATERIALS AND METHODS

2.1 | Primers and probes

Real-time primers and probes were designed based on evaluations of available *M. ovipneumoniae* 16S rRNA gene sequences in the GenBank database (accessed 1/2/2020), and partial 16S rRNA gene sequences provided by the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) for *M. ovipneumoniae* and *M.* sp. nov. Sequences were aligned using ClustalX version 2.1 (http://www.clustal.org/clustal2/), and resulting alignments were evaluated for optimum primer and probe design sites in BioEdit version 7.1.9.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Candidate sites with the greatest number of matched sequences to the mycoplasma target regions were chosen for further analyses (Table 1). Although two different *M.* sp. nov. genotypes/sequence types were identified, type A (*M*novA) and type B (*M*novB), our goal was to differentiate *M.* sp. nov. from *M. ovipneumoniae*. Since *M*novA and *M*novB differentiation was not a goal, probes for both of these targets utilized the same reporter dye (VIC). Because of the high sequence similarity between *M. ovipneumoniae* and *M.* sp. nov. binding sites, and relatively low GC content in the probe-designing region, minor groove binder (MGB) probes (Thermo Fisher Scientific, Waltham, MA) that are designed to provide increased specificity were utilized for *M*novA and *M*novB targets. A multi-species mammalian 18S ribosomal RNA (rRNA) gene, using Cy5 reporter dye, was also included in the assay to serve as an internal control to monitor for nucleic acid extraction efficiencies and possible PCR inhibition (Bai et al., 2018).

2.2 | Real-time PCR optimization

All assay development and optimization were performed using a small subset of DNA samples provided by USDA-ARS. Sample nucleic acid ($n = 12$) from sheep and goats, previously tested positive for *M. ovipneumoniae* ($n = 6$) or *M.* sp. nov. ($n = 6$) by cPCR-seq (Highland et al., 2018),

TABLE 1 Primers and probes used in the real-time polymerase chain reaction for detection and differentiation of *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) and a novel respiratory-associated Mycoplasma species (*M.* sp. nov.) from ovine and caprine specimens

Target	Primer/probe	Sequence	Fluorescent dye	Quencher
M. ovipneumoniae	[a]Probe	AGGAAATGATTTAGTCTTG	FAM	NFQ
	Forward primer	ATGTAAACTGCTGTTGTAAGGGAAG	NA	NA
	Reverse primer	CTGGCACATAGTTTGCCGT	NA	NA
M. sp. nov.	[a]Probe (*M*novA)	AAATGACCTAGTCTTGAC	VIC	NFQ
	[a]Probe (*M*novB)	AAATGATCTGGTCTTGAC	VIC	NFQ
	Forward primer (*M*novA)	GGATGTAAACTGCTGTTGTAAAGG	NA	NA
	Forward primer (*M*novB)	AGGGATGTAAACTGCTGTTATAAGG	NA	NA
	Reverse primer	CTGGCACATAGTTTGCCGT	NA	NA
18S rRNA internal control	Probe	AAGGAATTGACGGAAGGGCA	Cy5	BHQ2
	Forward primer	GGAGTATGGTTGCAAAGCTGA	NA	NA
	Reverse primer	GGTGAGGTTTCCCGTGTTG	NA	NA

[a]Minor groove binder probes.

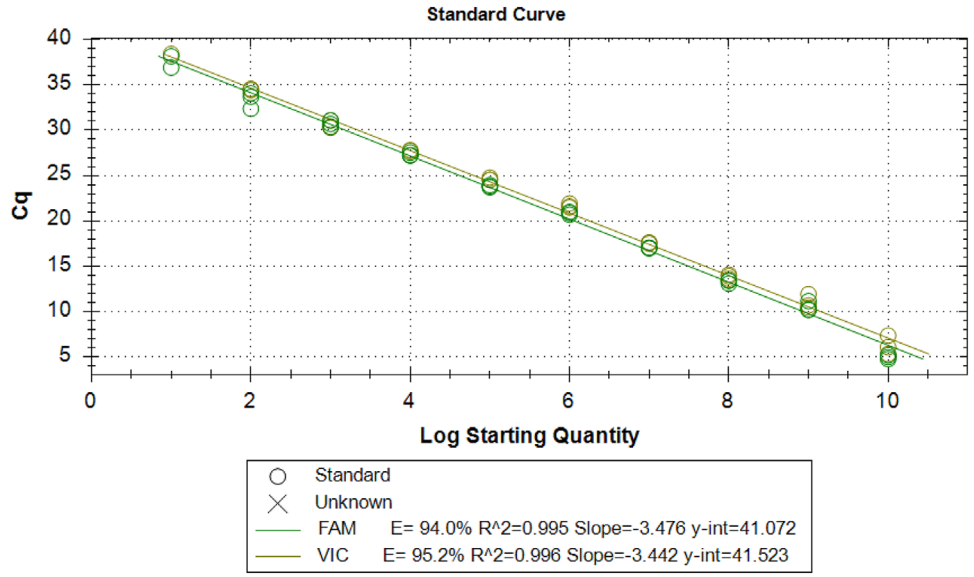

FIGURE 1 Standard curve for serial log diluted *Mycoplasma ovipneumoniae* (FAM) and *Mycoplasma* sp. nov. (VIC) targets tested in triplicate by real-time multiplex PCR

was randomly selected and tested by qPCR using a temperature gradient consisting of the following annealing temperatures: 55°C, 55.7°C, 57°C, 59°C, 61.4°C, 63.3°C, 64.5°C, and 65°C.

2.3 | Real-time PCR parameters

The qPCR consisted of 0.5 μM of *M. ovipneumoniae* and *M.* sp. nov. primers, 0.5 μM of each mycoplasma target probe, 0.25 μM of 18S rRNA primers and probe, 10 μl of 2x iQ Multiplex Powermix (Bio-Rad, Hercules, CA), and 5 μl of DNA template for a total reaction volume of 20 μl. Assay running conditions were selected based on the temperature gradient experiment described above, and consisted of 95°C for 5 min, followed by 45 cycles of 95°C for 15 s and 60°C for 50 s. Negative template controls were included in all qPCR testing to monitor for potential cross-contamination. All qPCR assays were performed using the BioRad (Hercules, CA) CFX96 Real-Time System. Mean ($\bar{x}$) *Ct* values of samples positive by qPCR for *M. ovipneumoniae* and *M.* sp. nov. targets were determined.

2.4 | Analytical specificity of the real-time PCR assay

Analytical specificity of the qPCR assay was tested with nucleic acid from ovine and caprine samples submitted to Kansas State Veterinary Diagnostic Laboratory (KSVDL) for small ruminant abortion qPCR panel testing, and that had tested positive for *Coxiella burnetii* ($n = 12$), *Toxoplasma gondii* ($n = 8$), Cache Valley virus ($n = 3$), and *Chlamydophila abortus* ($n = 2$). Nucleic acid from bovine ocular swabs that previously tested positive by qPCR (Zheng et al., 2019) for *Mycoplasma bovis* ($n = 42$) and *Mycoplasma bovoculi* ($n = 37$) were also tested.

2.5 | Analytical sensitivity of the real-time PCR assay

Recombinant plasmids carrying the *M. ovipneumoniae*, *M*novA, and *M*novB targets were constructed for use as positive amplification controls. Target sequence fragments were ligated into pUC57-Amp plasmid vectors by Genewiz (South Plainfield, NJ, USA), then transformed into Mix&Go competent cells (Zymo, Irvine, CA, USA). Transformed cells were then spread-plated onto the LB agar plates with X-Gal and ampicillin and incubated at 37°C for 14 h. White colonies, indicative of plasmid insertion, were sub-cultured to LB broth with ampicillin (Teknova, Hollister, CA, USA). Plasmid DNA, extracted using QIAprep Spin Miniprep Kit (Valencia, CA, USA), served as positive amplification controls and were used for standard curve analysis.

To provide a natural host 18S rRNA internal control template, plasmid DNA was serially diluted in ovine DNA that had previously tested negative by qPCR for all mycoplasma targets. Each dilution was tested in triplicate by the multiplex qPCR, then correlation coefficients and PCR efficiencies were determined from the resulting standard curves (Figure 1). To determine whether multiplexed PCR conditions contributed to any loss in assay sensitivity, individual standard curves for *M. ovipneumoniae* and *M.* sp. nov. were also created as described above, using singular PCR reaction mixtures. End-point cycle threshold (*Ct*) values (limit of detection, or LOD) for each target were calculated for multiplex and singular standard curves, and based on the average *Ct* of the most dilute sample that still generated a signal for at least two of the three replicates. Concentrations of serially diluted plasmids were measured by a ThermoFisher Nanodrop spectrophotometer (Waltham, MA) and target copy numbers corresponding to endpoint LOD *Cts* were calculated as described in Hamill et al. (2022).

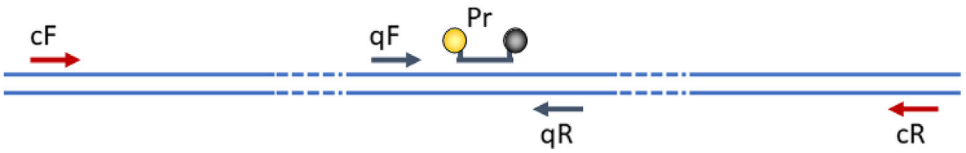

FIGURE 2 Conventional PCR flanking primers (cF and cR) used to amplify and sequence regions of *Mycoplasma ovipneumoniae* and *Mycoplasma* sp. nov. targets that contain the real-time PCR primer (qF and qR) and probe (Pr) binding site

2.6 | Application of the real-time PCR assay and comparison with conventional PCR followed by sequencing for detection of *M. ovipneumoniae* and *M. sp. nov.* from domestic sheep and goat samples

USDA-ARS provided nucleic acid from DS (n = 153) and DG (n = 194) nasal swabs that had been previously tested for *M. ovipneumoniae* and *M.* sp. nov. by the Highland et al. (2018) cPCR-seq assay. Sample nucleic acids were described by USDA-ARS as positive for either *M. ovipneumoniae* (n = 117) or *M.* sp. nov. (n = 138) or negative for both targets (n = 92). All nucleic acids were tested by qPCR and samples were determined as positive or negative based on resulting *Ct* values. Results from qPCR and cPCR-seq testing were compared. Samples with discordant results were further investigated by two cPCR reactions, each utilizing primers flanking one of the mycoplasma qPCR target regions (Figure 2). The cPCR flanking primers used for *M. ovipneumoniae* were Movi-cF1, 5′-GCGCAACATTAGTTAGTTGGTAG-3′ and Movi-cR1, 5′-CCCACGCTTTCGTCCA-3′. The cPCR flanking primers used for *M.* sp. nov. were Mcl-cF1, 5′-TTAGTTGGTGAGGTAATGGCTC-3′ and Mcl-cR1, 5′-CCCACGCTTTCGTCCC-3′. The PCR products were visualized using QIAxcel capillary electrophoresis (Valencia, CA, USA) and samples producing the expected amplicon size (~500 base pairs) for one or both mycoplasma targets were submitted to GENEWIZ (South Plainfield, NJ, USA) for Sanger sequencing confirmation. Only sequencing data with a satisfactory quality score (≥40) were considered for analysis. Raw sequence files were trimmed, assembled, and aligned using CLC Main Workbench version 21.0.3 (Valencia, CA, USA), then searched via BLAST (basic local alignment search tool; Altschul et al., 1990) using BLASTN algorithm against nr/nt (nucleotide collection) dataset for the highest nucleotide (nt) identity match.

2.7 | Statistical analyses

Overall agreement between the qPCR and cPCR-seq assays was assessed by the Cohen's kappa statistic and 95% confidence intervals using the kappa calculator (http://vassarstats.net/kappa.html). Kappa values were interpreted based on the Landis and Koch's (1977) scale.

3 | RESULTS

3.1 | Real-time PCR optimization

During initial assay development and optimization, 100% (6/6) of samples that were previously positive by cPCR-seq for *M. ovipneumoniae* were also positive by qPCR for *M. ovipneumoniae* at all annealing temperatures tested. Among the six samples previously positive for *M.* sp. nov. by cPCR, five of six samples were positive for *M.* sp. nov. by qPCR-seq at all annealing temperatures tested. The remaining sample was positive only for *M. ovipneumoniae* at all annealing temperatures tested. To further investigate the discordant result, the sample was subjected to cPCR of the mycoplasma flanking regions and subsequent sequencing confirmation. The sample had a 100% (473/473 nts) identity match to *M. ovipneumoniae* (MN028333.2). Among the annealing temperatures tested in the gradient experiment (55°C, 55.7°C, 57°C, 59°C, 61.4°C, 63.3°C, 64.5°C), optimum qPCR amplification of *M. ovipneumoniae* and *M.* sp. nov. targets occurred at both 59°C and 61.4°C, therefore 60°C was selected for application to all remaining samples.

3.2 | Analytical specificity of the real-time PCR assay

None of the nucleic acid from samples positive for *Mycoplasma bovis* (n = 42), *Mycoplasma bovoculi* (n = 37), *Coxiella burnetii* (n = 12), *Toxoplasma gondii* (n = 8), Cache Valley virus (n = 3), and *Chlamydophila abortus* (n = 2) were positive using this newly developed qPCR assay.

3.3 | Analytical sensitivity of the real-time PCR assay

To generate standard curve data, plasmids carrying the *M. ovipneumoniae* and *M.* sp. nov. targets were extracted, serially diluted in ovine DNA that had previously tested negative for each mycoplasma target, then dilutions were tested in triplicate by qPCR, both in singular and multiplex conditions. For all standard curves, correlation coefficients for *M. ovipneumoniae* and *M.* sp. nov. targets were >0.99 and PCR amplification efficiencies were between 94.0% and 102.3% (Table 2). The average end-point *Ct* for each target was ~37 and based on the highest dilution that still generated a signal for at least two of the three replicates tested. The calculated plasmid copy numbers corresponding to the end-point *Cts* for *M. ovipneumoniae* and *M.* sp. nov. targets were 5.4 and 3.1 copies per microlitre, respectively. The 18S rRNA spike-in was consistently amplified across all dilutions tested, in both singular and multiplex conditions, without any cross-reactivity observed to either mycoplasma target. Overall, comparison data show that multiplex conditions do not reduce assay sensitivity.

TABLE 2 Analytical sensitivity comparison of the real-time polymerase chain reaction assay, in singular and multiplex conditions, for detection of *Mycoplasma ovipneumoniae* and novel respiratory-associated *Mycoplasma* species (*M.* sp. nov.) plasmid DNA

	Singular		Multiplex	
	M. ovipneumoniae	*M.* sp. nov.	*M. ovipneumoniae*	*M.* sp. nov.
PCR efficiency	99.3%	102.3%	94.0%	95.2%
Correlation coefficient (R^2)	0.997	0.998	0.995	0.996
[a]Plasmid copy number (per μl) limit of detection	5.4	3.1	5.4	3.1
Average *Ct*s of dilutions tested in triplicate				
10^{-1}	6.9	8.6	5.0	6.3
10^{-2}	11.4	11.8	10.5	11.0
10^{-3}	14.8	15.4	13.3	13.9
10^{-4}	18.5	18.4	17.0	17.6
10^{-5}	21.4	21.9	20.9	21.7
10^{-6}	24.7	25.3	23.8	24.6
10^{-7}	27.8	28.4	27.3	27.6
10^{-8}	31.1	31.9	30.7	30.6
10^{-9}	34.7	35.3	33.4	34.5
10^{-10}	37.2	37.4	37.5	37.4

[a]It corresponds to the endpoint *Ct* (10^{-10} dilution) of each target.

3.4 | Application of the real-time PCR assay and comparison with conventional PCR followed by sequencing for detection of *M. ovipneumoniae* and *M.* sp. nov. from domestic sheep and goat samples

All sample nucleic acids were tested by qPCR and based on previous standard curve analysis, samples were determined as either positive ($Ct > 0$ and ≤ 37), suspect ($Ct > 37$ and ≤ 39), or negative ($Ct = 0$ or > 39). No target amplification was observed in any of the negative template controls included in each PCR run. All samples were positive for the 18S rRNA internal control gene ($Ct < 30$), indicating successful extraction efficiencies and low PCR inhibition.

Among samples previously test positive by cPCR-seq for one of the two mycoplasma targets, qPCR was in agreement for 88.0% (103/117; $\bar{x}$ $Ct = 26.1$) and 89.9% (124/138; $\bar{x}$ $Ct = 28.3$) of *M. ovipneumoniae* and *M.* sp. nov. positive samples, respectively (Table 3). Cohen's kappa statistic indicated "almost perfect" agreement between the qPCR and cPCR for detection of *M. ovipneumoniae* ($\kappa = 0.81$) and *M.* sp. nov. ($\kappa = 0.84$), where values of 0 and 1 represent agreement due to random chance and perfect agreement, respectively. As analyzed by species, "almost perfect" agreement was also observed for detection of *M. ovipneumoniae* positive samples from DS ($\kappa = 0.85$) and *M.* sp. nov. from DG ($\kappa = 0.89$). "Substantial" agreement of the assays was observed for detection of *M. ovipneumoniae* positive samples from DG ($\kappa = 0.75$) and *M.* sp. nov. from DS ($\kappa = 0.74$). The qPCR detected both mycoplasma targets in two of 67 (3.0%) *M. ovipneumoniae* positive DS and in one of 40 (2.5%) *M.* sp. nov. cPCR-seq positive DS. Co-positives were detected by qPCR in a slightly higher proportion of DG, including in five of 50 (10.0%) *M. ovipneumoniae* positive samples and in four of 98 (4.1%) *M.* sp. nov. positive samples. The qPCR detected *M. ovipneumoniae* and *M.* sp. nov. in 6.5% (6/92; $\bar{x}$ $Ct = 32.9$) and 4.3% (4/92; $\bar{x}$ $Ct = 33.14$) of total samples, respectively, that previously tested negative for both targets by cPCR-seq.

Samples with discordant results were further investigated by two cPCR reactions, each utilizing primers flanking one of the mycoplasma qPCR target regions (Figure 2). All samples tested were described by one of the following PCR result profiles: (i) *M.* sp. nov. positive samples by cPCR that were qPCR co-positive or positive for *M. ovipneumoniae*, (ii) *M. ovipneumoniae* positive samples by cPCR that were qPCR co-positive or positive for *M.* sp. nov. and (iii) samples negative for both mycoplasma targets by cPCR but positive to any mycoplasma target by qPCR. If samples produced the expected amplicon band size (~500 base pairs) for either mycoplasma target, then PCR product was subjected to Sanger sequencing confirmation.

Of nine samples testing positive for *M.* sp. nov. by cPCR, each had *M. ovipneumoniae* detected by qPCR, and five of these had both mycoplasmas detected; *M. ovipneumoniae* was confirmed by sequencing in four of the nine samples, each of which had produced lower *Ct*s (25.2, 25.3, 29.2. 32.2) compared to the majority of remaining samples that were not sequence confirmed (*Ct*s of 29.3, 32.3, 33.8, 35.8, 36.5). *M.* sp. nov. was sequence confirmed in four of the five co-positive samples. Of eight samples testing positive for *M. ovipneumoniae* by cPCR, six were co-positive by qPCR and two were negative for *M. ovipneumoniae* but positive for *M.* sp. nov. Among the co-positive samples, five of six were confirmed for both targets by sequencing. The two samples positive for only *M.* sp. nov. (*Ct*s of 33.6 and 36.6, respectively) did not produce bands by either cPCR reaction, therefore were not sequence confirmed. Of seven samples testing negative for both targets by cPCR, three were positive by qPCR for *M. ovipneumoniae* (*Ct*s of 30.1, 34.2 and 36.3) and four were positive for *M.* sp. nov. (*Ct*s 31.1, 32.2, 33.1

TABLE 3 Detection of *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) and a novel respiratory-associated *Mycoplasma* species (*M.* sp. nov.) from ovine and caprine specimens by real-time polymerase chain reaction (qPCR), and agreement of samples positive and negative by partial 16S rRNA conventional PCR (cPCR)

Sample host (No. samples)	Partial 16S rRNA cPCR sample status			No. (%) samples positive and negative by qPCR		Kappa statistic (95% CI)	[a]Strength of agreement
				Positive	Negative		
Domestic sheep ($n = 153$)	*M. ovipneumoniae*	Positive	67	63 (94.0)	4 (6.0)	0.85 (0.76–0.95)	Almost perfect
		Negative	46	4 (8.7)	42 (91.3)		
	M. sp. nov.	Positive	40	30 (75.0)	10 (25.0)	0.74 (0.60–0.88)	Substantial
		Negative	46	1 (2.2)	45 (97.8)		
	Co-positive		0	3			
Domestic goat ($n = 194$)	*M. ovipneumoniae*	Positive	50	40 (80.0)	10 (20.0)	0.75 (0.62–0.88)	Substantial
		Negative	46	2 (4.3)	44 (95.7)		
	M. sp. nov.	Positive	98	94 (95.9)	4 (4.1)	0.89 (0.81–0.97)	Almost perfect
		Negative	46	3 (6.5)	43 (93.5)		
	Co-positive		0	9			
Total ($n = 347$)	*M. ovipneumoniae*	Positive	117	103 (88.0)	14 (12.0)	0.81 (0.73–0.89)	Almost perfect
		Negative	92	6 (6.5)	86 (93.5)		
	M. sp. nov.	Positive	138	124 (89.9)	14 (10.1)	0.84 (0.77–0.91)	Almost perfect
		Negative	92	4 (4.3)	88 (95.7)		
	Co-positive		0	12			

[a]Based on the scale proposed by Landis and Koch (1977).

and 35.1); none were co-positive. One sample (*Ct* 30.1) was sequence confirmed for *M. ovipneumoniae*, but all others produced low sequence quality scores (<40) with high background signal.

4 | DISCUSSION

Multiple diagnostic methods are available for detection of *M. ovipneumoniae*, yet not all are suitable for routine testing applications. Although culture methods are still considered by some to be the gold standard for diagnosis (Weiser et al., 2012; Wang et al., 2020), these methods are not only time consuming (~1–3 weeks) and labour-intensive, but generally less sensitive than PCR-based methods (Weiser et al., 2012; Jennings-Gaines et al., 2016). Prior to the discovery of *M.* sp. nov. (Herndon et al., 2021), several PCR assays for *M. ovipneumoniae* detection were developed in which the primers and/or probes share either a high and/or 100% sequence identity match to *M.* sp. nov. (Lauerman, 1998; Lawrence et al., 2010; Manlove et al., 2019; McAuliffe et al., 2003; Yang et al., 2014; Ziegler et al., 2014). Some of these assays have been reported to cause false positive results when testing for *M. ovipneumoniae*, reportedly due to detection of either an "unidentified *Mycoplasma* like organism" (Manlove et al., 2019) or *M.* sp. nov. (Herndon et al., 2021).

In the current study, we harnessed the advantages of qPCR, which include high sensitivity, high specificity via probe-based amplification, and rapid turn-around time to results, to create a novel assay for detection and differentiation of *M. ovipneumoniae* and *M.* sp. nov. in DS and DG samples. The target profiles of the sample nucleic acid used for diagnostic validation in the current study were previously determined by cPCR in combination with Sanger sequencing confirmation (Highland et al., 2018), therefore we compared the performance of our qPCR assay to previous cPCR results. Although qPCR is generally more sensitive than cPCR (Biassoni & Raso, 2014), Cohen's kappa statistic indicated "almost perfect" agreement between the qPCR and cPCR for overall detection of each target from the total population of samples, and "almost perfect" and "substantial" agreement for detection of each target from DS and DG, respectively, which suggests high sensitivity and high specificity of the cPCR-seq procedure in Highland et al. (2018). A small portion of these few discrepant data were a result of the qPCR not detecting one of the mycoplasma targets that was previously positive by cPCR. It is possible that target load in these samples was already low at the time of extraction, and since nucleic acid were tested by cPCR as fresh extracts but by qPCR after multiple freeze/thaws, nucleic acid degradation may have contributed to these discrepant data (Schaudien et al., 2007; Shao et al., 2012). The other type of discrepant data were due to disagreement between assays on the target profiles of mycoplasma positive samples or qPCR detection of a mycoplasma target from a sample that was previously negative for both targets by cPCR. All samples that were successfully sequenced had identity matches that confirmed the qPCR result. The majority of samples that did not sequence well also had relatively high *Cts* by qPCR. From our experience, and based on a previous finding (Noll et al., 2020), sample *Cts* at or below the middle to low 30s are required for successful Sanger sequencing of PCR product. Therefore, it is likely that these

samples are true positives, based on qPCR testing, yet target concentrations are below the limit of sequencing detection.

To conclude, our qPCR is novel in that it is the first assay specifically designed to detect and differentiate *M. ovipneumoniae* and *M.* sp. nov. The increased sensitivity and specificity of this qPCR assay should allow for a reduction of false positive test results that have been associated with previous assays that were designed prior to the discovery of *M.* sp. nov. Although results from the cPCR-seq procedure in Highland et al., 2018 were in high agreement with the qPCR, our assay, by comparison, is capable of providing rapid results with decreased time and labour associated with the additional steps required with cPCR and sequence confirmation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STATEMENT

The research project did not involve animal handling. All samples were previously extracted and only nucleic acids were submitted to KSVDL for testing. None of the authors were involved in the sample collection process.

DATA AVAILABILITY STATEMENT

Primer and probe sequences are the primary data and are included in this manuscript. A summary of standard curve data is also included; raw data are available upon request.

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