ADVANCEMENTS IN MOLECULAR DIAGNOSTICS FOR DETECTION OF *MYCOPLASMA GALLISEPTICUM* AND *MYCOPLASMA SYNOVIAE* IN POULTRY

by

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(Under the Direction of Naola Ferguson-Noel)

ABSTRACT

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are economically relevant respiratory pathogens of poultry which may have analogous clinical presentations to respiratory diseases of several different etiologies. The overarching goal of this research was to facilitate rapid, sensitive, and specific diagnostic testing for poultry respiratory pathogens. There are several hurdles to overcome in achieving that goal as there are different standards and recommendations for different types of pathogens – from collection of samples in the field to processing in the diagnostic lab. In this research, we investigated several of the practical limitations to simplification and unification of molecular testing for multiplex testing of avian respiratory pathogens. The effect of transportation/preparation media on MS and MG quantitative PCR of tracheal swabs was evaluated and brain heart infusion (BHI) broth (already recommended for transport of several agents of poultry respiratory disease) was determined to be the ideal choice. The effect of swab location on MS, MG, and infectious laryngotracheitis virus (ILTv) detection by qPCR was evaluated and choanal cleft swabs were determined to be superior to tracheal and oropharyngeal swabs for detection of the pathogen DNA. Next, two highly

sensitive duplex qPCRs for detection of ILTv and either MS or MG were developed in progress toward developing more complicated multiplex assays. And, finally, a novel multiplex targeted nanopore sequencing panel using MS and MG strain typing genes was developed for use on the accessible MinION nanopore sequencer along with a user friendly bioinformatic workflow. The outcomes of this research allow the poultry industry to maximize the benefits of costly diagnostic assays, set scientifically based standards in different laboratories for sample collection and handling, and meet demanding turnaround times for conclusive diagnosis of respiratory diseases.

INDEX WORDS: *Mycoplasma synoviae*, *Mycoplasma gallisepticum*, diagnostic, sampling, qPCR, nanopore sequencing

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DEDICATION

For my family and friends, both near and far, who have provided their unwavering support and love:

For my mom, Regina, who teaches me strength and resilience no matter the situation. Thank you for showing me that joy can be found as long as you choose to look for it.

For Charles, because anybody can be a father, but it takes something indescribably special to be a dad. Thank you for always being there for all of us.

For my sisters and brothers, Rebekah, Patrick, Sarah, Daemon, and Phoebe, who always listen and make me laugh.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Mycoplasma genus

Members of genus *Mycoplasma* are ubiquitous bacteria belonging to phylum Tenericutes, class Mollicutes, order Mycoplasmatales, and family *Mycoplasmataceae*. With over 120 discovered species, Mycoplasmas generally demonstrate host-specificity for humans, animals, plants, and arthropods. They are in a unique subset of bacteria which lack a cell wall and are instead bound solely by a plasma membrane. This cell wall deficiency enables Mycoplasmas to exhibit pleomorphism whereby they cannot be defined by typical bacterial morphology such as cocci or bacilli. Without a cell wall, mycoplasmas are resistant to antibiotics, such as penicillin, that target cell walls. They also require specific nutrients, like cholesterol, to grow effectively [1]. Additionally, the smallest free-living organisms are represented in this genus, with most cells measuring less than 1 μm. Mycoplasmas also have the smallest genomes of all free-living organisms ranging between 580 and 1350 kb [1-3]. These genomes have a low G+C content between 23 to 40% [4, 5]. The above features are a result of reductive evolution from gram-positive, spore-forming clostridial species. Essential genes responsible for DNA replication, transcription, and translation were retained and genes used for cell wall synthesis and energy metabolism were either totally eliminated or significantly reduced [6-8]. Generally, mycoplasmas tend to be restricted to a single host species but some mycoplasma species may have a larger host range [9]. While there are many mycoplasmas known to infect avian species,

those pathogenic in poultry and therefore cause for industry concern include *Mycoplasma synoviae* (MS), *Mycoplasma gallisepticum* (MG), *Mycoplasma meleagridis* (MM), and *Mycoplasma iowae* (MI). For the purposes of this research, focus will be on mycoplasmas pathogenic in chickens, *Mycoplasma synoviae* and *Mycoplasma gallisepticum*.

Mycoplasma synoviae

Mycoplasma synoviae (MS) has worldwide prevalence in commercial poultry flocks and, while found predominantly in chickens and turkeys, has also been found naturally in ducks [10], geese [11], pheasants and partridges [12], and many more avian species [13-19]. MS infections typically present as subclinical upper respiratory disease, but infections that become systemic can result in infectious synovitis primarily in the joints and tendon sheaths [20]. Clinical signs of birds with infectious synovitis include pale or bluish-red combs, lameness, swelling in the joints followed by increased lethargy, dehydration, and emaciation. With the upper respiratory presentation of MS infection, chickens can have minor rales or remain asymptomatic [20]. MS has also been shown to cause eggshell apex abnormalities [21-23], though eggs produced by broiler breeders with MS infections tend to be less susceptible to these abnormalities [24].

The genome of MS is approximately 800-850 kb [25, 26]. Typical genes or regions used for identification and strain differentiation for MS include *vlhA*, *nanA*, *ugpA*, *tufA*, and the 16S-23S intergenic spacer region. MS *vlhA* (partial variable lipoprotein hemagglutinin A) is a commonly used gene for MS strain typing [27-30]. MS *vlhA* encodes two major membrane antigens, MSPA and MSPB [31]. In MS, the *vlhA* gene family is localized to a single region of the genome and each gene in the family consists of a conserved region, semi-variable region, and highly-variable region. Within the conserved region, there is a 410-530 bp segment with only one copy in the entire genome which contains the only promotor in the MS *vlhA* gene family.

There are then multiple copies of the rest of the conserved region, semi-variable regions, and highly-variable regions making up pseudogenes of the *vlhA* gene family in MS [27]. MS *nanA* (N-acetylneuraminate lyase) and MS *ugpA* (UTP-glucose-1-phosphate uridylyltransferase) are housekeeping genes that have been previously used in a multi-locus sequence typing (MLST) scheme published by Dijkman et al. [32] and showed the highest genetic variability among the 5 genes used in that assay. MS *tufA* (elongation factor Tu) is a highly conserved housekeeping gene with only one copy in Mycoplasma genomes that also contains variable regions that are species specific [33]. *Mycoplasma synoviae* has two 16S-23S rRNA IGSRs (intergenic spacer regions, alternatively called ITS) which can exhibit intercistronic heterogeneity; between MS species there are a few polymorphisms present as well as differing amounts of A/T in poly-A and poly-T regions of the IGSRs [34]. Between 23 avian mycoplasma species, the IGSRs have shown high inter-species variation; however, for the four pathogenic avian mycoplasma species, there is a low intra-species variation [35].

Mycoplasma gallisepticum

Mycoplasma gallisepticum (MG) is distributed worldwide in commercial poultry and infections with MG result in economic losses due to condemnations, poor feed efficiency, and reduced egg production [36]. MG generally occurs naturally in chickens and turkeys, though naturally occurring infections have also been seen in pheasants [37, 38], partridges [38, 39], peafowl [37, 38], quail [40], ducks [10], geese [41], sparrows and pigeons [13], and much more [17, 42-44]. In chickens, adults with MG infections present clinical signs of rales, coughing, and nasal discharge with resultant loss of adequate feed consumption and reduction in weight. Layer flocks will experience declining egg production [36]. Turkeys typically experience worse clinical signs than those described for chickens as well as infectious sinusitis and dyspnea [36]. Egg

transmission of MG from breeders results in embryo mortality and reduced hatchability [45-49]. Severity of clinical signs for MG infections tend to worsen in the winter [50] and in younger birds [51].

The *Mycoplasma gallisepticum* genome is approximately 1,000 kb [52-54]. Typical genes are genomic regions used for identification or train differentiation of MG include the 16S-23S rRNA intergenic spacer region, *vlhA*, and *mgc2*. MG has only one 16S-23S rRNA IGSR that has been shown to be highly variable between MG strains such that single-locus typing of MG isolates is possible [55, 56]. MG has 43 *vlhA* genes spread across 5 loci and making up about 10% of the genome [52]. High-resolution melting curve analysis of MG strains using the *vlhA* gene has been shown to differentiate all strains [57]. The *mgc2* (cytadhesin) gene has exhibited usefulness in both MG identification as well as MG strain differentiation [58-60]. In terms of differentiation, *mgc2* genes can have differing insertions and deletions resulting in gene size polymorphisms, particularly in the proline-rich, surface-exposed carboxyterminal-encoding region [58].

Gallid herpesvirus type 1

Gallid herpesvirus type 1 (GaHV-1) is a viral species belonging to order Herpesvirales, family *Herpesviridae*, subfamily *Alphaherpesvirinae,* and genus *Iltovirus*. GaHV-1 is the causative agent of infectious laryngotracheitis (ILT), a respiratory infection of chickens that can result in increased mortality and decreased egg production [61]. The virus may also be more simply referred to as ILTv, or infectious laryngotracheitis virus. Severe ILT presents with wet rales, nasal discharge, and significant conjunctivitis which leads to dyspnea and blood-stained expectorant [62]. Epithelial damage and hemorrhaging mucosa in the larynx and trachea can produce mucoid plugs in the trachea that may lead to asphyxiation [63]. Mild ILT can present

with slight conjunctivitis and tracheitis, nasal discharge, mild rales, and stunted growth [64-66]. Natural infection, in addition to chickens, has been reported in turkeys [67], pheasants, and peafowls [68].

The approximately 151 kb genome of GaHV-1 is composed of linear, non-segmented, double-stranded DNA. The DNA characteristically contains a unique short (U_S) region of 13 kb and unique long (U_L) region of 111 kb which are flanked by inverted repeats [61]. The GaHV-1 genome contains 12 identified glycoprotein-coding genes [69] with glycoproteins J and C being the most abundant of the surface glycoproteins [70-73]. Typical genes utilized for identification of GaHV-1 via qPCR or sequencing include gC, TK, ICP4, and UL15 [61]. The GaHV-1 gC gene encodes a herpes simplex virus glycoprotein C gene homologue of 1242 bp in length, which shows limited interspecies homology when compared to other herpesvirus gC proteins [74]. The ILTv ICP4 gene is 4386 bp and is located within the inverted repeat region [75]. The herpes simplex virus homologue of GaHV-1 ICP4 encodes a regulatory protein of HSV-1 responsible for switching gene expression from immediate-early to early and late expression as well as for downregulation of some genes [75]. Though it is a nonessential gene, the thymidine kinase, TK, gene lies among a cluster of highly conserved genes of GaHV-1 and has been useful in differentiating between herpesvirus subfamilies [76]. The UL15 gene is highly conserved in herpesviruses and is essential for DNA cleavage and packaging in HSV-1 [77, 78].

Differential Diagnoses for MS, MG, and ILTv Infection

In addition to potential for confused diagnoses between MS, MG, and ILTv, other poultry pathogens may also present clinical signs and lesions similar to those of MS, MG, and ILTv which can convolute accurate and timely diagnosis of flocks experiencing respiratory issues or synovitis. MS or MG infections complicated by respiratory viruses, such as infectious bronchitis

virus (IBV) or Newcastle disease virus (NDV), as well as *Escherichia coli* can result in chronic respiratory disease (CRD) presenting as severe airsacculitis [36].

Infectious bronchitis virus (IBV) infections during the first week of age are characterized by respiratory signs such as rales, coughing, sneezing, dyspnea, conjunctivitis, and swollen sinuses, and can result in lethargy and decreased weight gain. Respiratory signs are less obvious in chickens six weeks and older, though layers may be affected by decreased egg production [79].

Infections with viscerotropic isolates of NDV are characterized by listlessness, depression, conjunctivitis, mucosal discharge from the mouth, and difficulty breathing [80]. Young birds vaccinated with the NDV LaSota live attenuated vaccine strain may experience respiratory distress after its application [81].

Low pathogenicity avian influenza (LPAI) viruses often present with mild to severe respiratory distress characterized by rales, rattles, coughing, sneezing, and severe lacrimal secretions. Non-respiratory signs associated with LPAI infections include depression and reduction in feed and water consumption. Layers may experience decreased egg production. Respiratory signs are less noticeable with infections of high pathogenicity avian influenza (HPAI) viruses, but can include coughing, sneezing, and rales [82].

The diphtheritic presentation of avian poxvirus causes similar respiratory signs to those seen with ILTv infection with gross lesions forming in the mouth, esophagus, and/or trachea. This leads to difficulty breathing and reduced feed and water consumption. It also can result in stunted growth and decreased egg production [83].

Infectious coryza, caused by *Avibacterium paragallinarum* infection, predominately presents as inflammation of the upper respiratory tract. Signs of this inflammation include nasal

discharge, conjunctivitis, facial edema, and swollen wattles. Rales, coughing, sneezing, and difficulty breathing may result with infections that reach the lower respiratory tract [84].

Chronic fowl cholera resulting from *Pasteurella multocida* infection often presents as localized infections that cause swelling of joints, footpads, wattles, sternal bursae, sinuses, and conjunctiva. Respiratory infections of *P. multocida* can result in rales and dyspnea [85].

Diagnostic Review for MS, MG, and ILTv Detection

Mycoplasma Isolation: For acute phases of MS and MG infections, bacteria may be isolated from airsacs and sinuses showing gross lesion of airsacculitis or synovitis, but bacterial load tends to diminish within a few weeks. For chronic infection, it is more reliable to isolate from the upper respiratory tract (trachea, choanal cleft) where the organisms tend to persist [86, 87]. For identification by culture, anywhere from 5-100 swabs may be taken depending on the stage of infection or the suspected pathogenicity of the strains [87]; sample size for detection by PCR is reflective of flock size and swab samples tend to be pooled. Mediums for growth of mycoplasmas consist of protein-rich animal serum (swine or horse typically) supplemented with a yeast-derived solution. Nicotinamide adenine dinucleotide (NAD) is also a necessary component for propagation of *Mycoplasma synoviae* [1]. Several medias have been described that readily facilitate mycoplasma growth, including Frey's medium [88], a modified Frey's medium [1], a medium described by Bradbury [89], and SP-4 broth [90]. After incubation at 37°C to exponential growth phase (typically indicated by color change), a secondary isolation is made by transfer of culture to agar forms of the media which are further incubated 3-10 days until colonies form [1, 87]. Mycoplasma species may then be identified by direct immunofluorescence of colonies [91] or growth inhibition of inoculum [92].

ILTv Isolation: GaHV-1 may be collected for virus isolation from swabs of trachea or conjunctiva and from tissues collected from the larynx and lungs. Two to 6 days post infection, during the acute disease stage, GaHV-1 can be reliably isolated from tracheal epithelium [93-95] by 7-8dpi [96]. The virus may be propagated in embryonated chicken eggs or in several types of avian cell cultures [61]. Chicken embryo liver (CELi) and chicken kidney (CK) cells have been shown to be more susceptible to GaHV-1 infection than chicken embryo lung (CELu) cells, chicken embryo kidney (CEK) cells, and chicken embryos [97]. Isolates are confirmed by immunohistochemistry (IHC) staining, fluorescent antibody (FA) staining, or PCR. Using virus isolation methodologies, conclusive diagnosis of GaHV-1 may take 1-2 weeks [61].

Serological tests: Of serological tests available for detection of ILTv antibodies, enzyme-linked immunosorbent assays (ELISAs) tend to be more advantageous than agar gel immunodiffusion (AGID) or virus neutralization (VN) in terms of sensitivity, turn-around time, and throughput. However, because of antigenic homogeneity among ILTv strains, conventional ELISA assays are ineffective in differentiating carriers and vaccinated chickens [61]. However, flocks vaccinated with recombinant viral vector vaccines containing ILTv glycoproteins may be differentiated from naturally infected flocks using a combination of glycoprotein-specific ELISAs [98, 99].

For serological detection of MS and MG, serum plate agglutination (SPA) assays and ELISAs are useful for screening flocks due to their high sensitivity whereas the highly specific hemagglutination inhibition (HI) assay is typically used as a confirmatory test when serum reacts on SPA or ELISA [87]. Confirmation with the HI assay is necessary because SPA tests may result in nonspecific reactors [100, 101] and ELISAs are prone to false positive reactions [20, 36]. However, HI assays tend to be more complicated and time consuming than SPAs and

ELISAs. Progression of infection also has an effect on serological results. Because the SPA test detects IgM antibodies, positive results may be seen 7-10 days post-infection [87, 102]; however, measurement of IgY antibodies by HI assays means positive results may not be seen until 2-3 weeks after infection onset [87].

PCR-based DNA detection: Inestimable conventional polymerase chain reaction (PCR) assays have been developed for detection of MS, MG, and ILTv DNA targets since the methodology was introduced. Conventional PCRs have also been further modified into restriction fragment length polymorphism (RFLP) PCR assays for MS, MG, and ILTv strain differentiation. More recently, quantitative PCR (qPCR) assays have also been developed with greater sensitivity and real-time detection capabilities than conventional PCRs. For instance, in comparing ELISA, conventional PCR, and SYBR Green qPCR for ILTv detection, viral DNA was able to be detected down to 102 copies with qPCR while conventional PCR could only detect down to 1660 copies [103]. Quantitative PCRs can be detected either by SYBR Green dye, where the fluorescent dye binds with any produced dsDNA with each successive cycle, or TaqMan probes, where a third target specific sequence carrying a fluorescent label is bound and released by the DNA as the polymerase processes amplicons. While there has been documentation that these two detection methods can be equally effective [104, 105], the characteristic ways in which the two methods work tend to make TaqMan probe based assays more specific. Quantitative PCRs are also more amenable to multiplexing as detection is by fluorescence-based reading by sensitive machinery as opposed to visually assessed on a gel [106]. Consequently, a number of duplex qPCRs have been developed to detect two pathogens simultaneously in real-time. Table 1.1 lists some of the above published PCR-based assays for DNA detection.

Non-PCR-based DNA detection: There have also been several assays described which do not use PCR for DNA detection. Historically, DNA probes have been used to detect MS [107-109], MG [108-110], and ILTv [111, 112] though they have predominantly been displaced by conventional and quantitative PCR assays which are prodigiously more sensitive [113]. To circumvent the need for thermocycling machinery which may not be readily available to laboratories with limited funds, isothermal amplification procedures have also been developed. Loop-mediated isothermal amplification (LAMP) procedures, which are conducted under a constant temperature, have been described for MS [114], MG [115], and ILTv [116, 117]. However, because multiple primers must be used, design of LAMP assays can be expensive and difficult [118, 119]. There has also been evidence that, for ILTv, LAMP assays can lose sensitivity compared to qPCR at late stages of viral infection [120]. Additionally, a novel isothermal polymerase spiral reaction (PSR) has been developed [121] which limits the number of primers and probes that would typically be needed in LAMP techniques. Cycling probe technology (CPT), an isothermal technique resulting in linear amplification, has also been used in genotyping and differentiating MS field isolates from the MS-H vaccine [122].

Sequencing: More in-depth genomic analysis than the aforementioned techniques has been particularly useful in molecular characterization of MS, MG, and ILTv. Multilocus sequence typing (MLST) characterizes bacterial species using several housekeeping genes to create allelic profiles based upon differences in nucleotides between each housekeeping gene. MLST assays have been described for both MS [32, 123, 124] and MG [125-127] characterization. Given the high conservation of housekeeping genes and their role in survivability of the cell, this is a very conservative approach for defining strains. There are also sequencing techniques targeting genes which are known to be highly discriminatory between

isolates from independent outbreak events of epidemiological importance. These may involve sequencing of one or multiple amplicons of interest. Table 1.2 lists common genomic regions used for targeted sequencing approaches as well as housekeeping genes targeted for MLST analyses.

Next- and Third-Generation Sequencing

Next-generation sequencing (NGS) platforms scaled up traditional Sanger sequencing approaches by sequencing small DNA fragments in a massively parallel manner such that whole genomes can be constructed. This has led to the proliferation of whole genome sequences of unnumerable organisms that can readily be shared. Most developed sequencing techniques for MS, MG, and ILTv characterization utilize NGS platforms, such as Illumina [124, 128] and Ion Torrent [129, 130].

Over the past few decades, rapid advancements have been made in nucleic acid sequencing technologies which have broadened the capacities of next generation sequencing for use in rapid diagnostics. These third-generation technologies, such as Oxford Nanopore Technologies (ONT) or Pacific Biosciences, not only provide the means to obtain much longer reads, but also have the added benefits of lowered capital investments and improved portability potential. Generally, a major drawback of these sequencing technologies has been single read inaccuracies and therefore the requirement for high coverage applications. Error analysis of nanopore sequencing reveals that most of these errors occur in homopolymer regions. When these regions move through the pore, the current amplitude does not change and must be inferred by the reading software. This can introduce erroneous insertions and deletions leading to frameshifts which are only reduced by a significant number of rereads of the same area (approximately 140 reads) [131].

MinION nanopore sequencing utilized in Chapter 5 was carried out using the PCR barcoding kit (SQK-PBK004, ONT). Using this kit (Figure 1.1), DNA is end-prepped by enzymatically adding 5′ phosphorylated and 3′ dA-tailed ends to the DNA to prepare them for barcode adapter ligation. ONT provided barcode adapters are ligated to the end-prepped amplicons through the action of a T4 DNA ligase which catalyzes the joining of 3′-OH to 5′-phosphate. The barcode adapters contain primer binding sites to which barcode primers will anneal to enable amplification of input amplicons joined with barcodes. The barcode primers contain a 5' tag which facilitates ligase-free attachment of rapid sequencing adapters. This also enables the majority of sequencing to be performed on those sequences which have gained a barcode. Sequencing adapters carry a processive enzyme (helicase) which will regulate translocation of DNA through the pore. It is active in solution, but the adapter sequence has specialized bases which prevent the enzyme from working until it is in contact with the nanopore [132]. Using a flow cell priming kit, tethers are added to the flow cell which will bring DNA strands to the membrane in order to improve DNA capture by the nanopores. Libraries are mixed with a sequencing buffer and loading beads. The sequencing buffer provides fuel for optimal translocation of DNA through the nanopores while loading beads (Sepharose beads) loosely bind to DNA and pellets it toward the membrane for greater DNA concentration near the nanopores [132].

Sequencing occurs as the helicase motor protein attached to the sequencing adapters comes into contact with the nanopore, unzips the DNA strand, and feeds ssDNA through the nanopore. As the ssDNA moves through the nanopore, the amino acid residues of the Reader section of the nanopore contact the ssDNA and are able to discriminate between different nucleotides. Specific combinations of nucleotides create characteristic disruptions in the electric

current which are collected by the MinKNOW software to define orders of nucleotides. ONT basecallers convert these raw signals into base calls using a recurrent neural network (RNN) which is continually learning and applying learned parameters to improve predictions of reads. These reads are written into FASTQ and/or FASTA files for further analysis [133, 134].

Scope of Research

Sensitive tests that allow very early detection of the aforementioned avian pathogens are an important part of the control of these infections and preventing widespread epidemics. As quantitative PCR protocols are amenable to high throughput testing, they have the potential to be used not only as confirmatory tests but also as screening tests for the early detection of avian pathogens. However, some of the advantages of qPCR may be reduced due to inappropriate collection, handling, and processing of samples [135, 136]. In routine poultry diagnostics, swabs are often submitted and processed for multiple tests (e.g., respiratory viruses and MG and MS). Consideration needs to be made when deciding on the optimal site to collect the sample from and choosing the appropriate media for transport and sample processing for amplification and detection of genomes from various respiratory diseases of poultry. In an ideal scenario, diagnosticians would be able to perform multiple PCR tests from the same pool of swabs. This would greatly improve animal welfare as separate sets of swabs would not need to be taken from a flock of birds to perform each individual test. In turn, the costs of tests would be lowered for labs, and therefore for customers. All of this would save time for the customers taking samples and the labs running the tests. Multiplex qPCRs will shorten the wait time and costs to obtain results and may contribute to more effective passive surveillance of flocks. Finally, sequencing technologies can be modified to allow even more samples to be processed at once for more streamlined and comprehensive diagnosis. For instance, targeted sequencing allows specific

regions of interest to be amplified for better sequence detection and it has the potential for thousands of targets to be amplified in a single reaction tube [137].

Objectives

Objective 1: Transport and Extraction Media Effect on MG & MG Quantitative PCR

Rationale: In routine poultry diagnostics, swabs are often processed for multiple tests (e.g., respiratory viruses and MG and MS) and careful consideration needs to be made when choosing the appropriate media for swab transport and preparation for multiple qPCR tests. Mycoplasmas are typically processed in phosphate buffered saline (PBS) or PCR grade water but are often transported for diagnostic testing in brain-heart infusion (BHI) broth or modified Frey's mycoplasma media (FMS). Meanwhile, a modified PBS containing calf serum and antibiotic-antimycotic solutions is often used to prepare tracheal swabs for ILTv qPCR [138, 139] and BHI has been determined to be the ideal media for transfer of avian influenza (AI) samples [140]. Solidifying whether a single media type may be utilized for transport and preparation of samples for detection of relevant respiratory pathogens via qPCR or qRT-PCR will be helpful in limiting the number of samples needed when multiple pathogens are to be tested. Limiting sampling will aid in reducing contact with potentially ill flocks and prevent additional costs that could be incurred by oversampling.

Hypothesis: We expect PBS to perform better as a transport and extraction preparation media than BHI, PCR grade water, FMS, and modified PBS when evaluating the sensitivity of MS and MG quantitative PCR.

Objective 2 – Swabbing Locations and Detection by MS, MG, and ILTv Quantitative PCR

Rationale: There is increasing need for rapid detection of all relevant respiratory pathogens in order to properly diagnose flocks suspected of disease. For this purpose, the ability

to test for multiple pathogens from a single sample would be beneficial. Typically, tracheal swabs are taken for MS, MG, and ILTv detection, while oropharyngeal swabs are taken for AI. Determining if a swab of a single location will be adequate to successfully detect each of these avian respiratory pathogens will improve diagnostic testing in terms of time and money and will enhance animal welfare and ease of sampling for poultry growers. Here, the effect of swab collection sites for detection of MS, MG, and ILTv via quantitative PCR was tested.

Hypothesis: Typically, tracheal swabs are taken for MS, MG, and ILTv detection, while oropharyngeal swabs are taken for AI. So, we expect tracheal swabs to perform best in terms of sensitivity of MS, MG, and ILTv qPCR compared to swabs of choanal cleft and oropharynx.

Objective 3 – Novel Multiplex Quantitative PCRs for MS, MG, and ILTv

Rationale: Due to the economic significance of MS, MG, and ILTv for the poultry industry, rapid detection of the causative agent of disease is necessary for growers and veterinarians to take appropriate actions. Additionally, surveillance of flocks for diseases requires testing to be cost effective as well as rapid, especially in cases of move birds. Currently, there are no active surveillance programs for ILTv as serological testing is unreliable (vaccinated flocks cannot easily be differentiated from infected flocks) and PCR is too expensive. Often swabs are submitted to test for multiple pathogens and rapid detection of these respiratory pathogens is necessary for timely diagnosis of flocks suspected of respiratory disease. Combining testing for MS, MG, and ILTv via multiplexed quantitative PCRs would allow for relatively inexpensive and prompt results.

Hypothesis: Novel multiplex quantitative PCRs for detection of MS, MG, and ILTv will be as sensitive as currently used single target qPCRs.

Objective 4 – Multiplex, Targeted Sequencing as a Diagnostic Test for MS and MG

Rationale: While quantitative PCR has become the gold standard for rapid detection of pathogens, there are limitations to the assay that may render sequencing techniques as preferable alternatives. For instance, most quantitative PCR thermocyclers can detect five targets at most. With targeted sequencing techniques, up to four-thousand primer pairs have been shown to be effective in detecting targets of interest [137]. Additionally, the cost of sequencing has become increasingly reasonable and therefore can be more readily implemented in a diagnostic setting. The Oxford Nanopore Technologies (ONT) MinION Mk1B sequencer has an accessible price tag of $1000USD. Over the past few years, nanopore sequencing has been shown to be useful for successful typing of several avian pathogens [141-145]. While whole genome nanopore sequencing has been effective in this regard, using nanopore sequencing in conjunction with gene targeting would allow us to hone in on genes we know to be valuable for identification and typing without the data overload that can come with whole genome analysis [146]. We can gain additional speed and information by also multiplexing the targeted sequencing approach to encompass multiple relevant pathogens potentially isolated from the same sample.

Hypothesis: Highly specific primers targeting genes of avian respiratory pathogens (beginning with pathogenic mycoplasmas of chickens*, M. synoviae* and *M. gallisepticum*) can be utilized to develop a diagnostic multiplex sequencing scheme which will identify and type avian respiratory pathogens in a more accurate and higher capacity than quantitative PCR.

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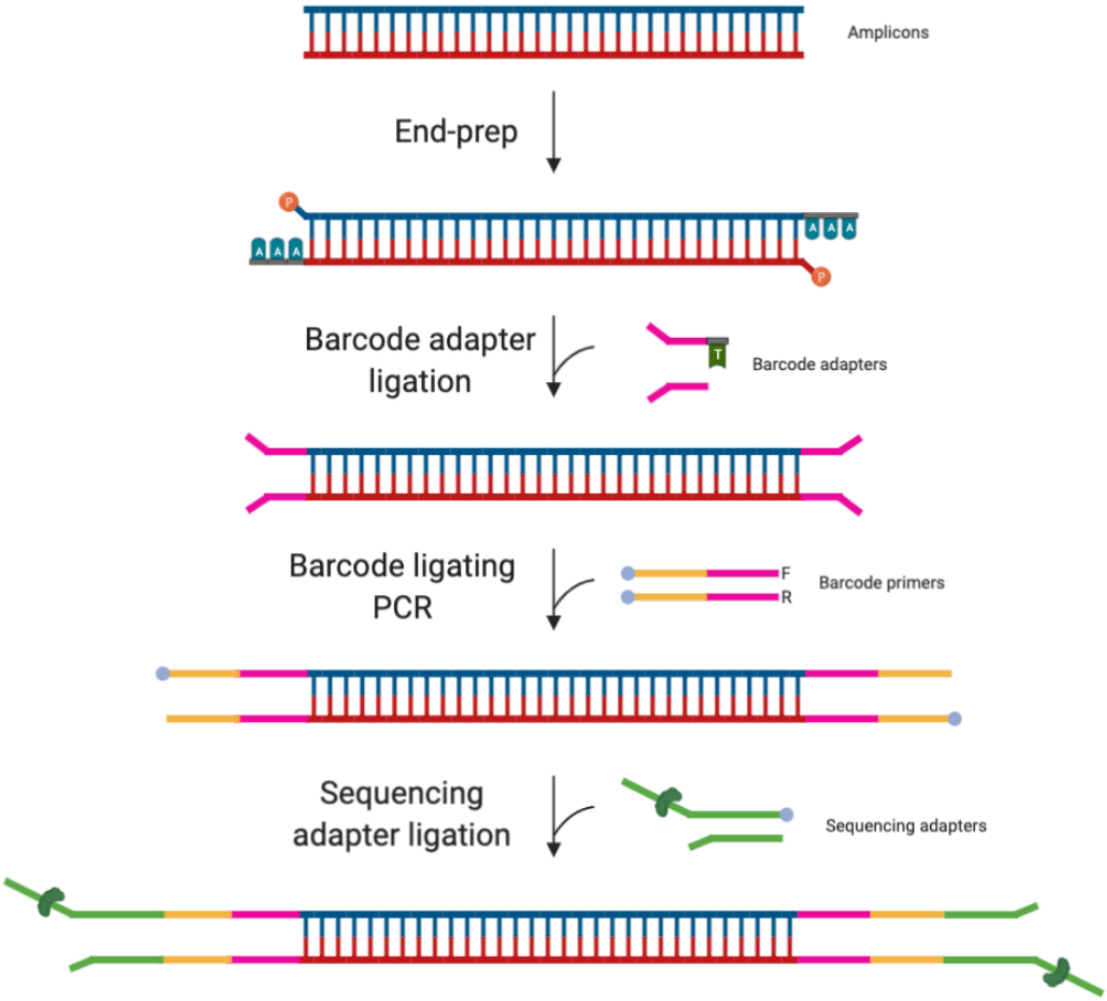
Table 1.1: **Selected PCR-based assays for detection of MS, MG, and ILTv**. Conventional PCRs, restriction fragment length polymorphism (RFLP) PCRs, and quantitative PCRs (single target and multiplex) for each agent and their targets are summarized.

	Agent	Target	Reference
Conventional PCR	MS	16S rRNA	[147]
	MS	*vlhA*	[28]
	MS	16S-23S rRNA intergenic spacer region	[148]
	MG	16S rRNA	[60]
	MG	*mgc2*	[60]
	MG	LP	[60]
	MG	*gapA*	[60]
	MG	16S-23S rRNA intergenic spacer region	[55]
	ILTv	TK	[149, 150]
RFLP-PCR	MS	*vlhA*, 16S rRNA	[151, 152]
	MG	*mgc2*, lipoprotein (lp), 16S rRNA	[152-154]
	ILTv	Multi-loci	[155-158]
Quantitative PCR	MS	16S-23S rRNA intergenic spacer region	[159]
	MG	*mgc2*	[159]
	MG	MGLP (MGA0319)	[59, 160]
	MG	16S rRNA	[161]
	MG	*gapA*	[162]
	ILTv	gC	[163]
	ILTv	UL15	[103]
	ILTv	TK	[164]
Multiplex qPCR	MS/MG	16S rRNA	[165]
	MS/MG	MS *vlhA*/MG *mgc2*	[106]
	ILTv/chicken	ILTv UL44/ chicken *α2*-collagen	[166]

Table 1.2: **Common genomic regions used for targeted sequencing or MLST analyses of MS, MG, or ILTv**.

Agent	Target	Reference
MS	*vlhA*	[29, 30, 167, 168]
	16S-23S rRNA intergenic spacer region	[34]
	nanA	[32]
	uvrA	[32]
	lepA	[32]
	ruvB	[32]
	ugpA	[32]
	gmk	[123]
	ppa	[123]
	nagC	[123]
	atpG	[123]
	efp	[123]
	recA	[123]
	adk	[123]
MG	*pvpA*	[58]
	gapA	[58]
	mgc2	[58]
	MGA_0312	[58]
	16S-23S rRNA intergenic spacer region	[55]
	atpG	[126]
	dnaA	[126]
	fusA	[126]
	rpoB	[126]
	ruvB	[126]
	uvrA	[126]
ILTv	ICP4	[169-172]
	gB	[144, 172]
	gM	[144]
	UL47/gG	[144]
	gG	[170]
	gD	[170]
	gJ	[170, 172]
	UL54	[172]
	UL52	[172]
	ICP18.5	[172]

Figure 1.1: **Library preparation for amplicon nanopore sequencing with PCR barcoding**. Figure adapted from the ONT SQK-PBK004 workflow illustration and created with BioRender.com.



CHAPTER 2

OPTIMAL MEDIA FOR TRANSPORT AND SAMPLE PREPARATION FROM TRACHEAL SWABS FOR THE DETECTION OF *MYCOPLASMA SYNOVIAE* AND *MYCOPLASMA GALLISEPTICUM* VIA QUANTITATIVE PCR [1]

[1] Jude, Rachel and Naola Ferguson-Noel. To be submitted to *Avian Diseases*.

Key words: *Mycoplasma gallisepticum*, MG, *Mycoplasma synoviae,* MS, transport media, BHI, PCR, real-time PCR

Abbreviations: BHI = brain-heart infusion; C_T = cycle threshold; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; PBS = phosphate-buffered saline; PCR = polymerase chain reaction.

Summary

Avian mycoplasmosis in commercial poultry, resulting from *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) infection, is the cause of severe economic losses worldwide. Early detection is a key component of a Mycoplasma control program and quantitative PCR can be an important tool in this respect. In this study, five common media used in the transport and preparation of avian mycoplasma samples were evaluated for their effect on diagnostic sensitivity of MG and MS quantitative PCRs. The media tested included mycoplasma media (modified Frey's broth (FMS)), brain heart infusion (BHI) broth, PCR grade water, phosphate buffered saline (PBS), and a modified PBS (mPBS). In this study, there were significant differences in the detection of MG and MS from cultures or tracheal swabs prepared in the various types of media ($P < 0.05$). With the results of this study in mind, BHI should be the media of choice in transporting and preparing samples for MS and MG qPCR.

Introduction

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) are economically significant respiratory pathogens with worldwide prevalence in poultry. MG and MS can be transmitted vertically *in ovo* and horizontally by contact with infected birds and fomites [1, 2]. Control of these pathogens has primarily been achieved by isolation, biosecurity, and maintaining breeding stock free of infection. Implementing robust programs for control of these pathogens relies heavily on early detection via sensitive and rapid molecular diagnostics.

Routinely, MS and MG infection is screened via a combination of serological tests, including serum plate agglutination (SPA), hemagglutination-inhibition (HI), and enzyme-linked immunosorbent assay (ELISA) [3]. Serological results are then confirmed through isolation and identification of the organisms or through DNA based detection methods [2, 4]. Diagnosis by culture and PCR requires swabbing of the trachea, choanal cleft, air sacs, or joint lesions [3]. Detection of MS and MG genomes by conventional PCR has been previously described [5-7]. However, the development and implementation of quantitative PCR assays [8-11] have allowed for much more rapid and sensitive detection of MS and MG genomes. High throughput capabilities of quantitative PCR protocols also allows for their use in early detection screening of pathogenic avian mycoplasma – not just as confirmatory tests. However, inappropriate collection, handling and processing of samples may dampen the benefit of quantitative PCR assays to detect minute amounts of the pathogen genome [12, 13]. Swab transport systems should be able to maintain the viability of the microorganisms present, and the swab should allow for release of a sufficient quantities of the specimen materials well as preserve the integrity of nucleic acids for amplification (PCR) testing.

Another consideration is that, in routine poultry diagnostics, swabs are often processed for multiple PCR tests (e.g., respiratory viruses in addition MG and MS). So, care needs to be taken when choosing the appropriate media for swab transport and preparation for optimal detection of many different pathogens. The primary goal of this study was to compare five media that may be used to transport and/or prepare tracheal swab samples for molecular diagnostics. Brain heart infusion (BHI) broth, mycoplasma media (modified Frey's broth (FMS)), PCR grade water, phosphate buffered saline (PBS), and a modified PBS (mPBS) were evaluated for the effect on sensitivity of MG and MS quantitative PCR. We expect PBS to perform better as a transport and extraction preparation media than BHI, PCR grade water, mycoplasma media (FMS), and modified PBS when evaluating the sensitivity of MS and MG qPCR.

Materials & Methods

Media. Brain heart infusion (Oxoid Ltd. Basingstoke, Hampshire, England), phosphate buffered saline (1X sterile filtered, HyClone Laboratories, Inc. Logan, Utah), modified phosphate buffered saline, mycoplasma media (FMS), and nuclease-free molecular grade water (HyPure™, HyClone Laboratories, Inc. Logan, Utah) were prepared and/or stored under sterile conditions. The modified phosphate buffered saline consisted of 98% 1X sterile PBS, 2% calf serum (Gibco, Grand Island, New York), and 2% 100X antibiotic/anti-mycotic containing streptomycin, amphotericin B, and penicillin (Gibco, Grand Island, New York). The modified Frey's broth (FMS) included swine serum, L-cysteine, and nicotinamide adenine dinucleotide (NAD) and was prepared according to Ferguson-Noel and Kleven [3].

Mycoplasma Isolates. Well characterized cultures of MG strain R_{low} [14] and MS WVU 1853 [15] were obtained from the Mycoplasma laboratory repository in the Department of Population Health at the University of Georgia, Athens, GA. The visibly growing cultures were

serially diluted in FMS to obtain five dilutions of 10^{-2} to 10^{-6} of each *Mycoplasma sp.* Samples for extraction were obtained by adding 100µL of each diluted culture to 900µL of each media to be tested (BHI, water, PBS, mPBS, and FMS).

Tracheal Swabs. Tracheas from chickens previously determined to be positive for MS (n=30) and MG (n= 30) were collected from experimentally and naturally infected chickens and turkeys. Tracheas were kept frozen at -70°C until they were used in this study. The majority of tracheas originated from chickens that had been inoculated with virulent strains of MS (K1968) or MG (R_{low}) and were originally utilized to obtain antisera. The remaining tracheas utilized in this study were obtained from MG or MS positive chickens and turkeys submitted to the Poultry Diagnostic and Research Center (PDRC) diagnostic laboratory. Detailed information on the source of the tracheas can be found in Table 2.1. The tracheas were thawed to room temperature before processing. Each trachea was swabbed four times and the swab resuspended into 1.5 mL of each media that had not been ruled out by the culture portion of the trial (BHI, water, PBS, and mPBS). Swabs were not pre-moistened and the order in which the tracheas were swabbed for each particular media was rotated throughout. To simulate transport time, the preparations were stored 24 hours at 4°C before they were extracted.

Nucleic Acid Extraction. Two hundred microliter aliquots of each media were extracted in triplicate using the Omega Mag-Bind® Viral DNA/RNA extraction kit (Omega Bio-Tek, Inc., Norcross, Georgia) on the MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems, Foster City, California) following the manufacturer's instructions. In the final step, nucleic acid was eluted in 50µL of the provided elution buffer.

Quantitative PCR. qPCR was performed in triplicate on all extracts on the Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies, Foster City, California)

using a 25μL assay of 12.5μL 2X QuantiFast Probe PCR Master Mix with ROX dye (Life Technologies, Carlsbad, California), 1μL reverse primer (12.5 μM), 1 μL forward primer (12.5 μM), 3μL nuclease free molecular grade water, 2.5 μL probe (1 μM), and 5μL template DNA. Primers and probes for MS and MG qPCR have previously been developed [10, 16]. The thermal profile used for both MS and MG amplification and C_T determination has been described [10]. To make the assays quantitative, plasmids were constructed containing the genome targets (MGA_0319 (MG) and 16S–23S rDNA ISR (MS)) and standard curves were developed for each qPCR. The procedures used in constructing the DNA controls and standard curves for quantitation have been described in detail elsewhere [17, 18]. The mean C_T value for each sample was converted to an estimate of mean genome copy numbers (MCN) in the sample using the standard curves.

Statistics. C_T values were converted to mean genome copy numbers (MCNs) for all statistical analysis except when measuring positive rates. Positive rates between medias used in the culture dilution study were compared using the Cochran Q test. If differences were found via the Cochran Q test, the positive rates between pairs of medias at the same dilution were compared via McNemar's test. Differences in MCN values between positive media (MS and MG) and between dilutions of the medias were tested using a linear mixed model. For the tracheal swab data, a linear mixed model tested for differences in MCN values between media used for MS and MG separately. Each linear mixed effects model included a fixed factor for media and a random intercept for each chicken to account for within chicken correlation. Multiple comparisons were adjusted for alternatively using Tukey's test and Dunnett's test. The analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC 27513).

Results

Cultures. The percent of positive qPCR results over culture dilutions 10^{-2} to 10^{-6} are illustrated in Figures 1A and 1B. The log10 MCN (mean genome copy number) for serial dilutions of pure cultures of R_{low} (MG) and WVU 1853 (MS) in the five available liquid media used in common swab transport systems are presented in Table 2.2. For the MG 10^{-5} dilution and the MS 10^{-6} dilution, Cochran's Q test was significant and therefore McNemar's paired comparisons were possible (when results were not 100% or 0%). For MS, the cultures were consistently 100% positive until reaching the 10^{6} dilution, where all medias resulted in a drop in positive results by qPCR. However, McNemar's test indicated there were no significant differences between paired media at this dilution (10^{-6}). With respect to FMS and BHI (both 0% at the 10^{-6} dilution), the MCNs for MS were higher overall for BHI at all other dilutions (Table 2.2). For MG, notable differences in positive cultures can be seen at the 10^{-5} dilution, with FMS and PBS medias reading all negative on real-time PCR. MG MCNs were lowest for these two media at the 10^{-5} dilution. Additionally, at this dilution, water performed significantly better than BHI ($P<0.05$, McNemar's test). Of the five media tested, FMS was identified overall as the worst performing when combining the data for both MS and MG cultures in terms of percent positive cultures detected together with average MCN log 10 values. The differences were relatively small incrementally, with FMS resulting in 68% positive overall and BHI, PBS, mPBS and water resulting in 70%, 71%, 76% and 81% positive respectively.

Tracheal Swabs. All samples exhibited C_T values <37.5 and were therefore considered positive. Figure 2.2 reports the mean genome copy number (MCN) recovered for tracheas prepped in each media. For MS, the MCN values for PBS and mPBS were not significantly different from each other. MCN values for BHI were significantly different from all other media

(P< 0.01, Tukey test). The MS qPCR assays using BHI as the preparation media were more sensitive and those using water less sensitive than qPCRs of all other media for detection of MS from tracheal swabs. For MG detection, the results with PBS and mPBS were again not significantly different from each other (P<0.01); however, they performed worse than water and BHI. As with detection of MS from tracheal swabs, BHI again outperformed all other media significantly (P< 0.01, Tukey test) for MG detection.

Discussion and Conclusion

In this experiment we investigated two different sample types – pure cultures and tracheal swabs, and although *Mycoplasma* isolates are not a common sample type submitted to poultry diagnostic labs for PCR testing, conducting the first part of this trial with isolates allowed us to compare relatively "clean" and well-defined samples while avoiding the variables that can be encountered when testing tracheal swabs (a much more common sample type).

After evaluation of MCN values obtained from MG and MS cultures diluted in the different media, FMS was eliminated from further testing with known MS or MG positive tracheal swabs. FMS was likely inhibitory due to the media containing multiple components, including a pH indicator dye, phenol red. The Applied Biosystems® 7500 Fast Real-Time PCR System requires a passive reference dye, ROX, to be used, so the addition of phenol red may have increased background fluorescence and caused measurements to be inaccurate. As the culture samples were prepared by diluting mycoplasma FMS cultures in the various medias, combining of media components from both FMS and BHI may have resulted in enhanced inhibition of qPCR of BHI prepared cultures when a similar effect was not noticeable with PBS or mPBS. Examples of inhibitors include proteins [19] and heme [20] but PCR inhibition is a

complex phenomenon; the same inhibitory substance(s) might not always be equally inhibitory for all PCR reactions or initial sample types [21].

For preparation of MG cultures for extraction and quantitative PCR, water performed significantly better than all other tested media in terms of log10 MCN values. For MG detection from the trachea, water performed slightly better than PBS and mPBS (but not BHI), however it performed worse than all three of the other medias for preparation of MS infected trachea.

Although we estimated the titers of the undiluted isolates to be in the range of 7.0-8.0log10 CCU/ml we did not attempt to titrate the isolates more accurately. We have shown that for glucose fermenting *Mycoplasma* spp. the correlation between color changing units (viable *Mycoplasma* cells) and genome copy numbers is poor once the bacteria are visibly growing (unpublished data). It is likely that the acid production resulting in the pH shift associated with visible growth also increases the rate of *Mycoplasma* cell death, so that the titer of viable cells remains similar over time even as the number of genomes (viable and non-viable cells) continues to increase.

For the tracheal swabs tested, it should be noted that there were slight variations in the strains of MS, the avian species with MG, and the age of the birds (MS and MG). The age and species of the birds could have a slight skewing effect on the results should the size of the trachea swabbed be a contributing factor for the load of mycoplasma in the tissue. A larger trachea may have little or no variation in qPCR results when swabbed four times, whereas smaller tracheas could result in lower amounts of mycoplasma being picked up by later swabs. Differences in strains of MS are unlikely to have caused inconsistencies in this study, as only one was S-56 while all others were K1968.

There is inherent variability in swabbing tracheas, and it is extremely difficult to consistently recover the same amount of material on each swab; in this research the same operator swabbed all of the tracheas in an attempt to reduce some of this variability. Including a sufficiently large sample size may also aid in reducing the effects of this variability. Each trachea in this study was swabbed 4 times (once for each media tested) in a rotating order to reduce bias. We have previously shown that frozen tracheas can be swabbed multiple times (more than 7) without a significant reduction in the quantity of Mycoplasma detected in subsequent swabbings (unpublished data).

It should also be mentioned that processes downstream from swab transport media may affect DNA recovery or PCR amplification. There may be differences in DNA extraction efficiency among samples or efficiency of amplification differences among individual PCR reactions [22]. We did not include internal PCR controls or attempt to quantify the actual amount of DNA (including chicken DNA) that was collected or released by the different swab samples. It may be useful to include internal controls in futures studies to more accurately estimate potential inhibitory effects of the media on mycoplasma nucleic acid quantity and integrity so the differences in qPCR results can be better associated to the status of the nucleic acid. The tracheal samples in this study were stored for 24 hours at 4°C to simulate overnight transportation and the mycoplasma culture samples were not stored before the extraction procedure. Different storage times and/or temperatures may greatly affect the integrity of nucleic acid from the samples. It is possible the nucleic acid may degrade at different rates in different transport media over time and at different temperatures [23, 24].

In this study, we showed that water gave the best results with the broth cultures whereas BHI was superior to the other media types with tracheal swab samples. This demonstrates the

one of the possible pitfalls associated with using "artificial" samples in this research; there may be factors in "real" samples that cannot be easily recognized or replicated.

In concluding this study, we have validated that BHI should be the media of choice in transporting and preparing samples for MS and MG qPCR. While Mycoplasma DNA was amplified from the tracheal samples in all of the transport media, the water, PBS, and modified PBS appear to inhibit the qPCR amplification for both MS and MG by one to three C_T values (the equivalent of approximately 0.3 to 0.8 MCNlog10). Historically, BHI has been used as a component in standard medium for mycoplasma growth [25], so it is possible it performed better by elevating the growth of mycoplasma slightly during the overnight refrigeration storage used to mock transportation.

This information will allow the poultry industry to maximize the benefits of costly diagnostic assays and set scientifically based standards in different laboratories for sample handling for MG and MS quantitative PCR. BHI has already been confirmed as a superior media in which to transfer avian influenza (AI) samples compared to PBS [26]. BHI (or PBS) is also recommended for Infectious Bronchitis virus testing [27]. Using BHI as the transport medium will reduce diagnostic costs when the poultry industry is sampling for both mycoplasma infections and other respiratory pathogens. In further studies, we would like to also verify that BHI would be an adequate medium in which to transport other relevant respiratory pathogens, such as avian infectious laryngotracheitis (ILT) herpesvirus.

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Table 2.1: **Bird and strain information for collected tracheas**. Information pertaining to each trachea or group of trachea in terms of strain of MS or MG, species and age of bird(s), and number of trachea selected from each group for testing. Note the trachea from the chicken with S-56 and the two turkey tracheas with R_{low} were from diagnostic cases. All other tracheas were from chickens used in previous research and/or antisera panels.

	Strain	**Species**	**Age (weeks)**	**No. of Tracheas**
MS	K1968	Chicken	8	1
	K1968	Chicken	15	5
	K1968	Chicken	8	4
	S-56	Chicken	5.4	1
	K1968	Chicken	8	19
MG	R_{low}	Chicken	11	5
	R_{low}	Turkey	34	2
	R_{low}	Chicken	24.2	23

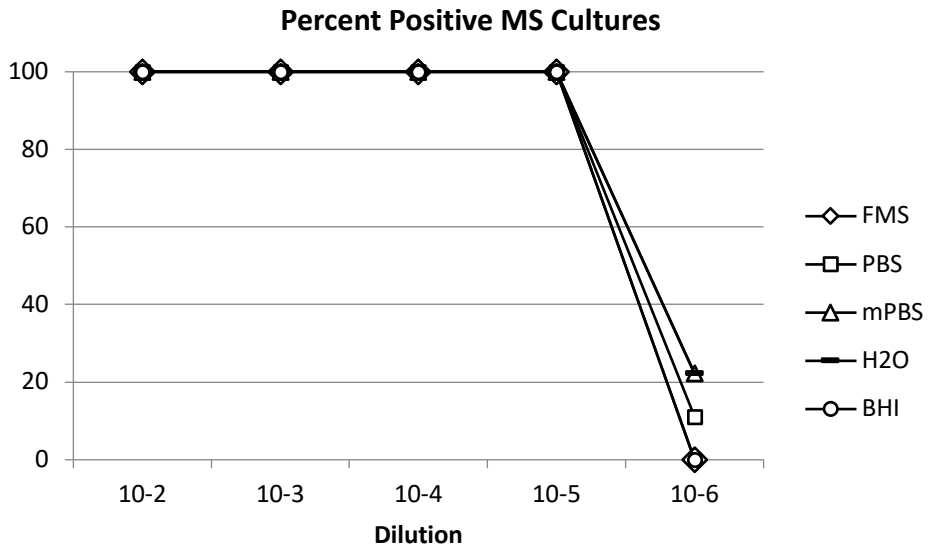
Table 2.2: **Effect of media on cultures detected via qPCR**. Number positive and mean genome copy numbers (MCNs) from quantitative PCR of MG and MS cultures (mean of nine replicates) in each media.

Organism	Dilution Factor	FMS	PBS	mPBS	Water	BHI
MS	**10^{-2}**	9/9[A] (3.76 ± 0.10)[B]	9/9 (4.17 ± 0.08)	9/9 (4.27 ± 0.03)	9/9 (4.15 ± 0.05)	9/9 (3.99 ± 0.03)
	10^{-3}	9/9 (2.66 ± 0.09)	9/9 (3.11 ± 0.05)	9/9 (3.15 ± 0.07)	9/9 (3.17 ± 0.06)	9/9 (2.78 ± 0.12)
	10^{-4}	9/9 (1.59 ± 0.05)	9/9 (1.84 ± 0.08)	9/9 (2.14 ± 0.03)	9/9 (2.08 ± 0.05)	9/9 (1.58 ± 0.04)
	10^{-5}	9/9 (0.50 ± 0.13)	9/9 (0.81 ± 0.16)	9/9 (0.87 ± 0.14)	9/9 (0.90 ± 0.10)	9/9 (0.71 ± 0.21)
	10^{-6}	0/9	1/9 (0.05)	2/9 (0.07 ± 0.10)	2/9 (0.07 ± 0.10)	0/9
MG	**10^{-2}**	9/9 (3.23 ± 0.19)	9/9 (3.68 ± 0.15)	9/9 (3.59 ± 0.10)	9/9 (4.07 ± 0.05)	9/9 (3.09 ± 0.09)
	10^{-3}	9/9 (1.77 ± 0.67)	9/9 (2.35 ± 0.14)	9/9 (2.28 ± 0.11)	9/9 (2.61 ± 0.13)	9/9 (2.32 ± 0.16)
	10^{-4}	7/9 (0.77 ± 0.45)	9/9 (1.25 ± 0.35)	7/9 (0.82 ± 0.48)	9/9 (1.30 ± 0.20)	6/9 (0.68 ± 0.54)
	10^{-5}	0/9	0/9	5/9 (0.22 ± 0.21)	7/9 (0.32 ± 0.28)	3/9 (0.16 ± 0.28)
	10^{-6}	0/9	0/9	0/9	1/9 (0.04)	0/9
Total Positive (MG+MS)		61/90 (68%)	64/90 (71%)	68/90 (76%)	73/90 (81%)	63/90 (70%)

[A]No. positive/No.tested
[B]Mean (genome) Copy No. Log 10 (± SD)

Figure 2.1. **Percent positives detected via qPCR of cultures prepared in media**. Percent positives via (A) MS and (B) MG qPCR of extracted culture dilutions 10^{-2} to 10^{-6} in the original five tested media: FMS, PBS, mPBS, water, and BHI.

A



B

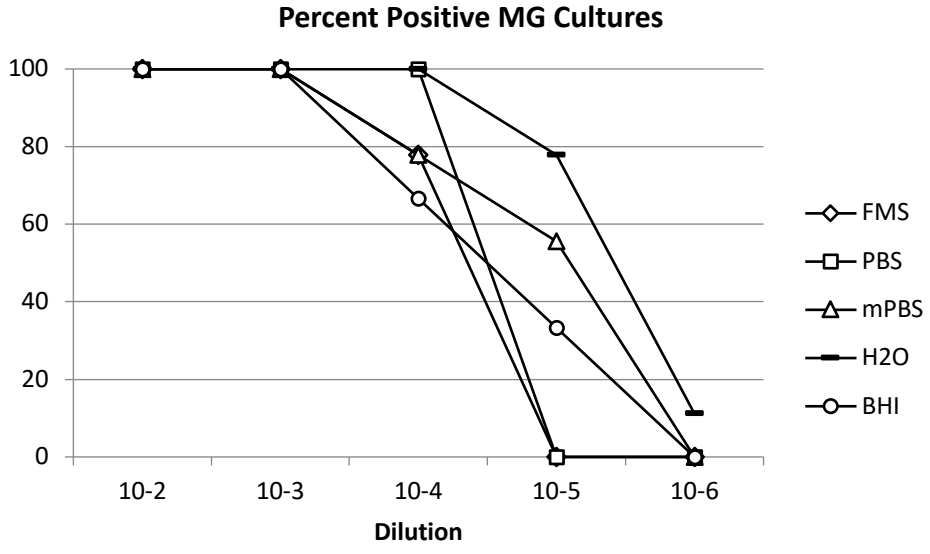
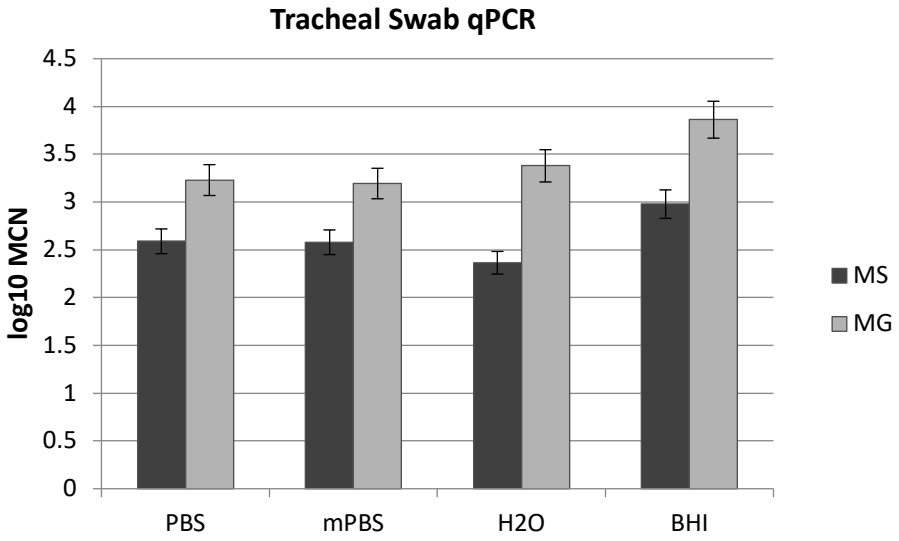


Figure 2.2: **Effect of preparation media on tracheal swabs detected via qPCR**. Mean genome copy number (log10) for MS and MG qPCR of tracheal swabs in the four-remaining media: PBS, mPBS, water, and BHI.



CHAPTER 3

OPTIMAL SWAB COLLECTION SITE FOR *MYCOPLASMA SYNOVIAE*, *MYCOPLASMA GALLISEPTICUM*, AND INFECTIOUS LARYNGOTRACHEITIS VIRUS DETECTION BY QUANTITATIVE PCR [1]

[1] Jude, Rachel and Naola Ferguson-Noel. To be submitted to *Avian Diseases*.

Key words: *Mycoplasma gallisepticum*, MG, *Mycoplasma synoviae,* MS, infectious laryngotracheitis virus, ILTv, PCR, real-time PCR, swab, choanal cleft

Abbreviations: C_T = cycle threshold; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; PBS = phosphate-buffered saline; PCR = polymerase chain reaction, MCN = mean genome copy number

Summary

Mycoplasma synoviae (MS), *Mycoplasma gallisepticum* (MG), avian influenza (AI), and infectious laryngotracheitis virus (ILTv) are economically significant respiratory pathogens affecting chickens. There is increasing need for rapid detection of relevant respiratory pathogens in order to properly diagnose flocks suspected of disease. For this purpose, the ability to test for multiple pathogens from a single sample would be beneficial. Here, the effect of swab collection sites for the detection of MS, MG, and ILTv via quantitative PCR was tested. As a whole, the choanal cleft was determined to be the optimal site for MS, MG, and ILTv detection in terms of percent positives returned via qPCR. Determining that a swab of a single location will be adequate to successfully detect each of these pathogens will improve diagnostic testing in terms of time and money, will enhance animal welfare, and limit the burden of sampling for poultry producers.

Introduction

Mycoplasma synoviae (MS), *Mycoplasma gallisepticum* (MG), avian influenza (AI), and infectious laryngotracheitis virus (ILTv) are economically significant respiratory pathogens affecting chickens. Control of these pathogens relies on exceptional biosecurity and prompt identification of causative agents of disease. Rapid, accurate diagnosis of affected flocks is essential to informed action in terms of treatment, isolation, or elimination of flocks. The development and implementation of quantitative PCR assays facilitates sensitive screening for early detection of these pathogens [1-4].

In routine poultry diagnostics, swabs are often processed for multiple PCR tests (e.g., respiratory viruses in addition MG and MS) and but there is no consistency or empirical information on sampling sites for all of these pathogens. Additionally, the ability to test for multiple pathogens from a single sample would be beneficial. Determining if a swab of a single location will be adequate to successfully detect each of these avian respiratory pathogens will improve diagnostic testing in terms of time and money, will enhance animal welfare, and ease sampling burden for poultry growers. Typically, tracheal swabs or tracheal scrapings are taken for MS, MG, and ILTv nucleic acid detection [5], while oropharyngeal swabs are taken for AI [6-8]. For chronic infections of MS and MG, it is more reliable to isolate from the upper respiratory tract (trachea, choanal cleft) where the organisms tend to persist as opposed to isolation from lesions [9, 10]. Here, the effect of swab collection sites for detection of MS, MG, and ILTv via quantitative PCR was tested. It was hypothesized that tracheal swabs would perform best in terms of sensitivity of MS, MG, and ILTv quantitative PCR compared to swabs of choanal cleft and oropharynx.

Materials & Methods

Inoculates: Cultures of *Mycoplasma synoviae* strain K6677 and *Mycoplasma gallisepticum* strain R were obtained from the Mycoplasma laboratory depository in the Department of Population Health at the University of Georgia, Athens, GA. R-strain is a well characterized virulent MG strain [11]; K6677 is a recently isolated virulent MS strain (unpublished data). Isolates were grown to exponential phase denoted by color change in Frey's modified broth medium at 37°C. For ILTv, the Zoetis Laryngo-Vac® modified-live freeze-dried vaccine was reconstituted according to the manufacturer's instructions for 1 dose per 100 µL.

Birds and Inoculations. At one and three weeks of age, 43 broiler-type chickens were inoculated with virulent MS strain K6677. The MS inoculation consisted of 100 µL applied to the right eye and 100 µL into the trachea. At 3 weeks of age, 39 broiler-type chickens were inoculated at with MG R strain. Birds were inoculated with MG R strain via approximately 1 mL aerosol spray per bird. The birds were then co-infected intraocularly (left eye) with 100 µL of the Zoetis Laryngo-Vac® modified-live freeze-dried vaccine at 5 and 6 days post final MS or MG inoculation, respectively.

Sampling and Processing. Two and six weeks post final MS and MG inoculation, the birds were swabbed in three sites (trachea, choanal cleft, and oropharynx, see Figure 3.1) in random, rotating order. Swabs were stored at -20°C until they were prepared by gently vortexing in 1.5 mL of PBS. Total DNA was extracted via the MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems, Foster City, California) and Omega Mag-Bind® Viral DNA/RNA extraction kit (Omega Bio-Tek, Inc., Norcross, Georgia) according to the manufacturer's instructions at a final elution of 50 µL.

Quantitative PCR assays were performed on extracted DNA samples in triplicate (each

sample was run 3 times for each of the assays) on the Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies, Foster City, California) using previously developed assays for MS, MG, ILTv, and collagen detection [1-3]. C_T values above 37 were considered negative. To make the assays quantitative, plasmids were constructed containing the genome targets as standard DNA controls. The procedures used in constructing the DNA controls and standard curves for relative quantitation have been described in detail elsewhere [12]. Using the straight-line formulas developed from the plasmid standard curves for each target, the mean C_T value for each sample (mean of three C_T values) was converted to an estimate of mean genome copy numbers (MCN) in the sample.

Statistical Analysis. Cycle threshold (C_T) values were calculated for the triplicate results from each swab and the mean C_T values for each collection site was determined. Log10 mean genome copy numbers (MCNs) were determined from the C_T values using in-house calculations of standard curves [12, 13] for the quantitative PCR assays used regularly in diagnostic detection at PDRC [1, 3]. The log10 MCNs were compared using the Tukey-Kramer HSD test with the significance level set at $\alpha = 0.05$. These analyses were performed using JMP® Statistics Made Visual (SAS Institute Inc., Cary, NC 27513) and SAS V 9.2 (Cary, NC 27513). The results were analyzed separately for all three groups of birds.

Results

Mycoplasma synoviae. All swabs at 2- and 6-weeks post MS inoculation were positive for all sampling locations. However, the proportion of weakly positive samples (C_T >30) was higher in the samples from the oropharynx at both time points, with 63% and 90% of the swabs being weak positive or negative at 2- and 6-weeks post inoculation respectively; compared to only 12% and 20% for the choanal cleft swabs, and 7% and 34 % negative or weak positives

from the trachea at these time points. The mean C_T values of MS positive swabs was lower for the trachea (27.2) and choanal cleft (28.0) sites than the oropharynx (30.7); and, correspondingly, estimates of the amount of MS DNA detected (MCNlog10) was significantly higher in the swabs from the trachea and choanal cleft (3.4 log10 and 3.1 log10 respectively) compared to swabs from the oropharynx (2.3 log10) ($P<0.05$) . Similar results were seen in samples collected at six weeks post inoculation. Significantly higher MCN values were obtained from the trachea and choanal cleft swabs (2.6 log10 and 2.9 log10 respectively) compared to swabs from the oropharynx (1.7 log10) ($P<0.05$) . (Table 3.1 and Figure 3.4).

Mycoplasma gallisepticum. At 2 weeks post MG inoculation, with the exception of one tracheal swab, tracheal, choanal cleft and oropharyngeal swabs were positive for MG. Similar to the MS results, the tracheal swabs taken during early in the infection yielded the lowest mean C_T value (25.5) and significantly more MG DNA was detected in tracheal swabs compared to those from the oropharynx and choanal cleft (3.9 log10 vs 3.4 and 2.9 log10) (($P<0.05$). Yields of MG DNA from choanal cleft swabs were also significantly higher than for oropharyngeal swabs ($P<0.05$). Again, the highest proportions of weakly positive samples were seen in oropharyngeal swabs (18%).

At 6 weeks post MG inoculation, the choanal cleft swabs returned a greater percentage of positives (94%) compared to tracheal and oropharyngeal swabs (53% and 35%, respectively); and MCN was also higher (2.1 log10) for the choanal cleft swabs compared to the oropharyngeal and tracheal swabs (1.0 log10 and 0.6log10, respectively). The differences in percent positive as well as MCN were statistically significant when the choanal cleft swabs were compared to the other sampling sites ($P<0.05$). Ninety-four percent of samples from the

oropharynx samples were weakly positive or negative compared to 85% and 68% of swabs from the trachea and choanal cleft, respectively.

Infectious laryngotracheitis virus. Several of the swabs at 1- and 5-weeks post ILT vaccination were negative or weak positives ($C_T > 30$). No ILTv was detected from any sample site in the birds that were co-inoculated with MG. The results from birds co-infected with MS are presented in Table 3.1 and Figure 3.4. At both early and late time points, the choanal cleft swabs had the highest percentage of positives (68% and 71% respectively) and highest MCN values (0.6 log10) compared to tracheal and oropharyngeal swabs. The difference in percent positives and MCN values between the choanal cleft and the trachea were not significant at 1 week post vaccination, however at 5 weeks post vaccination the number of positives from the trachea dropped to 21%, compared to 71% from the choanal cleft; the difference was statistically different at this time point ($P<0.05$). With respect to oropharyngeal swabs, there were statistical differences in percent positives as well as MCN values compared to both trachea and choanal cleft sites at 1 week post inoculation. At 5 weeks post inoculation, oropharyngeal swabbing yielded slightly better (but not significant) results than swabs from the trachea ($P<0.05$).

Discussion and Conclusion

Results from this study indicate that, unless all of the samples are 100% positive (as with the MS samples), choanal cleft swabs yield the highest percentage of positives among the sampling sites during both early and late infection with both MG and ILT. In the early sampling, the trachea seems to be comparable to swabs from the choanal cleft; these swabs yielded higher MCN values for MG and MS, indicating the detection of more MG or MS DNA in swabs from this site, and with MG the result was statistically significant ($P<0.05$). Understanding the replication dynamics and pathogenesis of the organisms involved in the disease syndrome will

aid in determining the preferred site for sampling in an early or later stage of the infection. *Mycoplasmas* tend to be found in high numbers on mucosal surfaces bound to epithelial cells, although both MG and MS are also capable of intracellular invasion [14, 15]. *Mycyoplasma* infection tends to result in marked thickening of the mucous membranes of affected respiratory tract tissues (as a result of infiltration with mononuclear cells)[16]. Mycoplasma can generally be detected in the upper respiratory tract (generally at lower levels in chronic infections for the life of the flock[17]. Conversely, infections with ILTv result in sloughing, degeneration, and necrosis of the tracheal epithelium [18]. It has been reported that during the acute phase of the disease, (2–6 dpi), the virus is commonly isolated from the tracheal epithelium and then the virus disappears from the trachea [19, 20]; however, low levels of viral genomes can be detected by PCR in tracheal swabs 20 to 60 days after vaccination [21, 22]. These factors may lead to differences in recovery of pathogen DNA from different locations and time in the infectious process. We saw that more DNA could be detected by qPCR from the trachea for MG and MS earlier in the infection, but during late infection the choanal cleft site was superior in terms of higher proportion of positives and lower proportions of weakly positive (high C_T) results. For ILT, the results were slightly different – earlier in the infection the trachea and choanal cleft seemed to be equally effective sampling sites; however, after clearance of the lytic ILTv infection the choanal cleft was significantly better than the trachea ($P<0.05$). Different samples have been evaluated for the detection of ILTv DNA by PCR including tracheal scrapings [23], tracheal swabs [24], and conjunctival swabs [25]. It may be difficult to draw conclusions for ILTv detection from this study as nearly all samples were negative for all swab sites at both one- and five-weeks post vaccination. These results can be attributed to the nature of an ILTv infection via vaccination since viral DNA is shown to peak in the trachea between 4 and 6 days

post vaccination or infection [20, 26-28] but tends to disappear from this location by 7-8dpi [19]. Alternatively, ILTv detection could have been lower than expected because of the co-inoculation with either MS or MG. It will be necessary to repeat the ILT portion of this study and have samples taken at even earlier time points for more realistic and reliable results. This said, given the positive results that we did generate, choanal cleft swabs appear to be best in terms of both percent positives and MCN log10 at both 1- and 5-weeks post ILT vaccination. Contrary to these results, a recent study evaluating detection of the Serva ILTv vaccine indicated that choanal cleft swabs would result in fewer positive samples than tracheal swabs, but that genome copies were comparable [29].

Oropharyngeal swabbing performed poorly across the board and is therefore not recommended for MS, MG, or ILTv detection. While oropharyngeal swabs are preferred for isolation of avian influenza due to concerns of invasiveness and skill needed to properly collect tracheal swabs, tracheal swabs were equivalent to oropharyngeal swabs for detection of avian influenza via RT-qPCR [7, 8]. It should be noted that oropharyngeal sampling involves swabbing of the choanal cleft in addition to general swabbing around the oral cavity. So, it is possible that oropharyngeal swabs performed poorly compared to tracheal and choanal cleft swabs if excess mucus buildup had an inhibitory effect on the qPCR or diluted out total pathogen concentration in the samples. We included qPCR protocols targeting chicken collagen [30] in this study (data not shown); this did not show differences in the detection of the chicken gene target that suggested an enhanced PCR inhibition from the oropharynx as compared to trachea or choanal cleft swabs as chicken collagen C_T values were not significantly different among the sites.

As all of testing was conducted in birds that were inoculated with two organisms (MG and ILTv or MS and ILTv), it is possible that co-infection may have altered the usual pathogenic

process compared to a single pure inoculation, not only with ILTv but also with MG or MS. However, we found similar results (unpublished data) when sampling birds inoculated with different strains of MS. The ease of method should be considered when recommending a preferred sampling site; tracheal swabbing requires more skill and time than choanal cleft or oropharyngeal swabbing. In our previous research with MS, samples were taken at necropsy and there was concern that the results would not extrapolate well to taking samples from live chickens in a poultry house. However, it is much more likely for poultry in commercial farms to be infected with multiple pathogens and vaccine strains at the same time so that the protocol conducted here may be more indicative of infections seen in commercial poultry.

There is inherent variability in the swabbing process, and it is extremely difficult to consistently recover the same amount of material even when following the same methodology; in this research the same operator swabbed all of the animals in an attempt to reduce some of this variability. Each animal in this study was swabbed 3 times (once for each site tested) at each sampling time and it is possible that the process of swabbing may increase the presence of PCR inhibitors (such as blood). The chickens in this study were sampled using a rotating order of sampling sites to reduce this potential effect. We found tracheal swabbing to be the most invasive and difficult sampling site.

These results alone are compelling enough to recommend choanal cleft swabbing for diagnostic purposes as it is also arguably the easiest and quickest sampling method. However, if a flock is likely in the early stages of infection, it may be more practical to take tracheal swabs, which return higher genome copies at two weeks post infection. Circumstances will need to dictate if tracheal swabs will be preferred over choanal cleft swabs.

As can be seen from this study, it would be a practical, time saving approach to uniformly swab choanal clefts for diagnostic submissions of MS, MG, and ILTv. Recently, it was demonstrated that choanal cleft swabs and tracheal swabs were comparable in detection of infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) [31], thus adding to the opportunity to narrow down sampling to a singular site for molecular detection of multiple pathogens.

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Table 3.1: **Percent positives, mean C_T values, and MCN values for early and late swabbing of the choanal cleft, trachea, and oropharynx**. Significance of MCN values: a, b, or c.

Time point	Site	MS			MG			ILT		
		No. Positive/No. Tested (% positive)	Mean Ct value^	MCN log10	No. Positive/No. Tested (% positive)	Mean Ct value^	MCN log10	No. Positive/No. Tested (% positive)	Mean Ct value^	MCN log10
Early	Choanal cleft	43/43 (100%)	28.0 ± 1.5	3.1 ± 0.5 [a]	39/39 (100%)	27.4 ± 1.4	3.4 ± 0.5 [b]	28/41 [a] (68%)	34.7 ± 1.8	0.6 ± 0.6 [a]
	Trachea	**43/43** (100%)	**27.2 ± 1.8**	3.4 ± 0.6 [a]	**38/39 (97%)**	**25.5 ± 1.3**	3.9 ± 0.8 [a]	20/41 [a] (49%)	35.8 ± 1.8	0.5 ± 0.6 [a]
	Oropharynx	43/43 (100%)	30.7 ± 2.0	2.3 ± 0.6 [b]	39/39 (100%)	28.8 ± 1.7	2.9 ± 0.6 [c]	7/41 [b] (17%)	35.2 ± 1.9	0.2 ± 0.4 [b]
Late	Choanal cleft	**41/41** (100%)	**28.6 ± 1.5**	2.9 ± 0.5 [a]	**32/34[a] (94%)**	**31.0 ± 2.2**	**2.1** ± 0.9 [a]	**24/34[a] (71%)**	**34.8 ± 2.0**	0.6 ± 0.6 [a]
	Trachea	41/41 (100%)	29.7 ± 2.8	2.6 ± 0.9 [a]	18/34[b] (53%)	32.2 ± 2.6	1.0 ± 1.1 [b]	7/34[b] (21%)	35.6 ± 2.9	0.2 ± 0.4 [b]
	Oropharynx	41/41 (100%)	32.4 ± 1.8	**1.7** ± 0.6 [b]	12/34[b] (35%)	32.2 ± 1.7	0.6 ± 0.9 [b]	12/34[b] (35%)	36.0 ± 2.6	0.3 ± 0.6 [b]

Figure 3.1: **Dissection of chicken's mouth and throat indicating project defined swabbing locations**. In green, the interior upper portion of the trachea. In blue, the choanal cleft. In red, the oropharynx – including the exterior of the larynx, the choanal cleft, and generally around the oral cavity.

Figure 3.2: **Percent positives for swabs of choanal cleft, trachea, and oropharynx for early and late MS, MG, and ILT infections**. wpi=weeks post inoculation, wpv=weeks post vaccination

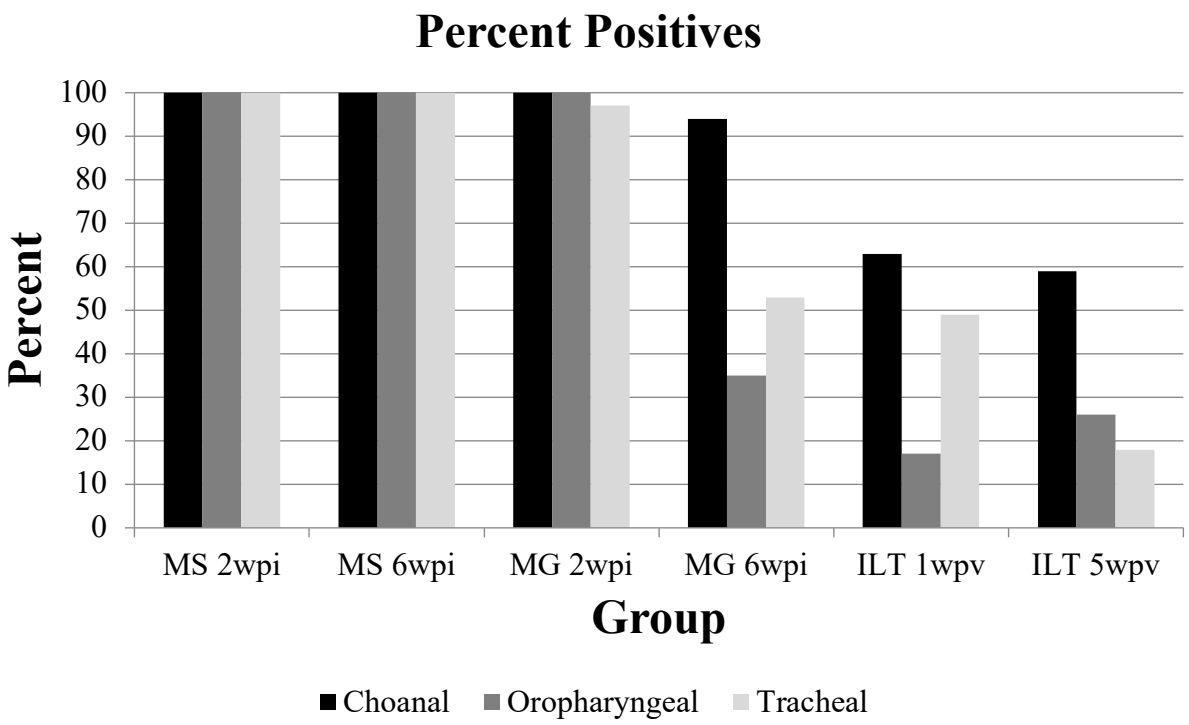


Figure 3.3: **Percent weak positives and negatives for swabs of choanal cleft, trachea, and oropharynx for early and late MS, MG, and ILT infections**. wpi=weeks post inoculation, wpv=weeks post vaccination. Weak positives were determined as C_T values greater than 30.

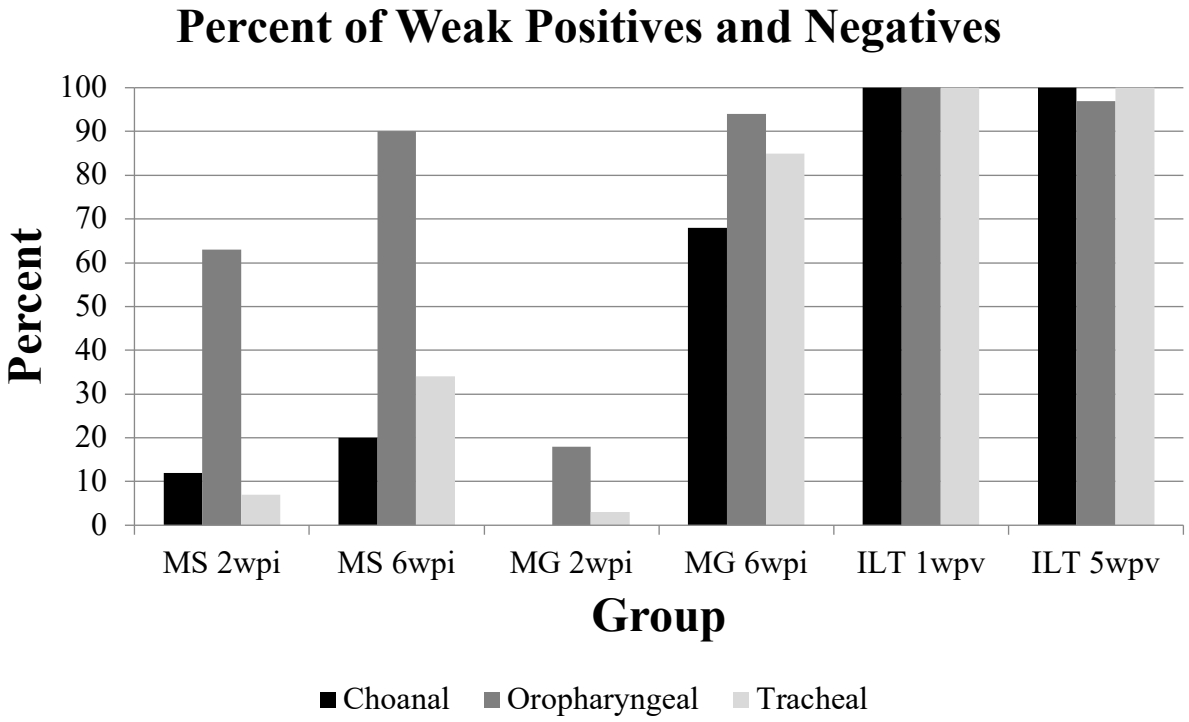
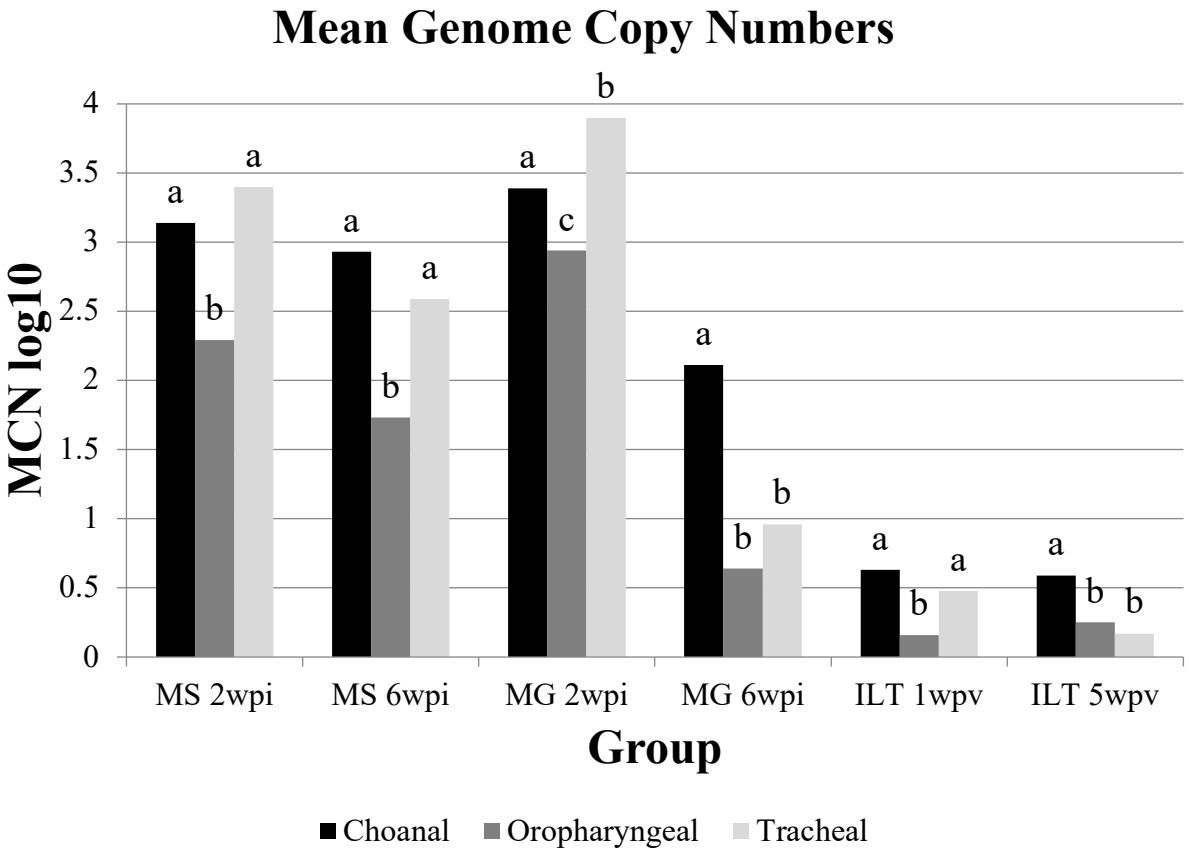


Figure 3.4: **Mean genome copy numbers for swabs of choanal cleft, trachea, and oropharynx for early and late MS, MG, and ILTv infections**. wpi=weeks post inoculation, wpv=weeks post vaccination. For each group, a, b, and c indicate significant differences between swabbing location as determined by the Tukey-Kramer HSD test, significance level $\alpha = 0.05$. MCN = log10 mean genome copy number.



CHAPTER 4

DEVELOPMENT OF TWO DUPLEX QUANTIATIVE PCR ASSAYS FOR DETECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS AND EITHER *MYCOPLASMA SYNOVIAE* OR *MYCOPLASMA GALLISEPTICUM* [1]

[1] Jude, Rachel and Naola Ferguson-Noel. To be submitted to *Avian Pathology*.

Key words: *Mycoplasma gallisepticum*, MG, *Mycoplasma synoviae,* MS, infectious laryngotracheitis virus, ILTv, PCR, real-time PCR, multiplex

Abbreviations: C_T = cycle threshold; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; PBS = phosphate-buffered saline; PCR = polymerase chain reaction, MCN = mean genome copy number

Summary

Mycoplasma synoviae (MS), *Mycoplasma gallisepticum* (MG), and infectious laryngotracheitis virus (ILTv) are economically significant respiratory pathogens affecting chickens. Rapid detection of all relevant respiratory pathogens is needed in order to properly diagnose flocks suspected of respiratory disease and also implement robust surveillance programs. In the preliminary steps to creating a novel respiratory panel for detection of these pathogens, duplex quantitative PCRs for simultaneous detection of ILTv and either MS or MG was developed. The duplex qPCRs target previously described and widely used genes, MG hypothetical lipoprotein (MGA_0319, MGLP), MS 16S-23S rRNA ISR, and ILTv glycoprotein C (gC). The multiplex qPCRs were validated against previously established individual quantitative PCR methods using (1) plasmids containing the genes of interest and (2) tracheal swabs from broilers experimentally infected with MS, MG, and ILTv vaccine. Adequate sensitivity and specificity of the duplex PCRs was confirmed by assessing the limit of detection for each target as well as testing the duplex qPCRs against all relevant poultry pathogens to assure no cross reactions occurred. These tests are expected to lower costs and shorten wait time for diagnostic qPCR results.

Introduction

MS, MG, and ILTv are respiratory pathogens of chickens that may present similar clinical signs depending on severity of disease [1-3]. Due to the economic significance of the pathogens for the poultry industry, rapid detection of the causative agent of disease is necessary for growers and veterinarians to take appropriate actions. Additionally, surveillance of flocks for diseases requires testing to be cost effective as well as rapid, especially in cases where birds are being moved. Currently, there are limited active surveillance programs for ILTv infection as serological testing is unreliable with vaccination programs in the mix [2] and qPCR is too expensive. Additionally, using virus isolation methodologies, conclusive diagnosis of GaHV-1 may take 1-2 weeks [2]. Combining testing for MS, MG, and ILTv would allow for relatively inexpensive and prompt results, while making implementation of active surveillance protocols possible.

Often swabs are submitted to test for multiple pathogens and rapid detection of these respiratory pathogens is necessary for timely diagnosis of flocks presenting with respiratory disease. While single target quantitative PCRs have been developed and are widely used, developing multiplex qPCRs will shorten the wait time and costs to obtain results when mixed infections are suspected. Recently, it was demonstrated that MS, MG, *E.coli* (APEC), and Salmonella detection could be achieved simultaneously via a multiplex conventional PCR using amplicons of varying lengths [4], however conventional PCRs lack the sensitivity and real-time capabilities of TaqMan quantitative PCR assays.

Single quantitative PCRs have been developed for all three of these pathogens targeting the MG MGLP [5], the MS 16S-23S rRNA ISR [6], and the ILTv glycoprotein C (gC) [7]. Here, duplex assays were developed using these targets for detection of ILTv and either MS or MG. A

duplex qPCR for both MS and MG detection was not developed here as commercial assays, such as BioChek Mg-Ms qPCR Test Kit (CP101; BioChek, Reeuwijk, Netherlands), and non-commercial [8-12] MS/MG duplex assays already exist. It was hypothesized that the novel multiplex quantitative PCRs for detection of MS, MG, and ILTv will be as sensitive and specific as currently used single target quantitative PCRs.

Materials & Methods

Quantitative PCR assays. The qPCRs target previously described and widely used genes, MG hypothetical lipoprotein (MGLP) [5], MS 16S-23S rRNA ISR [6], and ILT glycoprotein C (gC) [7]. These previously described qPCRs were modified to develop the duplex quantitative PCRs. For the MG/ILTv duplex qPCR, the MGLP probe fluorescent label was altered from FAM to Cy5. For the MS/ILTv duplex qPCR, the MS ISR probe fluorescent label was altered from FAM to TAMRA and an internal quencher, TAO, was added. The probe from the single target ILTv assay was not altered; the ILTv gC probe is labeled with the FAM fluorescent dye. The duplex assays' components and working concentrations can be seen in Table 4.1. The thermal profile used for duplex ILTv/MG or ILTv/MS amplification was as follows: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C, 15 sec; 60°C, 60 sec with optics ON. C_T values less than 37 for MS and MG and less than 35 for ILTv were considered for analysis. To validate this duplex quantitative PCR, all samples were analyzed against separate routinely used qPCR assays for MS, MG, and ILTv detection [5-7].

Plasmid construction. The MG MGLP, MS ISR, and ILTv gC targets were amplified via conventional PCR using the primers described in the previously published qPCR assays according to the manufacturer's instructions for the Invitrogen™ TOPO® TA Cloning Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and single, distinct bands for each

amplicon were observed via agarose gel electrophoresis. PCR products were cloned into the TOPO vector as directed and transformed in OneShot TOP10 Chemically Competent *E. coli*. Plasmid DNA was isolated using the PureLink Quick Plasmid Miniprep Kit. 10-fold dilutions of plasmids were amplified via the appropriate qPCR to determine mean copy numbers and standardized to 10^7 copies for 10-fold dilutions down to 10^0 copies.

Analytical sensitivity testing. Serial 10-fold dilutions of plasmids (10^7 to 10^0 copies) for target genes of MG, MS, and ILTv were run on both duplex quantitative PCR assays in three independent runs and were compared to the single MS, MG, and ILTv qPCR assays, noted above. Standard curves and equations were generated. Log10 mean genome copy numbers (MCNs) were determined from the C_T values using in-house calculations of standard curves for the single target quantitative PCR assays used regularly in diagnostic detection. Further, the detection limits of the assays were determined using 3 independent runs of low copy plasmid dilutions of 100, 75, 50, 25, 10, 5, and 1 copy.

Analytical specificity. Previous assays [5-7] have tested the targets against several avian Mycoplasma species as well as other relevant avian pathogens as single qPCRs, so repetition of this test was not necessary for development of the duplex assays. qPCRs were only positive for their intended targets according to these publications. See Table 4.2 for a full breakdown of agents used in specificity testing for each published single target qPCR.

Inoculates. Cultures of *Mycoplasma synoviae* strain K6677 and *Mycoplasma gallisepticum* strain R were obtained from the Mycoplasma laboratory repository in the Department of Population Health at the University of Georgia, Athens, GA. R-strain is a well characterized virulent MG strain [13]; K6677 is a recently isolated virulent MS strain (unpublished data). Isolates were grown to exponential phase by visible color change in Frey's

modified broth medium at 37°C. For ILTv, the Zoetis Laryngo-Vac® modified-live freeze-dried vaccine was reconstituted according to the manufacturer's instructions for a 2X dose.

Clinical samples. Three-week-old broilers were inoculated with 100µL each of MG strain R_{low} (n=43) via aerosol spray and MS strain K6677 (n=43) via eyedrop and footpad injection (50 µL each). Five- and 6-days post MS and MG inoculation, respectively, the birds were vaccinated intraocularly with a 2X dose (log_{10}6.32 $TCID_{50}$/mL) of Laryngo-Vac® ILT modified-live freeze-dried vaccine (Zoetis, Florham Park, NJ). 40 negative control birds were housed separately. At 4-, 7-, and 14-days post ILTv vaccination, tracheal swabs were collected from each bird. All swabs were stored at -80°C until extraction. Each sample (n=378) was extracted using the Omega Mag-bind Viral DNA/RNA extraction kit on the ABI MagMAX™ Express-96 Deep Well Magnetic Particle Processor following the manufacturer's instructions and DNA was eluted into 50µL of provided elution buffer. The DNA was amplified with the MG/ILTv and MS/ILTv duplex assays described here and the published single target qPCR assays [5-7].

Clinical sensitivity and specificity calculations. Results for the developed duplex assays for detection of MS, MG, and ILTv in the experimentally infected birds were used to determine the clinical sensitivity and specificity of the duplex assays with the single target assays from which they were derived as performance standards. True positives (TP) were positive for both assays and true negatives (TN) were negative for both assays. False positives (FP) were negative for the single target assays and positive in the duplex assays. False negatives (FN) were positive for the single target assays and negative for the duplex assays. Sensitivity was calculated as TP/(TP+FN) and specificity was calculated as TN/(FP+TN). Positive predictive values (PPV) and negative predictive values (NPV) were also reported, where PPV = TP/(TP+FP) and NPV=TN/(FN+TN).

Results

Linearity, efficiency, & reproducibility. Standard curves (Figure 4.1 A-G) were assembled from three independent runs of 10-fold dilutions of plasmids (10^7 to 10^0 copies) giving positive C_T results in each assay, both duplex and single target. Linear equations, R^2 values, and amplification efficiencies are reported in the graphs of each standard curve. All R^2 values were 0.99 or greater. The ILTv *gC* and MS ISR assays which were previously published gave amplification efficiencies of 90.7% and 96.9%, respectively. The MS ISR and ILTv *gC* duplex gave an efficiency of 113.9% for the ISR target. All other targets across all assays were within ±3% of perfect efficiency.

Analytical sensitivity: limit of detection. See Table 4.3. Using low plasmid concentrations, each assay was able to detect down to 25 copies of their intended targets in at least 2 of 3 repetitions. The MS and MG targets in the duplex assays lost effectiveness at 10 and 5 copies, respectively, and the MG target in the previously published assay was ineffective at reporting positive results at 1 copy per reaction.

Clinical sensitivity and specificity. Figure 4.2 (A and B) shows $\log_{10}$MCN between single target and duplex quantitative PCRs. There were no significant differences ($P<0.05$) in $\log_{10}$MCNs between single target and duplex quantitative PCRs for ILTv, MG, or MS detection. In MS and MG detection, there were no discrepancies between positive and negative results of the duplex PCR and the established individual quantitative PCRs for all tracheal samples. However, for the ILTv duplex qPCRs there were a few false positives when using the single target assays as references. See Tables 4.4 (A and B) and 4.5 (A and B) for the clinical sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) for the novel duplex assays versus the established single target assays.

Discussion and Conclusion

The development and validation of two duplex quantitative TaqMan PCRs for the detection of infectious laryngotracheitis virus and either *Mycoplasma gallisepticum* or *Mycoplasma synoviae* from artificial and clinical samples was described. The previously published single target assays using primers of all the targets demonstrated high specificity when tested against DNA from avian mycoplasma species and other high impact agents known to colonize the respiratory tracts of poultry, only amplifying DNA from organisms to which they were designed to target [5-7].

The limit of detection for the two duplex assays was evaluated in triplicate using plasmids carrying the genes of interest in low concentrations and both were able to detect down to 25 copies per reaction for each target. For detection of both MS and MG in the duplex assays, the limits of detection were higher than those previously reported for single target assays in the published reports and in this report. However, detection limits of 25 copies per reaction are adequate for diagnostic qPCR tests.

Reproducibility and reaction efficiency were determined using individual triplicate serial 10-fold dilutions of plasmids carrying the targeted genes from 10^7 to 10^0 copies. Standard curves indicated excellent reproducibility (R^2>0.99) that was consistent between single target and duplex assays for each target. In terms of amplification efficiency, most targets in both multiplex and single target assays had efficiencies within the target range of 90-110%. However, the MS ISR target in the MS/ILTv duplex qPCR had an efficiency of 113.9% indicating potential low levels of non-specific amplification or inhibitors. The concern with efficiencies of >100% is that weaker samples have artificially higher C_T values and therefore false negative reports are more likely. In the assessment of clinical samples that were positive for both MS and ILTv, MS was

isolated from tracheas at consistently moderate levels. Inclusion of more MS samples that were weaker may have also resulted in false negative results given this high efficiency.

Analysis of primers and probes for MS ISR and ILTv gC show the possibility of cross dimerization of the first 6 nucleotides on the 3' quencher-tagged ends of the probes. Given the amplification efficiency of the gC target in the MS/ILTv duplex was within 3% of perfect efficiency, it is unlikely that this cross dimerization was occurring at levels which could have affected the assay efficiency as this would also lock up probes for the gC target. The cross-dimerization of probes may also induce false negative results, especially with low template input. However, no false negatives were observed in clinical samples from birds that were positive for MS and ILTv, maintaining 100% clinical sensitivity for both ISR and gC targets.

When assessing clinical samples from birds that were positive for MS and ILTv or MG and ILTv, specificity for ILTv detection in the duplex assays was 94.2% and 97.4%, respectively, while specificity for MS or MG detection remained 100%. Consider that all the false positives for ILTv were detected at 7 days post vaccination when viral loads in the tracheas were beginning to wane, while MS or MG load remained consistently moderate to high throughout. Inclusion of more MS and MG samples from birds with low levels of infection may have induced false results that could have affected both clinical specificity and sensitivity of the assay.

Another explanation of these false positives for ILTv in the duplex assays goes back to the assessment of amplification efficiencies of the duplex assays versus the previously described ILTv single target assay. While the efficiency of gC in the single target assay falls within the acceptable range of 90-110% at 90.7%, it is still considerably lower than the gC amplification efficiencies for both of the duplex assays. Since the amplification reactions are nearly perfectly

doubling the gC template every cycle in the duplex assays and amplification is lagging behind in the single target assay, C_T values should be slightly lower for the duplex assays vs the single target assay. Hence, borderline ILTv negative samples reported by the single target assay may be considered weakly positive according to the duplex assays. Indeed, this effect can be seen in Figure 4.2 showing mean genome copy numbers (log10 MCN) for ILTv recovered from clinical samples in the single target and duplex assays. Though statistically insignificant, for both duplex assays the MCNs are slightly higher than those reported for the single target assay. It should be noted that the analysis of these duplex assays relies heavily on the notion that the previously published single target assays from which they were derived are impeccable in terms of sensitivity, specificity, reproducibility, and amplification efficiency.

The developed duplex qPCRs were designed with distinct fluorescent labels for each of the probes with the end goal in mind to develop a triplex qPCR for MS, MG, and ILTv detection. However, several attempts to develop this triplex failed, highlighting the limitations of qPCR. While the fluorescent dyes of the probes were selected to be in distinct channels on the ABI 7500 Fast (FAM in channel 1, TAMRA in channel 3, and Cy5 in channel 5), the master mix used for this assay also has a ROX reporter dye falling in channel 4. Crosstalk can be created when fluorescent signals from reporter dyes bleed over into adjacent channels. While the ROX dye could have been eliminated to create separation between these channels, it is a necessary component used to normalize fluorescent signals for precise quantification.

Another alternative in creating a triplex qPCR would be to investigate SYBR Green I based detection. Multiplex qPCRs have been developed using SYBR Green I for detection and subtyping of LPAI H9N2 [14], however melting curve analysis is a necessary, and sometimes convoluted, component to such assays. Additionally, with probe-based assays, there is an added

layer of specificity for a particular amplicon because it is necessary for three sequences to be compatible rather than two. This said, it would be more prudent to develop multiplex qPCRs for greater than two targets with the introduction of increasingly sophisticated qPCR platforms.

Acknowledgements

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Table 4.1: **Duplex assay recipe with final concentration of reagents**.

REAGENTS	FINAL CONCENTRATION
QuantiFast Multiplex Mastermix	1X
gC FWD	12.5 µM
gC REV	12.5 µM
MS or MG FWD	12.5 µM
MS or MG REV	12.5 µM
gC Probe (FAM)	1 µM
MS (TAMRA) or MG (Cy5) Probe	1 µM

Table 4.2: **Specificity of previously published qPCR assays for MS 16S-23S rRNA intergenic spacer region (ISR), MG MGLP, and ILTv gC**. Open circles (○) represent samples not tested for the assigned assay.

	MS 16S-23S rRNA ISR (Raviv, 2009)	MG MGLP (Callison, 2006)	ILTv gC (Callison, 2007)
Avian adenoviruses	○	-	-
Avian encephalomyelitis virus	○	-	-
Chicken anemia virus	○	-	-
Fowlpox virus	○	-	-
IBV	○	-	-
MDV	○	-	-
NDV	○	-	-
Reovirus-1133	○	-	-
ILTv	○	○	+
Psittacid herpesvirus	○	○	-
Columbid herpesvirus	○	○	-
Duck enteritis herpesvirus	○	○	-
Staphylococcus aureus	○	-	-
Lactobacillus acidophilis	○	-	-
Lactobacillus agilis	○	-	-
Haemophilus paragallinarum	○	-	-
Enterococcus gallinarum	○	-	-
Algaligenes faecalis	○	-	-
Escherichia coli-DH5	○	-	-
Acholeplasma laidlawii	-	-	-
Acholeplasma axanthum	○	-	-
M. gallisepticum	-	+	-
M. synoviae	+	-	-
M. anatis	-	-	○
M. anseris	○	-	○
M. buteonis	○	-	○
M. cloacale	-	-	○
M. columbinasale	-	-	○
M. columbinum	-	-	○
M. columborale	-	-	○
M. corogypsi	○	-	○
M. falconis	○	-	○
M. gallinaceum	-	-	○

M. gallinarum	-	-	○
M. gallisepticum (R strain)	○	+	○
M. gallisepticum (ts-11)	-	+	○
M. gallisepticum (6/85)	-	+	○
M. gallisepticum (F VAX)	○	+	○
M. gallopavonis	-	-	○
M. glycophilum	-	-	○
M. gypsis	○	-	○
M. imitans	-	-	○
M. iners	-	-	○
M. iowae	-	-	○
M. lipofaciens	-	-	○
M. meleagridis	-	-	○
M. pullorum	-	-	○
M. sturni	○	-	○

Table 4.3: **Detection limits of duplex vs single target qPCRs at dilutions of low plasmid concentrations**. At each concentration, 3 samples were processed for each assay and are reported as the number positive out of three.

Copies/ reaction	ILTv (gC)			MS (ISR)		MG (MGLP)	
	w/MS ISR	w/MG MGLP	Single	w/ILTv gC	Single	w/ILTv gC	Single
100	3/3	3/3	3/3	3/3	3/3	3/3	3/3
75	3/3	3/3	3/3	3/3	3/3	3/3	3/3
50	3/3	3/3	3/3	2/3	3/3	3/3	3/3
25	3/3	3/3	3/3	3/3	3/3	2/3	3/3
10	3/3	3/3	3/3	1/3	3/3	2/3	3/3
5	3/3	3/3	3/3	1/3	3/3	0/3	2/3
1	2/3	3/3	3/3	0/3	2/3	0/3	1/3

Table 4.4: **Clinical sensitivity, specificity, positive predictive value, and negative predictive value for MG (A) and ILTv (B) detection via a duplex ILTv/MG qPCR**.

4.4 A - MG detection for a duplex ILTv/MG qPCR

		Single qPCR (MG)		PPV	NPV
		+	-	TP/(TP+FP)	TN/(FN+TN)
Duplex qPCR (ILTv/MG)	+	117	0	100.0%	
	-	0	40		100.0%
Sensitivity	TP/(TP+FN)	100.0%			
Specificity	TN/(FP+TN)		100.0%		

4.4 B - ILTv detection for a duplex ILTv/MG qPCR

		Single qPCR (ILTv)		PPV	NPV
		+	-	TP/(TP+FP)	TN/(FN+TN)
Duplex qPCR (ILTv/MG)	+	43	3	93.5%	
	-	0	111		100.0%
Sensitivity	TP/(TP+FN)	100.0%			
Specificity	TN/(FP+TN)		97.4%		

Table 4.5: **Clinical sensitivity, specificity, positive predictive value, and negative predictive value for MS (A) and ILTv (B) detection via a duplex ILTv/MS qPCR**.

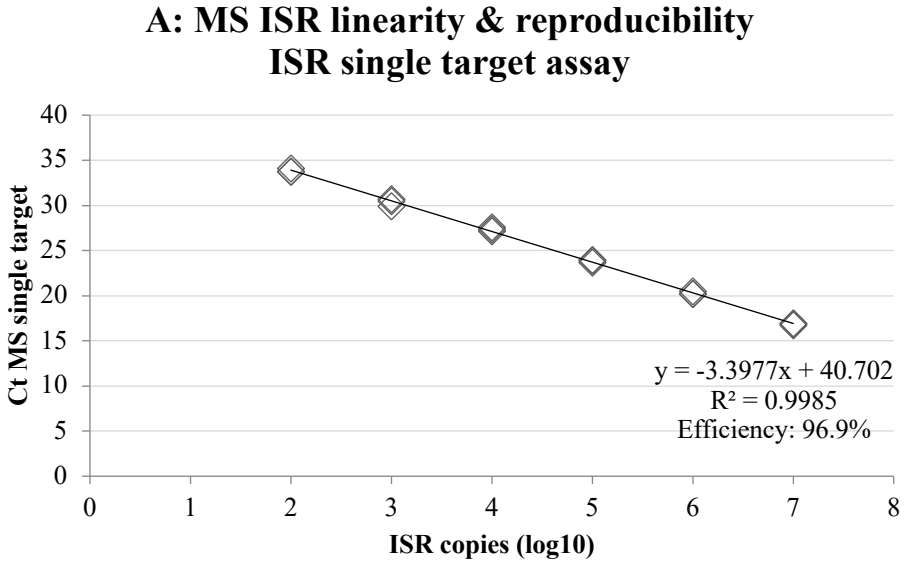
4.5 A - MS detection for a duplex ILTv/MS qPCR

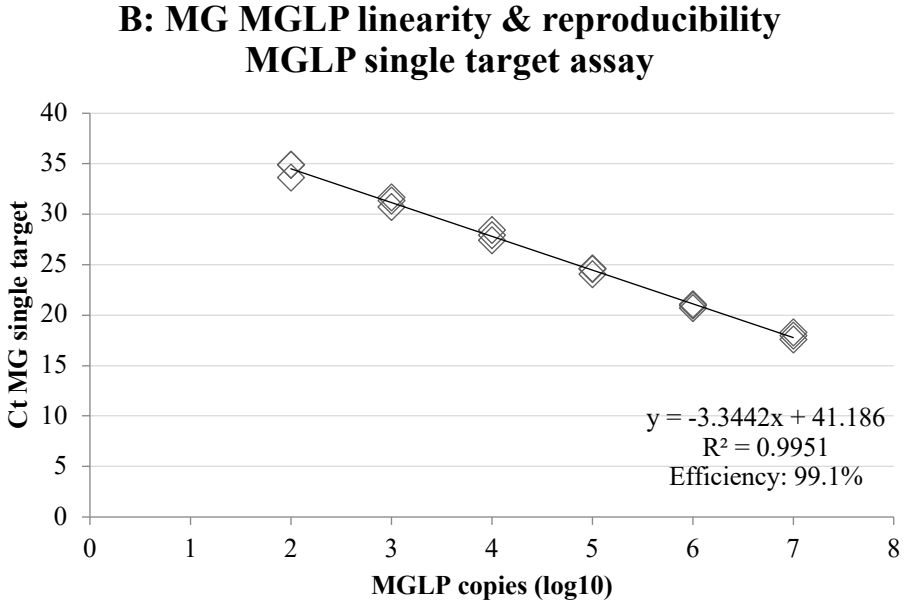
		Single qPCR (MS)		PPV	NPV
		+	-	TP/(TP+FP)	TN/(FN+TN)
Duplex qPCR (ILTv/MS)	+	128	0	100.0%	
	-	0	0		100.0%
Sensitivity	TP/(TP+FN)	100.0%			
Specificity	TN/(FP+TN)		100.0%		

4.5 B - ILTv detection for a duplex ILTv/MS qPCR

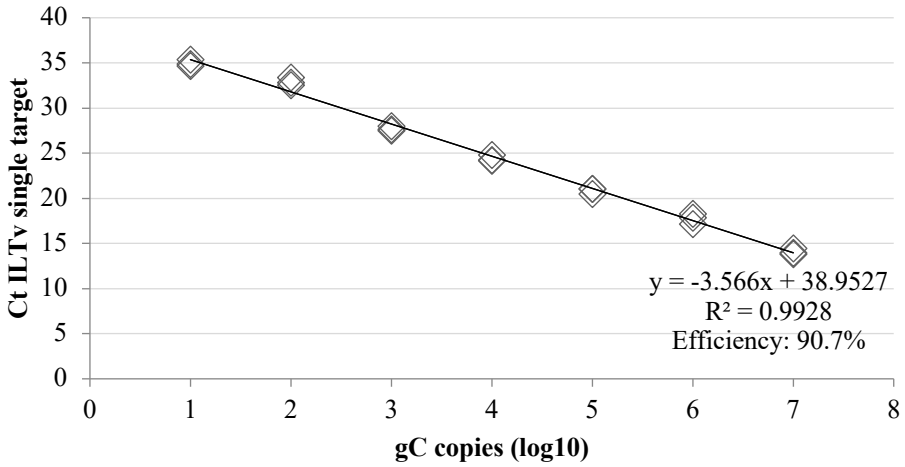
		Single qPCR (ILTv)		PPV	NPV
		+	-	TP/(TP+FP)	TN/(FN+TN)
Duplex qPCR (ILTv/MS)	+	64	6	91.4%	
	-	0	98		100.0%
Sensitivity	TP/(TP+FN)	100.0%			
Specificity	TN/(FP+TN)		94.2%		

Figure 4.1 (A-G). **Standard curves assembled from three independent runs of 10-fold dilutions of plasmids, both single target (A-C), MS/ILTv duplex (D-E), and MG/ILTv (F-G)**. Linear equations, R^2 values, and amplification efficiencies are reported with each standard curve. Plasmid dilutions were between 10^7 to 10^0 copies.

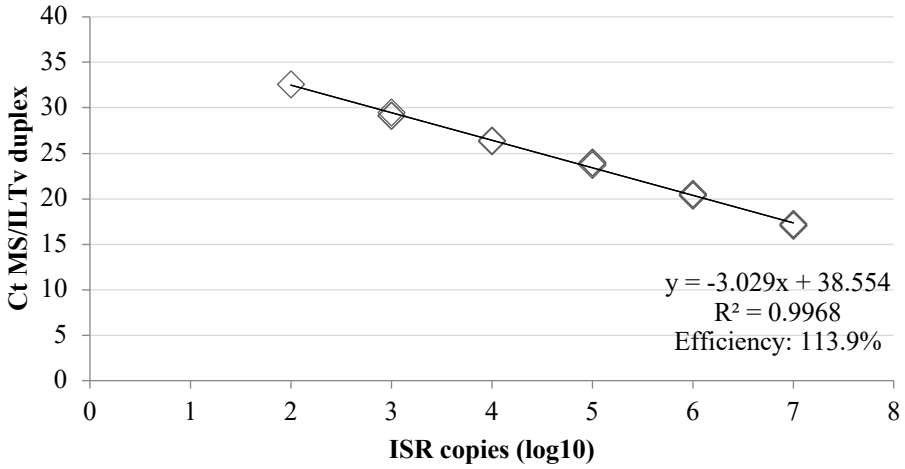




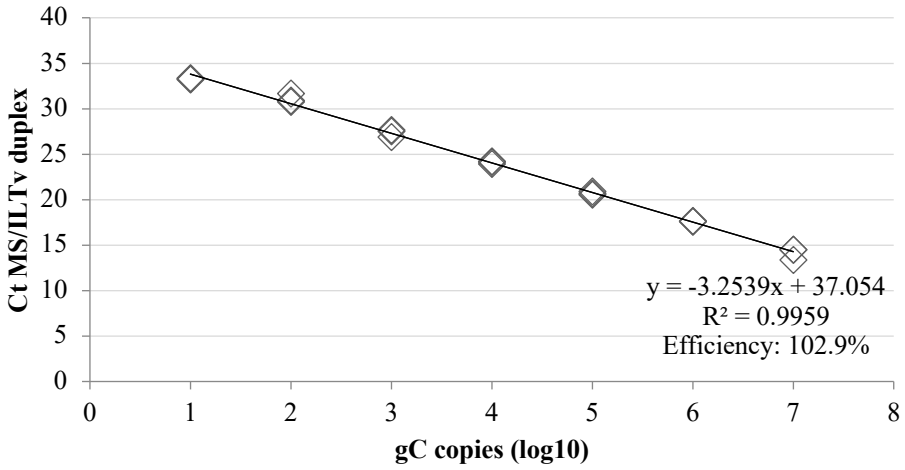
C: ILTv gC linearity & reproducibility
gC single target assay



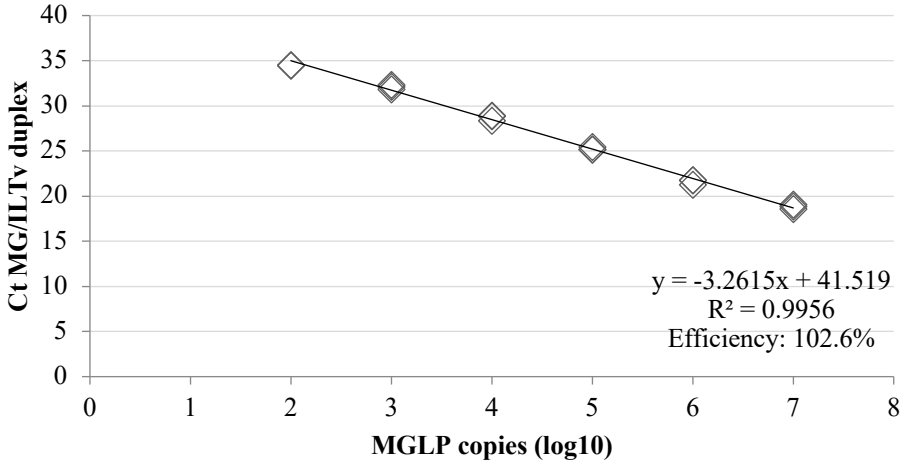
D: MS ISR linearity & reproducibility
ISR/gC multiplex assay



E: ILTv gC linearity & reproducibility
ISR/gC multiplex assay



F: MG MGLP linearity & reproducibility
MGLP/gC multiplex assay



G: ILTv *gC* linearity & reproducibility
MGLP/gC multiplex assay

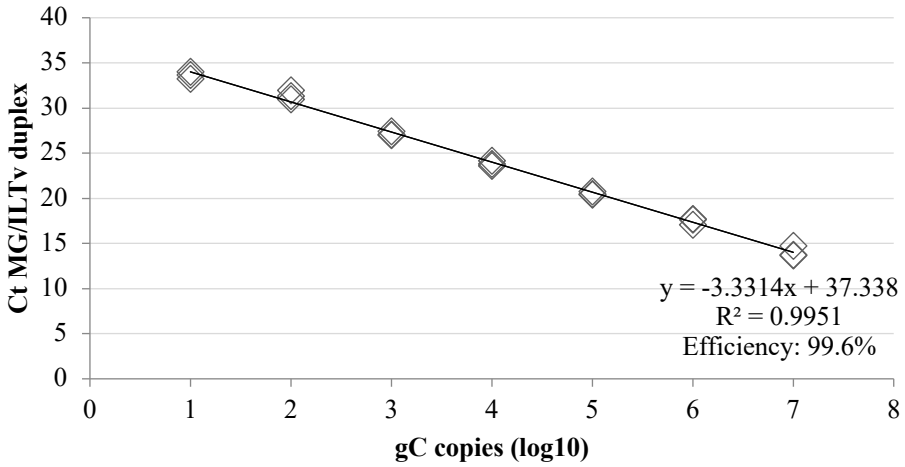
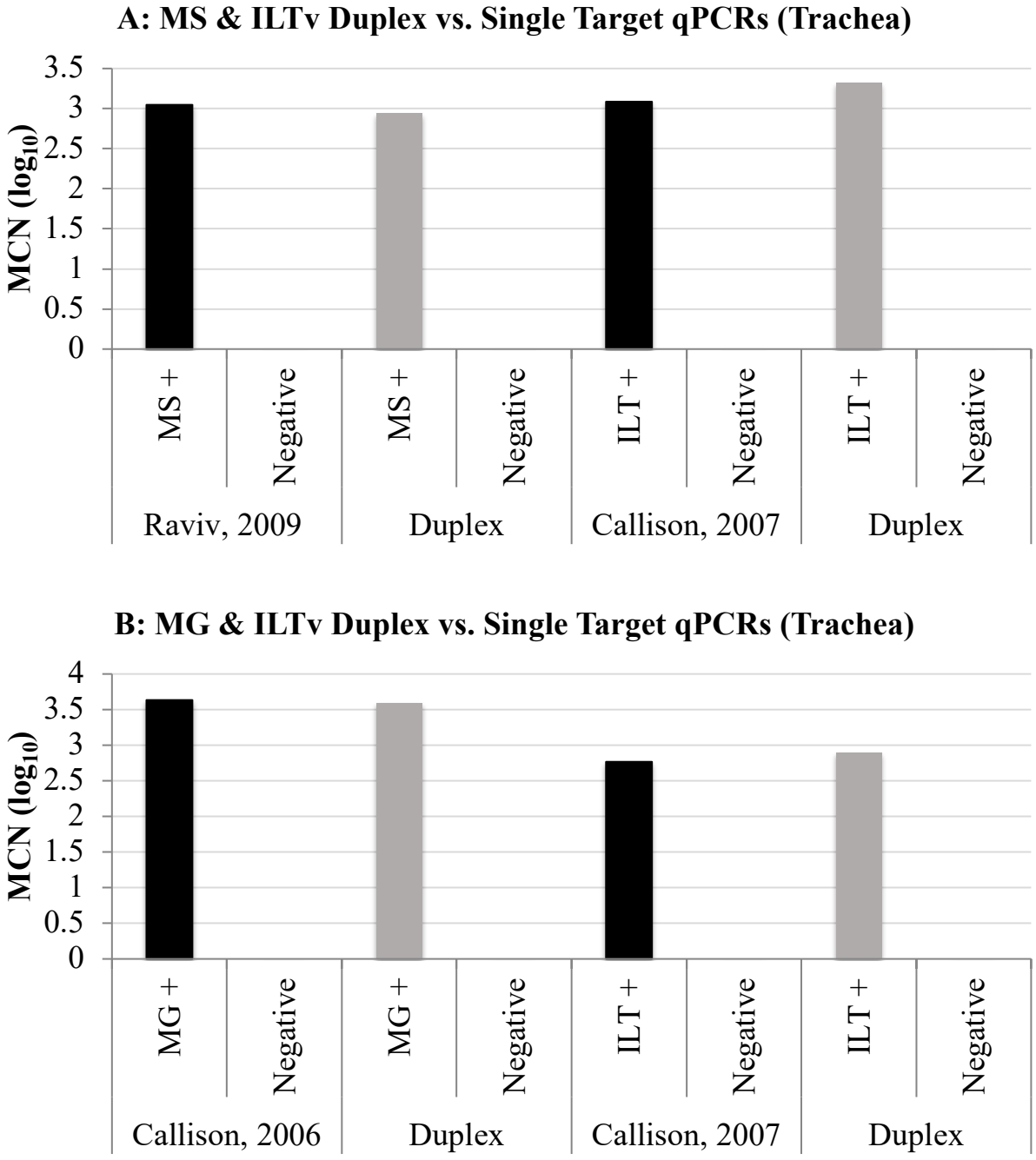


Figure 4.2: **Mean genome copy numbers detected for tracheal samples between single target and duplex qPCRs for ILTv and (A) MS or (B) MG detection**. MCN = log10 mean genome copy number.



CHAPTER 5

DEVELOPMENT OF A MULTIPLEX TARGETED NANOPORE SEQUENCING SCHEME FOR IDENTIFICATION AND CHARACTERIZATION OF *MYCOPLASMA SYNOVIAE* AND *MYCOPLASMA GALLISEPTICUM* [1]

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Key words: *Mycoplasma gallisepticum*, MG, *Mycoplasma synoviae,* multiplex, nanopore, MinION

Abbreviations: C_T = cycle threshold; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; PCR = polymerase chain reaction, MCN = mean genome copy number

Summary

Mycoplasma synoviae (MS) and *Mycoplasma gallisepticum* (MG) are economically significant respiratory pathogens affecting chickens. Limitations to quantitative PCR assays, which are standardly used for MS and MG detection, may be overcome with the use third-generation MinION sequencing. In the preliminary steps to creating a novel panel for detection of respiratory pathogens in chickens, a multiplex targeted nanopore sequencing scheme was developed and assessed for its ability to detect MS or MG and differentiate among MS and MG strains. Three targets each for MS and MG were selected based on their use in current gene targeted sequencing (GTS) applications and databases. In terms of analytical sensitivity, the assay was highly sensitive for MS but had limited sensitivity for most MG targets. Clinically, the assay had 100% specificity for both MS and MG, but the clinical sensitivity for MS was 92.2% and for MG was 71.4%. Overall, the designed assay is more useful for strain typing but lacks sensitivity to equally detect low levels of mycoplasma infection. MG *mgc2*, MG IGSR, MS *nanA*, and MS *ugpA* accurately classified field isolates by their known strain types and repeat samples from the same farms.

Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are pathogenic mycoplasmas affecting commercial poultry which lead to economic losses worldwide. MG and MS may be transmitted both vertically and horizontally [1, 2]. Maintaining infection-free breeding stocks, isolation, and biosecurity are important for control of MS and MG which can be facilitated with rapid and sensitive diagnostic tests. While quantitative PCR has become the gold standard for rapid detection of pathogens, there are limitations to the assay that may render sequencing techniques as a preferable alternative – especially when cause of disease is elusive and multiple tests may be needed.

Genomic sequencing has been particularly useful in molecular characterization of MS and MG. Multilocus sequence typing (MLST) characterizes bacterial species using several housekeeping genes to create allelic profiles based upon differences in nucleotides between each housekeeping gene. MLST assays have been described for both MS [3-5] and MG [6-8] characterization. Given the high conservation of housekeeping genes and their role in survivability of the cell, this is a very conservative approach for defining strains. There are also sequencing techniques targeting genes which are known to be highly discriminatory between isolates from independent outbreak events [9-15]. These may involve sequencing of one or multiple amplicons of interest. Most developed sequencing techniques for MS and MG characterization utilize NGS platforms, such as Illumina [5, 6] or Ion Torrent [16, 17]. However, over the past few decades, rapid advancements have been made in nucleic acid sequencing technologies which have broadened the capacities of sequencing for use in rapid diagnostics. Third-generation nanopore sequencing with the Oxford Nanopore Technologies (ONT) MinION sequencer may be practical for molecular diagnostics due to unique features such as real-time

data acquisition, low overhead costs, a compact laboratory footprint, and ease of use. However, the high error rate of nanopore sequencing necessitates the need for high sequencing coverage [18], which can be achieved by using a targeted sequencing approach [19-22].

Targets for MS and MG were selected based upon maintaining utility of the gene targeted sequencing (GTS) approach currently used for mycoplasma strain typing at PDRC, UGA. The main benefit of this is that a vast database of known strains has been established over many years and will not need to be totally rebuilt. Primers for the current targeted sequencing approach only produce amplicons of around 350-500 bp, so they were not used for this assay as longer amplicons were desired (800-1500bp was the general range). MS targets used in the diagnostic GTS are *vlhA*, *nanA*, and *ugpA* [4, 23]; MG targets used in the diagnostic GTS are mgc2 and IGSR [14, 15]. A number of targets were considered for the assay to be described; but targets ultimately selected for this assay for MS include *nanA*, *ugpA*, and *vlhA*, while targets for MG include *mgc2*, *vlhA*, and the 16S-23S rRNA intergenic spacer region (IGSR). An additional target, α2 collagen found in chicken epithelia, was investigated as an endogenous control, but was unable to be amplified in this multiplex.

MS *vlhA* (partial variable lipoprotein hemagglutinin A) is a commonly used gene for MS strain typing [11, 12, 24, 25]. In MS, the *vlhA* gene family is localized to a single region of the genome and each gene in the family consists of a conserved region, a semi-variable region, and a highly-variable region. Within the conserved region, there is a 410-530bp segment with only one copy in the entire genome and contains the only promotor in the *vlhA* gene family. There are then multiple copies of the rest of the conserved region, semi-variable regions, and highly-variable regions making up pseudogenes of the *vlhA* gene family [24]. The *vlhA* target selected for this assay has been previously designed as a 898bp amplicon by El-Gazzar *et al*. [24] with the

forward primer 45 bp from the start codon (only one copy) and the reverse primer within the semi-variable region (multiple copies, pseudogenes).

The remaining MS targets selected had not been previously developed. MS *nanA* (N-acetylneuraminate lyase) and MS *ugpA* (UTP-glucose-1-phosphate uridylyltransferase) have been previously used in a multi-locus sequence typing (MLST) scheme [4] and showed the highest genetic variability among the 5 genes used in that assay. These two genes are also currently used for strain differentiation by gene targeted sequencing (GTS) at the Poultry Diagnostic and Research Center (PDRC), UGA. MS *tufA* (elongation factor Tu) was selected as a general MS identifier as it is a highly conserved housekeeping gene with only one copy in Mycoplasma genomes that also contains variable regions that are species specific [26]. *Mycoplasma synoviae* has two 16S-23S rRNA IGSRs which can exhibit intercistronic heterogeneity and between MS species there is presence of a few polymorphisms as well as differing amounts of A/T in poly-A and poly-T regions [13]. Between 23 avian mycoplasma species, the IGSRs have shown high inter-species variation; however, for the four pathogenic avian mycoplasma species, there is a low intra-species variation [27]. MS *tufA* and MS IGSR were originally included as targets but were ultimately dropped in an attempt to prevent total inhibition of MG targets and because they are not represented in the established GTS database.

MG has only one 16S-23S rRNA IGSR that has been shown to be highly variable between MG strains such that single-locus typing of MG isolates is possible [15, 28]. MG has 43 *vlhA* genes spread across 5 loci and making up about 10% of the genome [29]. High-resolution melting curve analysis of MG strains using the *vlhA* gene has been shown to differentiate all strains [30]. The *mgc2* (cytadhesin) gene has been shown to be highly useful in both MG identification as well as MG strain differentiation [14, 31, 32]. In terms of differentiation, *mgc2*

genes can have differing insertions and deletions resulting in gene size polymorphisms, particularly in the proline-rich, surface-exposed carboxyterminal-encoding region [14].

While quantitative PCR (qPCR) has become the gold standard for rapid detection of pathogens, there are limitations to the assay that may render sequencing techniques as preferable alternatives. For instance, most qPCR thermocyclers can detect five targets at most. With targeted sequencing techniques, up to four-thousand primer pairs have been shown to be effective in detecting targets of interest [33]. Additionally, the cost of sequencing has become increasingly reasonable and therefore can be more readily implemented in a diagnostic setting. The ONT MinION Mk1B sequencer has an accessible price tag of $1000USD. Over the past few years, nanopore sequencing has been shown to be useful for successful typing of several avian pathogens [20, 22, 34-36]. While whole genome nanopore sequencing has been effective in this regard, using nanopore sequencing in conjunction with gene targeting would allow us to hone in on genes we know to be valuable for identification and typing without the data overload that can come with whole genome analysis [21]. We can gain additional speed and information by also multiplexing the targeted sequencing approach to encompass multiple relevant pathogens potentially isolated from the same sample locations. It was hypothesized that highly specific primers targeting genes of avian respiratory pathogens (beginning with pathogenic mycoplasmas affecting chickens*, M. synoviae* and *M. gallisepticum*) can be utilized to develop a diagnostic multiplex sequencing scheme which will identify and type avian respiratory pathogens in a more accurate and higher capacity than quantitative PCR.

Materials & Methods

Primer Design: In order to leave potential for addition of viral RNA species to the sequencing panel, the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase was used for the PCR master mix. This master mix calls for an annealing temperature between 55-66 °C. For simplicity and consistency, all primers were designed to have annealing temperatures within 3°C of 60°C. The master mix also requires final product sizes of 10kb or less; the aim was to design primers for final product sizes of between 800-1500bp and for product sizes of different targets to be separated by approximately 50bp or more. Primers were designed using the Primer3Plus and The National Center for Biotechnology Information (NCBI) tools.

First, sequences of each gene/region were downloaded from NCBI from the MS WVU 1853 (RefSeq: NZ_CP011096.1) and MG R_{low} (RefSeq: AE015450.2) genomes. Flanking sequences were also downloaded, and primers were developed upon those flanking sequences so the entire target could be amplified. See Table 5.1 for regions isolated from NCBI genomes for each target.

These sequences were then uploaded to Primer3Plus [23] with the specific regions to be targeted marked. Primer3Plus was set to pick both left and right primers, product size ranges 851-1000 and 1001-1500, primers size optimum length of 20bp, primer optimum Tm of 60.0, and primer optimum GC% of 50.0. The returned primers were analyzed, and one set selected based on how closely they met the given parameters. Each primer pair selected was also required to amplify products at least +/- 50bp in size from any other primer pair. Once primer pairs were selected, they were sent to the Primer3Manager and BLAST was selected to ensure query cover

and percent identity were both 100% only to the species for which they were designed. The targets, their sequences, melting temperatures (Tm), and product sizes are found in Table 5.2.

Mycoplasma Isolates: Well-characterized cultures of *Mycoplasma gallisepticum* strain R_{low} [37] and *Mycoplasma synoviae* strain WVU1853 [38] were obtained from the Mycoplasma laboratory repository in the Department of Population Health at the University of Georgia, Athens, GA and grown to stationary phase in a total of 300mL of modified Frey's broth (FMS) [39]. Cultures were pelleted and supernatant poured off. Pellets were resuspended in 3mL of phosphate buffered saline (PBS) and DNA extracted from 200 µL aliquots extracted using the Qiagen DNeasy Blood and Tissue Kit with 5 or more repetitive 100 µL elution steps to obtain as much product as possible. Additional mycoplasma species found in avian species, to be used in specificity testing, were also obtained from the University of Georgia Poultry Diagnostic and Research Center (PDRC) Mycoplasma laboratory depository and grown and isolated as above. All isolate species were confirmed by qPCR (when possible) or sequencing performed by the Georgia Genomics and Bioinformatics Core (GGBC, Athens, GA).

Diagnostic Samples: Previously tested diagnostic samples from the PDRC diagnostic lab were selected based upon the following requirements: must be tracheal swab submissions prepped in PBS; must be chicken samples from a variety of locations and ages; include samples which were MS+ (n=28), MG+ (n=33), MS/MG+ (n=36), or MS/MG- (n=14) via the MS and MG qPCRs used by the PDRC diagnostic lab [31, 40]. The selected diagnostic sample preparations were extracted using the Omega Mag-bind Viral DNA/RNA extraction kit on the ABI MagMAX^{TM} Express-96 Deep Well Magnetic Particle Processor following the manufacturer's instructions and DNA was eluted into 50µL H_2O. See Table 5.3 for description of

the selected diagnostic samples including mean genome copy numbers and strain types (where reported).

Quantification of samples: Previously described quantitative PCR assays for MS and MG detection [31, 40] in conjunction with the Qubit 4 fluorometer and Qubit 1x dsDNA HS Assay Kit were utilized to calculate the mean genome copy number (MCN)/µL for each sample.

Limit-of-detection (LOD): A limit-of-detection scheme was arranged using the highly concentrated DNA isolates of *Mycoplasma gallisepticum* strain R_{low}, *Mycoplasma synoviae* strain WVU1853, and mycoplasma-free chicken DNA isolated from tracheal swabs of backyard birds submitted to the PDRC diagnostic lab. MG R_{low} and MS WVU1853 DNA was titrated individually from 10^6 to 10^0 MCNs per 5 µL (assay template input volume). To mimic potential coinfections of MS and MG in chickens, final concentrations of 10^4 to 10^0 MCNs per 5 µL were spiked into mycoplasma-free chicken DNA (1:10) in a matrix consisting of 25 samples of high MS/high MG, high MS/low MG, low MS/high MG, and low MS/low MG. See Table 5.4 for the full dilution scheme. Dilutions of MG strain R_{low} were considered positive with read counts of 500+, 100% query coverage to MG strain R_{low} (AE015450.2), and pairwise identities of 98% or higher. Dilutions of MS strain WVU1853 were also considered positive with read counts of 500+, 100% query coverage to MS strain WVU1853 (CP011096.1), and pairwise identities of 98% or higher.

Conventional Multiplex PCR: Using MS WVU 1853 and MG strain R_{low} cultures, a multiplex assay was developed to include six total targets, three for MS and three for MG. Template DNA for the MS and MG 6 target multiplex was concentrated such that it was uniform for the concentration of DNA used for the single target amplifications of each MS or MG target. The assay was developed using the SuperScript III One-Step RT-PCR kit supplemented with

1U/reaction of Platinum Taq DNA Polymerase High Fidelity. Primers (x12) were diluted to a final concentration of 0.4 μM. The thermal cycling profile for the multiplex assays was as follows: one reverse transcription stage of 30 minutes at 55°C, one initial denaturation stage for two minutes at 94°C, 40 amplification cycles of 15 seconds at 94°C followed by 30 seconds at 55°C and 5 minutes at 68°C, and a final extension stage for 5 minutes at 68°C. 1.75% agarose gel electrophoresis was used for initial verification of amplification of single targets, though complete verification of the 6-target multiplex is not possible this way as amplicons overlap.

Library Preparation: Libraries were prepared of the multiplexed amplified products using the Oxford Nanopore Technologies PCR Barcoding Kit (SQK-PBK004) as follows:

End Prep: Each amplified DNA product was concentrated to 100 ng in 50 μL. End prep was performed using the NEBNext Ultra II End Repair/dA-Tailing Module. 7 μL Ultra II End-prep reaction buffer and 3 μL Ultra II End-prep enzyme mix was mixed into each 50μL 100 ng amplified DNA product and incubated at 20°C for 5 minutes then 65°C for 5 minutes on the Bio-Rad iCycler. Each sample is then magnetic bead-purified using AMPure XP beads (Agencourt) (1:1) according to the manufacturer's instruction. End-prepped DNA is quantified using the Qubit 4 fluorometer and Qubit 1x dsDNA HS Assay Kit.

Barcode Adapter Ligation: Next, PCR adapters are ligated and amplified by subsequent addition of 10 μL of the provided barcode adapter (BCA) and 25 μL of the NEB Blunt/TA Ligase Master Mix to 15 μL of the end-prepped DNA followed by a 10-minute incubation at room temperature. Each sample is then bead-purified (1:1) and quantified.

Barcoding via PCR: Barcodes for each sample are added by the following 50 μL PCR reaction: x μL of adapter ligated DNA at a final concentration of 0.2 ng/μL (or entire sample if 0.2 ng/μL final concentration cannot be met) , 25 μL NEB LongAmp Hot Start Taq 2X Master

Mix, 1 µL Barcode Primers (LWB 01-12), and 24-x µL nuclease-free water. The cycling conditions for the barcode amplification are as follows: initial denaturation of 3 minutes at 95°C, 14 amplification cycles of 15 seconds at 95°C, 15 seconds at 56°C, and 50 seconds at 65°C, and a final extension for 6 minutes at 65°C. Each barcoded library is then bead-purified (1:1) (with final resuspension in 10 µL of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl) and quantified as previously described.

Sequencing Adapter Ligation: 1.6 µL of barcoded libraries were pooled in groups of 6 different barcodes and brought to 10 µL total volume using 10 mM Tris-HCl pH 8.0 with 50 mM NaCl. While original ONT documentation specifies using equimolar concentrations of each library, equal volumes for each library were utilized instead to prevent bias for particular samples. Sequencing adapters were then ligated by adding 1 µL of RAP (ONT) to 10 µL of the pooled and barcoded DNA libraries and incubating for 5 minutes at room temperature.

Sequencing – Priming and Loading Flow Cells: For each sequencing reaction, a new R9.4.1 FLO-MIN006 MinION flow cell (previously checked for pore activity) was installed on the MinION Mk1B and primed using the ONT Flow Cell priming Kit (EXP-FLP002) according to the manufacturer's instructions. Using the flow cell priming kit, tethers are added to the flow cell which will bring DNA strands to the membrane. The pooled, barcoded DNA libraries were prepared for loading by mixing reagents as follows: 34 µL Sequencing Buffer (SQB), 25.5 µL Loading Beads (LB), 4.5 µL nuclease-free water, and 11 µL DNA library. This preparation was then loaded to the flow cell via the sample port in a dropwise fashion. After approximately half of the original available pores in the flow cells were exhausted or a 36-hour run time was reached, the run was paused, and flow cells washed using the flow cell wash kit (EXP-WSH004, Oxford Nanopore Technologies). A second round of 6 pooled barcoded libraries tagged with

different barcodes than 1st 6 libraries was then loaded as above, and the run restarted until all pores were exhausted or the 72-hour total run time was reached.

Computer Specs: Dell Precision 3520 with Intel Core i7-7820HQ (Quad Core 2.90GHz, 3.90GHz Turbo, 8MB 35W), Nvidia Quadro M620 w/2GB GDDR5, and 1TB M.2 PCIe Solid State Drive Class 40. Operating system: Ubuntu 18.04.3 LTS, 64-bit.

Sequencing – MinION Run: Sequencing runs were carried out using the MinKNOW software from ONT. The MinION Mk1B was connected to the computer via USB and flow cell type (R9.4.1 FLO-MIN006) and kit selection (SQK-PBK004) are input. In the basecalling tab, high-accuracy basecalling (HAC) and the demultiplex barcode options were selected. HAC will provide a more accurate basecalls (97%) compared to the fast basecalling option (92%) [41]. With these options selected, the run was started. Throughout the run, flow cell health was assessed. At the beginning, the number of active pores should be similar to those reported during the pre-run flow cell check. Experiments were run for up to 72 hours or until all pores were exhausted.

Bioinformatic Workflow: Following completion of live basecalling, passed reads were processed using Porechop (https://github.com/rrwick/Porechop) to further demultiplex barcodes and trim adapters [42]. Barcodes were demultiplexed using the –require_two_barcodes option. Porechop defaults to require only one barcode but changing to two barcodes only allows reads to be binned if they have good matches for barcodes on both ends of the read. The barcode threshold was also increased from 75% to 85% as this related to how strongly a barcode must match to known barcode sequences in order to be binned. Porechop defaults to trim adapters from the reads, so no changes were made in that regard. Finally, after reads were trimmed of

adapters and binned by barcode, Porechop was also be used to convert output files from FASTQ to FASTA. The input command was as follows:

```
Porechop -i INPUT [-b OUTPUT] --barcode_threshold
85 --require_two_barcodes --format fasta
```

Once reads were trimmed of adapters and barcodes and were binned according to barcode, the Geneious Prime suite (Biomatters Ltd.) was next used to extract sequences which were only in the size ranges from 500 to 2,000 bp. Reads were then further demultiplexed according to sequence identity in the Geneious Prime suite. A target driven binning step using a custom database of all target sequences was executed using the Geneious mapper at medium sensitivity and fine-tuning iteration of up to 5X, and minimum mapping quality set at 10 (90% confidence). Basecalled reads were aligned to their target sequences if present and clustered according to their alignments. Reads which could be assigned to a cluster were then taken forward into the next analysis step.

The size-selected and binned sequences were then mapped to their respective reference target sequence using the Geneious assembler at medium sensitivity, fine-tuning iteration of up to 5X, and minimum mapping quality of 30 (99.9% confidence). Consensus sequences of the assemblies were then megablasted against a local custom database of complete, annotated MS and MG genomes using the Geneious Prime suite. Results were given in a hit table and retrieve the matching genomic region with annotations. Annotation, pairwise identity to R_{low} (AE015450) or ATCC 25204 (CP011096), and the E value were recorded. Given the highly variable nature of the majority of the selected targets, cut-off pairwise identities and query coverages of diagnostic samples to MS and MG complete genomes in the local BLAST database were identified for each target by calculating average pairwise identities and standard deviations for positive samples. In

some cases, the reference target sequences containing regions know to be highly variable, such as the downstream portion of the MS *vlhA* target were edited to only include the semi-conserved regions to use for assembly when identification (and not strain differentiation) was the goal (*vlhA* herein named *vlhA*-U for the upstream portion of the gene).

Consensus sequences with query coverages in the normal range for their given target and built from 500+ reads were trimmed of ambiguous bases at their ends and then aligned using the MAFFT multiple align feature (MAFFT v7.450) [43, 44] in the Geneious Prime suite with the auto algorithm set, scoring matrix of 1PAM/k=2, and gap open penalty and offset values set at their default values of 1.53 and 0.123, respectively. Cladograms for each target were built using the PhyML plugin (PhyML 3.3.20180621) [45] in the Geneious Prime suite using the Hasegawa-Kishino-Yano (HKY85) substitution model and 100 bootstrap replicates.

Clinical sensitivity and specificity calculations. Results of the developed multiplex sequencing assay for detection of MS and MG diagnostic submissions of tracheal swabs were used to determine the clinical sensitivity and specificity of the multiplexed targeted nanopore sequencing assay with routinely used diagnostic qPCR assays for MS and MG detection as performance standards. True positives (TP) were positive for both assays and true negatives (TN) were negative for both assays. False positives (FP) were negative for qPCR and positive in the multiplex targeted sequencing scheme. False negatives (FN) were positive for the qPCR assays and negative for the multiplex targeted sequencing scheme. Sensitivity was calculated as TP/(TP+FN) and specificity was calculated as TN/(FP+TN). Positive predictive values (PPV) and negative predictive values (NPV) were also reported, where PPV = TP/(TP+FP) and NPV=TN/(FN+TN).

Results

Primer design and gel visualization of RT-PCR products: All primers run in a single target RT-PCR amplified their intended targets based on the size of bands returned. The target for MG *vlhA* also returned a band of approximately 650 bp when run against MS WVU 1853. There was also an additional band for the MG *vlhA* target when run against MG R_{low} strain. Targets were pooled for multiplex RT-PCRs of 1.) all MS targets, 2.) all MG targets, and 3.) all MS and MG targets. For the MS multiplex, all 5 targets returned bands at the expected size. For the MG multiplex, both bands that were previously seen with the *vlhA* targets showed up, however it is difficult to see if *mgc2* and IGSR are both present due to size similarities of the targets. The amplicons for MG targets were considerably weaker than MS targets, so the MS *tufA* and MS IGSR targets were eliminated from the assay. These two targets are not used for strain typing at PDRC and it was expected that their elimination would prevent amplification of MS targets from overwhelming that of MG targets. For the multiplex of all MS and MG targets, gel analysis revealed a base pair size profile that would be expected when comparing to multiplexes of individual Mycoplasma species. The alpha2 collagen target to be used as a control for chicken DNA also did not amplify in the multiplexed PCR and was eliminated. Using this and knowledge of current strain typing genes, final targets to be used for the multiplexed amplicon sequencing were determined to be *mgc2*, IGSR, and *vlhA* for MG and *ugpA*, *nanA*, and *vlhA* for MS.

Cut-off pairwise identities and query coverage: For MS *vlhA*-U, the average pairwise identity was calculated at 90.9 ± 2.6% (cut-off: 88.3%) and query coverage of at least 60% to the full MS *vlhA* target (query coverage for full MS *vlhA* target must be 98% or higher). For MS *nanA*, the average pairwise identity was calculated at 97.5 ± 0.4% (cut-off: 97.1%) and query

coverage of at least 98% after ambiguous nucleotides are trimmed from ends. For MS *ugpA*, the average pairwise identity was calculated at 95.9 ± 1.5% (cut-off: 94.4%) and query coverage of at least 98%. For MG *mgc2*, the average pairwise identity was calculated at 94.9 ± 1.6% (cut-off: 93.3%) and query coverage of at least 98%. For MG IGSR-F, the average pairwise identity was calculated at 97.9 ± 0.4% (cut-off: 97.5%) and query coverage of at least 98%.

Limit-of-detection (LOD), MG only: 10^6 to 10^0 copy dilutions of MG strain R_{low} were positive for MG *vlhA* and *mgc2* targets through 10^3 copies and for MG IGSR through 10^5 copies. While pairwise identity and query coverage were adequate for MG IGSR reads at 10^4 and 10^3 copies, read counts fell under 500 at 448 and 131 reads, respectively. Figure 5.1 shows read counts on a logarithmic scale for each MG target between 10^6 to 10^0 copies.

Limit-of-detection (LOD), MS only: 10^6 to 10^0 copy dilutions of MS strain WVU1853 were positive for all targets (*nanA*. *ugpA*, and *vlhA*-U) through 10^1 copies, with adequate pairwise identity, query coverage, and read counts. All MS targets at 10^0 copies of MS strain WVU1853 failed to produce any reads to build a consensus. Figure 5.2 shows read counts on a logarithmic scale for each MS target between 10^6 to 10^0 copies.

Limit-of-detection (LOD), MSMG bidirectional: Tables 5.5 (A-F). For MG *vlhA* and MG IGSR, only very high MG concentrations combined with low MS concentrations returned positive results. For MG *mgc2*, no samples were positive when MS was present. Generally, mock coinfection with MG had no effect on MS limits of detection across all MS targets.

Specificity: Table 5.6. Targeted sequencing of highly concentrated extractions of common avian mycoplasmas, aside from *M. synoviae* and *M. gallisepticum*, failed to produce consensus sequences matching the MS and MG targeted regions. Sequencing of MS WVU1853 only

produced consensus sequences matching MS *nanA*, MS *ugpA*, and MS *vlhA* while sequencing of MG R_{low} only produced consensus sequences matching MG *vlhA*, MG *mgc2*, and MG IGSR.

Diagnostic samples, identification capabilities: For classification of a positive result using this assay, sequencing must have produced a consensus sequence composed of 500+ reads for at least one target with adequate query coverage and pairwise identity to sequences in the local blast database. Positive and negative results of diagnostic samples (n=111) subjected to the targeted multiplex sequencing assay were compared to MS and MG qPCR results obtained just prior to library preparation to obtain counts of true positives, true negative, false positives, and false negatives. In terms of accurate MS detection, the assay was 92.2% sensitive and 100% specific with a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 90.4%. For MG, the assay was 71.4% sensitive and 100 % specific with a PPV of 100% and a NPV of 67.2%. See Table 5.7 A & B for calculations of these statistics. Full results for each diagnostic sample may also be found in Table 5.8.

Diagnostic samples, target proportions: Among all positive diagnostic samples, the proportions of reads returned for all six targets was calculated for MS positive, MG positive, and both MS and MG positive. See Figure 5.3. For samples only positive for MS, *ugpA* read counts were lower in proportion to *nanA* and *vlhA*-U reads. In samples positive for only MG, the *mgc2* target made up nearly half of the reads. In both MS and MG positive samples, MS reads made up 68.9% of all reads with similar proportions of reads for the MS targets as seen in the MS-only positive samples. Of the 31.1% of reads for MG targets in the MS/MG positive samples, 44.3% of reads were for MG *vlhA*, 41% for *mgc2*, and only 14.7% for IGSR. The average MCN for MS in the MS/MG positive samples was 2.90 log10 copies and for MG was 3.17 log10 copies.

Diagnostic samples, phylogenies: Cladograms of all diagnostic samples can be found for consensus sequences of each target: MS *nanA* (Figure 5.4), MS *ugpA* (Figure 5.5), MS *vlhA*-U (Figure 5.6), MG *mgc2* (Figure 5.7), and MG IGSR-F (containing 16S and 23S flanks) (Figure 5.8). A cladogram for MG *vlhA* was unable to be built due to poor homologies between consensus sequences resulting in failed alignments.

The cladogram for MG *mgc2* clustered repeat samples from the same farms together and separated samples that were previously classified as different *mgc2* strains. For MG IGSR, strains ts-11, 6/85, F-strain, and wildtypes 13, 40, 45, and 48 were represented in separate clusters. However, some strains of wildtype 2 clustered with strain HF-51, though the two strains did not demonstrate high divergence overall. Though in two separate clusters, farms previously classified as WT-2 did cluster together.

The cladogram for MS *nanA* clustered repeat samples from the same farms together and separated samples that were previously classified as different *nanA* strains. Farm 35 clustered in separate subtrees, however the samples were from separate houses and also had previously typed differently as N-39 and N-51. For MS *ugpA*, repeat samples from the same farms were clustered together and samples that were previously classified as different *ugpA* strains were distinct. Though nodes were not highly divergent for MS *vlhA*-U, strains were represented in separate subtrees, generally. MS *vlhA* S-13 clustered with S-56 using the upstream portion of the *vlhA*. This approach grouped MS *vlhA* strain S-56 in multiple closely homologous subtrees even though many of these samples were taken from the same farm (F18).

Discussion and Conclusion

This study outlines the development of a multiplex targeted nanopore sequencing protocol for detection of MS and MG as well as the assay's potential use as a strain typing method using genomic targets commonly used for differentiation of diagnostic submissions. This method was specifically developed for use on the ONT MinION sequencer, which may be an affordable, compact-footprint option for researchers or diagnostic labs wishing to perform sequencing in-house. It was also developed with the intention to offer flexibility for expansion of its detection abilities toward other relevant respiratory pathogens of chickens in the future.

This assay multiplexed 3 targets each for MS and MG in a single reaction. Analysis of limit of detection of the assay for either MS or MG revealed that while the assay effectively detected MS from 10^6 down to 10^1 copies per PCR reaction, the limit of detection in MG only samples was poor with detection of MG *vlhA* and *mgc2* targets through 10^3 copies and for MG IGSR only through 10^5 copies. In addition, a bidirectional dilution scheme to show the limit of detection in the event of coinfections revealed that amplification of the MG targets would be greatly suppressed when both MS and MG were present in the sample, except in cases where MG concentration is very high. Due to cost limitations, replicates for these limit of detection runs was not feasible. However, the addition of independent limit of detection runs would better elucidate the true limit of detection for this assay. When plotting MCN values from the qPCRs of clinical samples against the consensus read count for sequencing of each of the samples, for the MG samples a considerably higher copy count compared to MS samples would be needed to obtain enough reads for the samples to be considered positive (500+ reads). Electing to set the cutoff for 500 reads was a conservative choice to assure good coverage and overcome the higher error rate of nanopore sequencing [18, 46-48].

The clinical specificity for all diagnostic samples was 100% for both the MS and MG targets. Meanwhile, the assay was 92.2% sensitive for MS targets and only 71.4% sensitive for MG targets. Using this assay purely for identification would result in too many false negatives to be acceptable for use diagnostically. This assay may be more effective if the primer pool was limited to three targets for MS or MG separately, though the MG *vlhA* target would need to be redesigned to prevent artifact amplification.

Overall, the designed assay is more useful for strain typing. MG *mgc2*, MS *nanA*, and MS *ugpA* accurately classified field isolates by their known strain types and repeat samples from the same farms. Though MS *vlhA*-U and MG IGSR clustered a few strains together, all repeat farm samples clustered together as well. The upstream region of the MS *vlhA* gene includes tandem repeats encoding proline-rich repeats (PRR) [25] which can often convolute differentiation between strains. Additional studies have indicated the importance of using multiple genomic targets with MS *vlhA* to strengthen the discriminatory power of strain differentiation [4, 12, 24]. It may be necessary to utilize multiple targets and assign allelic profiles, as has been seen in multilocus sequence typing (MLST), with both MS and MG strain differentiation in this assay. Indeed, while farm 31 (F31-2014) previously identified as strain S-13 clustered with multiple samples identified as strain S-56 from farm 18 (F18-2019-[1-14], farm 31 was distinct from farm 18 in cladograms built from the *nanA* and *ugpA* genes. The same line of reasoning can be used to parse out poor differentiation of strains HF-51 and WT-2 using the MG IGSR target. With *mgc2*, farms 29 and 43 (IGSR WT-2) typed as strain WT-DD. Farms 26 and 28, which were also IGSR WT-2 on a separate branch, classified as *mgc2* types ts-11 and WT-S6, respectively.

Further, it may be beneficial to obtain the original Illumina sequences for the diagnostic samples to compare with the MinION sequencing results in this study. It is unknown how the MG IGSR strains HF-51 and WT-2, which grouped together with this assay, might have been differentiated using Illumina sequencing. Addition of Illumina sequencing data would also be beneficial in ascertaining the error rate of nanopore sequencing using amplicons in this assay. However, many studies have already illustrated the use of amplicon sequencing to overcome these error rates [19-22].

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Table 5.1: **Regions isolated from NCBI genomes for each target for the multiplex targeted nanopore sequencing assay**. Additionally, included are regions used for trimmed consensus sequences, MS vlhA-U (denoting the upstream portion of the MS vlhA) & MG IGSR. MG IGSR-F and MS IGSR-F indicates the full MS and MG IGSR targets with flanking 16S and 23S rRNA sequences. *M. synoviae* has two IGSRs that were both targeted by the primers.

Target	RefSeq	Region	Size (bp)
MS *tufA*	NZ_CP011096.1	806629-807813	1185
MS *nanA*	NZ_CP011096.1	238070-238906	837
MS *ugpA*	NZ_CP011096.1	405931-406983	1053
MS IGSR	NZ_CP011096.1	700125-700429 812170-812471	305 302
MS IGSR-F	NZ_CP011096.1	699862-700764 811907-812806	903 900
MS *vlhA*	NZ_CP011096.1	319232-320130	899
MS *vlhA*-U	NZ_CP011096.1	319232-319641	410
MG *vlhA* 4.01	AE015450.2	250927-252776	1850
MG *mgc2*	AE015450.2	222174-223058	885
MG IGSR	AE015450.2	335716-336380	665
MG IGSR-F	AE015450.2	335479-336823	1345

Table 5.2: **Prospective targets for the multiplex targeted nanopore sequencing assay**. Forward and reverse primer sequences, melting temperatures (Tm), and expected product size are noted. Targets denoted with an asterisk (*) indicate primers used for the final multiplex PCR.

Target	Primer Sequences (5'-3')	Tm (°C)	Product Size (bp)
MS *tufA*	Fwd: accattggacacgttgacca Rev: atccggcaccaacagttctt	59 59	1114
*MS *nanA*	Fwd: gagagcgtccaaagcaggat Rev: ctcccatggttagccccatc	60 61	997
*MS *ugpA*	Fwd: tggaatgcctgaaatgggact Rev: acgctagaaaataaaacgccagt	59 57	1481
*MS *vlhA* (El-Gazzar)	Fwd: ggccattgctcctrctgttat Rev: atgcwggaaayactgatgaagc	57-60 55-58	898
MS IGSR	Fwd: tttcgctcgccgctactaat Rev: tcttacgagtggggcaacac	59 59	903
*MG *vlhA*	Fwd: tacttcagcaccagcaccag Rev: taggtgttggttgagctgca	60 59	1052
*MG *mgc2*	Fwd: tgattgctatggtgggcttga Rev: agctccagaaactgctaatcca	59 58	1409
*MG IGSR	Fwd: gtctgggcgacttccatcaa Rev: tcggattgagggctgcaatt	60 59	1348
α2 collagen	Fwd: gccctaggggttgtgaagtc Rev: gcacgtgctgctaacacaaa	61 59	742

Table 5.3: **Selected diagnostic samples for clinical validation of the multiplex targeted nanopore sequencing assay**. The diagnostic reported diagnosis, the qPCR result and corresponding mean genome copy number (MCN) performed prior to library preparation, breed and location of the tested flocks, and the sequencing result from the diagnostic submission for strain differentiation are included. Farms were assigned names based on farm location, year submitted, and numbered when replicates were used. Farms with samples collected from different houses are denoted with an H next to the number of replicates. BB = broiler breeder, LB = layer breeder, L = layer, BY = backyard bird. Open circles (○) indicate no sequencing was reported in the diagnostic submission for the particular targets.

Farm-Year-#	Dx Report	qPCR Result	MS MCN	MG MCN	Breed	Location	Dx Sequence				
							MG *mgc2*	MG IGSR	MS *vlhA*	MS *nanA*	MS *ugpA*
F01-2018	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F02-2018	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F03-2018-1	NEG	NEG	-	-	LB	AL, USA	○	○	○	○	○
F03-2018-2	NEG	NEG	-	-	LB	AL, USA	○	○	○	○	○
F04-2018	NEG	NEG	-	-	LB	AL, USA	○	○	○	○	○
F05-2018	NEG	NEG	-	-	LB	AL, USA	○	○	○	○	○
F05-2020	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F06-2020	NEG	NEG	-	-	LB	AL, USA	○	○	○	○	○
F07-2020-1	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F07-2020-2	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F08-2020	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F09-2020-1	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F09-2020-2	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F10-2020	NEG	NEG	-	-	BB	AL, USA	○	○	○	○	○
F11-2016	MSMG	MS	2.65	-	L	PA, USA	failed	failed	S-15	○	○
F12-2014	MSMG	MS	1.47	-	BB	FL, USA	ts-11	ts-11	S-73	○	○
F13-2014-1	MSMG	MS	3.53	-	B	MD, USA	S-06	failed	S-02	○	○
F13-2014-2	MSMG	MS	4.12	-	B	MD, USA	S-06	failed	S-02	○	○
F14-2015-1	MSMG	MS	4.92	-	B	GA, USA	ts-11	HF-51	S-02	○	○
F15-2015-1	MSMG	MS	1.08	-	L	IA, USA	failed	failed	S-56	○	○
F15-2015-2	MSMG	MS	1.78	-	L	IA, USA	failed	failed	S-56	○	○
F15-2015-3	MSMG	MS	1.17	-	L	IA, USA	failed	failed	S-56	○	○
F16-2015-1	MSMG	MS	2.15	-	L	IA, USA	F-strain	F-strain	failed	○	○

F16-2015-2	MSMG	MS	1.23	-	L	IA, USA	F-strain	F-strain	failed	○	○
F16-2015-3	MSMG	MS	0.67	-	L	IA, USA	F-strain	F-strain	failed	○	○
F17-2015-1	MSMG	MS	3.11	-	L	IA, USA	failed	failed	S-56	○	○
F17-2015-2	MSMG	MS	2.12	-	L	IA, USA	failed	failed	S-56	○	○
F18-2019-1	MS	MS	2.89	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-2	MS	MS	2.48	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-3	MS	MS	1.48	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-4	MS	MS	1.38	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-5	MS	MS	2.45	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-6	MS	MS	1.64	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-7	MS	MS	2.03	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-8	MS	MS	0.73	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-9	MS	MS	2.26	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-10	MS	MS	2.70	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-11	MS	MS	2.06	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-12	MS	MS	2.17	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-13	MS	MS	0.68	-	BB	AL, USA	○	○	S-56	N-37	U-05
F18-2019-14	MS	MS	2.11	-	BB	AL, USA	○	○	S-56	N-37	U-05
F19-2020-1	MG	MS	2.39	-	L	AZ, USA	F-strain	F-strain	○	○	○
F20-2018-1	MG	MG	-	3.75	BB	FL, USA	ts-11	ts-11	○	○	○
F20-2018-2	MG	MG	-	3.85	BB	FL, USA	ts-11	ts-11	○	○	○
F21-2018-1	MG	MG	-	3.85	BB	FL, USA	ts-11	ts-11	○	○	○
F21-2018-2	MG	MG	-	4.47	BB	FL, USA	ts-11	ts-11	○	○	○
F21-2018-3	MG	MG	-	2.46	BB	FL, USA	ts-11	ts-11	○	○	○
F22-2018-1	MG	MG	-	3.67	BB	FL, USA	ts-11	ts-11	○	○	○
F22-2018-2	MG	MG	-	2.47	BB	FL, USA	ts-11	ts-11	○	○	○
F23-2018-1	MG	MG	-	3.75	BB	FL, USA	ts-11	ts-11	○	○	○
F23-2018-2	MG	MG	-	3.19	BB	FL, USA	ts-11	ts-11	○	○	○
F23-2018-3	MG	MG	-	4.01	BB	FL, USA	ts-11	ts-11	○	○	○
F21-2018-4	MG	MG	-	0.74	BB	FL, USA	ts-11	ts-11	○	○	○
F24-2018-1	MG	MG	-	2.98	BB	FL, USA	ts-11	ts-11	○	○	○
F24-2018-2	MG	MG	-	1.82	BB	FL, USA	ts-11	ts-11	○	○	○
F24-2018-3	MG	MG	-	1.32	BB	FL, USA	ts-11	ts-11	○	○	○
F25-2018-1	MG	MG	-	3.95	L	NY, USA	WT-S3	WT-45	○	○	○
F25-2018-2	MG	MG	-	2.00	L	NY, USA	WT-S3	WT-45	○	○	○
F25-2018-3	MG	MG	-	4.44	L	NY, USA	WT-S3	WT-45	○	○	○
F25-2018-4	MG	MG	-	3.65	L	NY, USA	WT-S3	WT-45	○	○	○
F26-2018-1	MG	MG	-	5.53	BB	SC, USA	ts-11	WT-2	○	○	○
F26-2018-2	MG	MG	-	5.39	BB	SC, USA	ts-11	WT-2	○	○	○
F26-2018-3	MG	MG	-	5.20	BB	SC, USA	ts-11	WT-2	○	○	○
F26-2018-4	MG	MG	-	5.31	BB	SC, USA	ts-11	WT-2	○	○	○
F26-2018-5	MG	MG	-	5.83	BB	SC, USA	ts-11	WT-2	○	○	○

F27-2019-1	MG	MG	-	4.16	BB	AL, USA	ts-11	WT-40	○	○	○
F27-2019-2	MG	MG	-	4.13	BB	AL, USA	ts-11	WT-40	○	○	○
F22-2019-1	MG	MG	-	3.52	BB	FL, USA	ts-11	ts-11	○	○	○
F28-2020-1	MG	MG	-	3.68	BB	MO, USA	WT-S6	WT-2	○	○	○
F28-2020-2	MG	MG	-	4.58	BB	MO, USA	WT-S6	WT-2	○	○	○
F28-2020-3	MG	MG	-	3.46	BB	MO, USA	WT-S6	WT-2	○	○	○
F29-2020-1	MG	MG	-	4.04	BB	AL, USA	WT-DD	WT-2	○	○	○
F29-2020-2	MG	MG	-	4.69	BB	AL, USA	WT-DD	WT-2	○	○	○
F29-2020-3	MG	MG	-	4.12	BB	AL, USA	WT-DD	WT-2	○	○	○
F30-2020	MG	MG	-	2.88	BB	FL, USA	ts-11	ts-11	○	○	○
F31-2014	MSMG	MSMG	2.90	2.72	B	ID, USA	S-06	WT-13	S-13	○	○
F32-2016	MSMG	MSMG	1.93	3.72	L	IA, USA	F-strain	F-strain	failed	○	○
F45-2016	MSMG	MSMG	3.22	2.06	L	IA, USA	failed	failed	S-30	○	○
F46-2016	MSMG	MSMG	3.65	4.41	L	IA, USA	F-strain	F-strain	S-30	○	○
F47-2016	MSMG	MSMG	3.57	2.22	L	PA, USA	F-strain	F-strain	S-50	○	○
F48-2016	MSMG	MSMG	4.34	3.85	L	PA, USA	6/85	6/85	S-72	○	○
F34-2017	MSMG	MSMG	2.04	1.68	BY	AL, USA	ts-11	WT-2	S-57	○	○
F35-2018-H1	MSMG	MSMG	3.72	2.75	L	IN, USA	F-strain	F-strain	S-15	N-39	U-45
F35-2018-H2	MSMG	MSMG	3.89	2.62	L	IN, USA	F-strain	F-strain	S-15	N-51	U-45
F35-2018-H3	MSMG	MSMG	3.62	2.95	L	IN, USA	F-strain	F-strain	S-15	N-39	U-45
F36-2019	MSMG	MSMG	3.10	3.10	L	PA, USA	F-strain	F-strain	S-70	N-28	U-54
F37-2019	MG	MSMG	4.06	3.36	L	NC, USA	F-strain	6/85	○	○	○
F38-2019	MSMG	MSMG	3.65	3.56	BY	AL, USA	WT-T3	WT-47	S-02	○	U-45
F39-2019	MG	MSMG	3.85	2.70	L	NC, USA	failed	6/85	○	○	○
F40-2020	MG	MSMG	2.27	3.87	L	CA, USA	ts-11	WT-48	○	○	○
F14-2015-2	MSMG	MSMG	5.07	4.24	B	GA, USA	ts-11	HF-51	S-02	○	○
F14-2015-3	MSMG	MSMG	4.65	3.88	B	GA, USA	ts-11	HF-51	S-02	○	○
F14-2015-4	MSMG	MSMG	4.89	5.80	B	GA, USA	ts-11	HF-51	S-02	○	○
F17-2015-3	MSMG	MSMG	3.41	1.89	L	IA, USA	failed	failed	S-56	○	○
F41-2016-1	MSMG	MSMG	3.34	3.75	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-2	MSMG	MSMG	3.64	3.88	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-3	MSMG	MSMG	3.53	3.76	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-4	MSMG	MSMG	2.13	1.87	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-5	MSMG	MSMG	3.54	3.87	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-6	MSMG	MSMG	2.64	2.35	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-7	MSMG	MSMG	3.35	3.14	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-8	MSMG	MSMG	2.07	1.83	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F41-2016-9	MSMG	MSMG	3.21	3.04	BB	CO, USA	WT-R3	HF-51	S-1	○	○
F42-2017	MSMG	MSMG	3.55	3.97	BY	AL, USA	ts-11/mix	WT-40	S-71	○	○
F43-2019-1H1	MG	MSMG	3.75	4.45	BB	AL, USA	WT-DD	WT-2	S-67	N-42	U-40
F43-2019-2H1	MG	MSMG	4.21	4.27	BB	AL, USA	WT-DD	WT-2	S-67	N-42	U-40
F43-2019-3H1	MG	MSMG	4.04	4.00	BB	AL, USA	WT-DD	WT-2	S-67	N-42	U-40

F43-2019-4H2	MG	MSMG	4.31	3.98	BB	AL, USA	WT-DD	WT-2	S-67	N-42	U-40
F43-2019-5H2	MG	MSMG	4.74	4.70	BB	AL, USA	WT-DD	WT-2	S-67	N-42	U-40
F44-2020	MG	MSMG	3.81	3.18	BB	GA, USA	F-strain	F-strain	○	○	○
F19-2020-2	MG	MSMG	1.37	3.77	L	AZ, USA	F-strain	F-strain	○	○	○

Table 5.4: **Bidirectional dilution scheme of DNA extracted from MS and MG cultures used to simulate the effect of the assay in cases of co-infection**. Numbers are in mean genome copy numbers present in one PCR reaction.

	MS 10,000	**MS 1,000**	**MS 100**	**MS 10**	**MS 1**
MG 10,000	MS 10,000	MS 1,000	MS 100	MS 10	MS 1
	MG 10,000	MG 10,000	MG 10,000	MG 10,000	MG 10,000
MG 1,000	MS 10,000	MS 1,000	MS 100	MS 10	MS 1
	MG 1,000	MG 1,000	MG 1,000	MG 1,000	MG 1,000
MG 100	MS 10,000	MS 1,000	MS 100	MS 10	MS 1
	MG 100	MG 100	MG 100	MG 100	MG 100
MG 10	MS 10,000	MS 1,000	MS 100	MS 10	MS 1
	MG 10	MG 10	MG 10	MG 10	MG 10
MG 1	MS 10,000	MS 1,000	MS 100	MS 10	MS 1
	MG 1	MG 1	MG 1	MG 1	MG 1

Table 5.5 (A-F): **Results of the bidirectional dilution scheme**. Positive and negative results for each target (MG *vlhA* = A, MG *mgc2* = B, MG IGSR-F = C, MS *nanA* = D, MS *ugpA* = E, and MS *vlhA*-U = F) were determined by pairwise identities to either MG strain R_{low} (AE015450.2) or MS strain WVU1853 (CP011096.1) and total consensus read count (500+ reads for positive identification).

A: MG *vlhA* results for bidirectional LOD - By pairwise & read count

	MS 10^4	MS 10^3	MS 10^2	MS 10^1	MS 10^0
MG 10^4	-	-	+	+	+
MG 10^3	-	-	-	-	-
MG 10^2	-	-	-	-	-
MG 10^1	-	-	-	-	-
MG 10^0	-	-	-	-	-

B: MG *mgc2* results for bidirectional LOD - By pairwise & read count

	MS 10^4	MS 10^3	MS 10^2	MS 10^1	MS 10^0
MG 10^4	-	-	-	-	-
MG 10^3	-	-	-	-	-
MG 10^2	-	-	-	-	-
MG 10^1	-	-	-	-	-
MG 10^0	-	-	-	-	-

C: MG IGSR-F results for bidirectional LOD- By pairwise & read count

	MS 10^4	MS 10^3	MS 10^2	MS 10^1	MS 10^0
MG 10^4	+	+	+	+	+
MG 10^3	-	-	+	+	+
MG 10^2	-	-	+	-	+
MG 10^1	-	-	-	-	-
MG 10^0	-	-	-	-	-

D: MS *nanA* results for bidirectional LOD - By pairwise & read count

	MS 10^4	MS 10^3	MS 10^2	MS 10^1	MS 10^0
MG 10^4	+	+	+	+	-
MG 10^3	+	+	+	-	-
MG 10^2	+	+	+	+	-
MG 10^1	+	+	-	+	-
MG 10^0	+	+	+	+	-

E: MS *ugpA* results for bidirectional LOD - By pairwise & read count

	MS 10^4	MS 10^3	MS 10^2	MS 10^1	MS 10^0
MG 10^4	+	+	+	+	-
MG 10^3	+	+	+	+	-
MG 10^2	+	+	+	-	-
MG 10^1	+	+	+	+	-
MG 10^0	+	+	+	+	-

F: MS *vlhA*-U results for bidirectional LOD - By pairwise & read count

	MS 10^4	MS 10^3	MS 10^2	MS 10^1	MS 10^0
MG 10^4	+	+	+	+	-
MG 10^3	+	+	+	+	-
MG 10^2	+	+	+	+	-
MG 10^1	+	+	+	+	-
MG 10^0	+	+	+	-	-

Table 5.6: **Specificity of the multiplex targeted sequencing assay**. Positive or negative qPCR results prior to library preparation is reported as well as positive and negative sequencing results for each target as measured by pairwise identities to either MG or MS sequences in the local BLAST database and total consensus read count (500+ reads for positive identification).

	qPCR results		Nanopore sequencing results					
	MS	MG	MG *vlhA*	MG *mgc2*	MG IGSR-F	MS *nanA*	MS *ugpA*	MS *vlhA*-U
A. laidlawii PG-8 K50997B	-	-	-	-	-	-	-	-
M. anatis (1340) K6312	-	-	-	-	-	-	-	-
M. cloacale 383	-	-	-	-	-	-	-	-
M. gallinaceum DD	-	-	-	-	-	-	-	-
M. gallinerum K285 LPG-16	-	-	-	-	-	-	-	-
M. gallopavonis K6401	-	-	-	-	-	-	-	-
M. imitans 4229 K5146	-	-	-	-	-	-	-	-
M. iowae Sr. J 693A-SU K285	-	-	-	-	-	-	-	-
M. meleagridis 1-529 K3060	-	-	-	-	-	-	-	-
M pullorum 496A K285	-	-	-	-	-	-	-	-
M. sp. 1220 K3922	-	-	-	-	-	-	-	-
M. synoviae WVU1853	+	-	-	-	-	+	+	+
M. gallisepticum R_{low}	-	+	+	+	+	-	-	-

Table 5.7 (A & B): **Calculations of clinical sensitivity and specificity of the multiplex targeted nanopore sequencing assay**. At least one target of three for either MS or MG must have been positive as measured by pairwise identities to either MG or MS sequences in the local BLAST database and total consensus read count (500+ reads for positive identification).

A: MS		qPCR		PPV	NPV
		+	-	TP/(TP+FP)	TN/(FN+TN)
Nanopore	+	59	0	100.0%	
	-	5	47		90.4%
Sensitivity	TP/(TP+FN)	92.2%			
Specificity	TN/(FP+TN)		100.0%		

B: MG		qPCR		PPV	NPV
		+	-	TP/(TP+FP)	TN/(FN+TN)
Nanopore	+	50	0	100.0%	
	-	20	41		67.2%
Sensitivity	TP/(TP+FN)	71.4%			
Specificity	TN/(FP+TN)		100.0%		

Table 5.8: **Full results of clinical samples tested with the multiplex targeted sequencing protocol**. Original diagnostic report, MS and MG qPCR result from just prior to library preparation, positive or negative results for each target, and overall assay results are given for each farm. Discrepancies between the nanopore sequencing result and the qPCR/diagnostic results are marked in grey.

Farm-Year-#	Dx Report	qPCR Result	MS Nanopore			MG Nanopore			Nanopore Result
			MS *ugpA*	MS *nanA*	MS *vlhA*-U	MG *mgc2*	MG IGSR-F	MG *vlhA*	
F01-2018	NEG	NEG	-	-	-	-	-	-	NEG
F02-2018	NEG	NEG	-	-	-	-	-	-	NEG
F03-2018-1	NEG	NEG	-	-	-	-	-	-	NEG
F03-2018-2	NEG	NEG	-	-	-	-	-	-	NEG
F04-2018	NEG	NEG	-	-	-	-	-	-	NEG
F05-2018	NEG	NEG	-	-	-	-	-	-	NEG
F05-2020	NEG	NEG	-	-	-	-	-	-	NEG
F06-2020	NEG	NEG	-	-	-	-	-	-	NEG
F07-2020-1	NEG	NEG	-	-	-	-	-	-	NEG
F07-2020-2	NEG	NEG	-	-	-	-	-	-	NEG
F08-2020	NEG	NEG	-	-	-	-	-	-	NEG
F09-2020-1	NEG	NEG	-	-	-	-	-	-	NEG
F09-2020-2	NEG	NEG	-	-	-	-	-	-	NEG
F10-2020	NEG	NEG	-	-	-	-	-	-	NEG
F11-2016	MSMG	MS	-	+	+	-	-	-	MS
F12-2014	MSMG	MS	+	+	+	+	-	-	MSMG
F13-2014-1	MSMG	MS	+	+	+	-	-	-	MS
F13-2014-2	MSMG	MS	+	+	+	-	-	-	MS
F14-2015-1	MSMG	MS	+	+	+	-	-	-	MS
F15-2015-1	MSMG	MS	-	-	-	-	-	-	NEG
F15-2015-2	MSMG	MS	-	-	-	-	-	-	NEG
F15-2015-3	MSMG	MS	+	-	-	-	-	-	MS
F16-2015-1	MSMG	MS	+	+	+	-	-	-	MS
F16-2015-2	MSMG	MS	-	-	-	-	-	-	NEG

F16-2015-3	MSMG	MS	-	-	-	-	-	-	NEG
F17-2015-1	MSMG	MS	-	+	+	-	-	-	MS
F17-2015-2	MSMG	MS	-	-	-	-	-	-	NEG
F18-2019-1	MS	MS	+	+	+	-	-	-	MS
F18-2019-2	MS	MS	+	+	+	-	-	-	MS
F18-2019-3	MS	MS	+	+	+	-	-	-	MS
F18-2019-4	MS	MS	+	+	+	-	-	-	MS
F18-2019-5	MS	MS	+	+	+	-	-	-	MS
F18-2019-6	MS	MS	+	+	+	-	-	-	MS
F18-2019-7	MS	MS	+	+	+	-	-	-	MS
F18-2019-8	MS	MS	+	+	+	-	-	-	MS
F18-2019-9	MS	MS	+	+	+	-	-	-	MS
F18-2019-10	MS	MS	+	+	+	-	-	-	MS
F18-2019-11	MS	MS	+	+	+	-	-	-	MS
F18-2019-12	MS	MS	+	+	+	-	-	-	MS
F18-2019-13	MS	MS	+	+	+	-	-	-	MS
F18-2019-14	MS	MS	+	+	+	-	-	-	MS
F19-2020-1	MG	MS	+	+	+	-	-	-	MS
F20-2018-1	MG	MG	-	-	-	-	-	-	NEG
F20-2018-2	MG	MG	-	-	-	-	-	-	NEG
F21-2018-1	MG	MG	-	-	-	+	+	-	MG
F21-2018-2	MG	MG	-	-	-	-	-	-	NEG
F21-2018-3	MG	MG	-	-	-	-	-	-	NEG
F22-2018-1	MG	MG	-	-	-	-	-	-	NEG
F22-2018-2	MG	MG	-	-	-	-	-	-	NEG
F23-2018-1	MG	MG	-	-	-	-	-	-	NEG
F23-2018-2	MG	MG	-	-	-	-	-	-	NEG
F23-2018-3	MG	MG	-	-	-	-	-	-	NEG
F21-2018-4	MG	MG	-	-	-	-	-	-	NEG
F24-2018-1	MG	MG	-	-	-	-	-	-	NEG
F24-2018-2	MG	MG	-	-	-	+	+	-	MG
F24-2018-3	MG	MG	-	-	-	-	-	-	NEG
F25-2018-1	MG	MG	-	-	-	-	-	-	NEG
F25-2018-2	MG	MG	-	-	-	+	+	-	MG

F25-2018-3	MG	MG	-	-	-	+	+	-	MG
F25-2018-4	MG	MG	-	-	-	-	-	-	NEG
F26-2018-1	MG	MG	-	-	-	+	+	-	MG
F26-2018-2	MG	MG	-	-	-	-	-	-	NEG
F26-2018-3	MG	MG	-	-	-	-	-	-	NEG
F26-2018-4	MG	MG	-	-	-	+	+	-	MG
F26-2018-5	MG	MG	-	-	-	+	+	-	MG
F27-2019-1	MG	MG	-	-	-	+	+	-	MG
F27-2019-2	MG	MG	-	-	-	+	+	-	MG
F22-2019-1	MG	MG	-	-	-	+	-	-	MG
F28-2020-1	MG	MG	-	-	-	+	-	-	MG
F28-2020-2	MG	MG	-	-	-	+	+	-	MG
F28-2020-3	MG	MG	-	-	-	+	+	-	MG
F29-2020-1	MG	MG	-	-	-	+	-	-	MG
F29-2020-2	MG	MG	-	-	-	+	+	-	MG
F29-2020-3	MG	MG	-	-	-	+	+	-	MG
F30-2020	MG	MG	-	-	-	-	-	-	NEG
F31-2014	MSMG	MSMG	+	+	+	+	+	-	MSMG
F32-2016	MSMG	MSMG	-	+	+	+	+	-	MSMG
F45-2016	MSMG	MSMG	+	+	+	-	+	-	MSMG
F46-2016	MSMG	MSMG	+	+	-	+	+	-	MSMG
F47-2016	MSMG	MSMG	+	+	+	+	+	-	MSMG
F38-2016	MSMG	MSMG	+	+	+	+	-	-	MSMG
F34-2017	MSMG	MSMG	+	+	+	+	-	-	MSMG
F35-2018-H1	MSMG	MSMG	+	+	+	+	-	-	MSMG
F35-2018-H2	MSMG	MSMG	+	+	+	+	+	-	MSMG
F35-2018-H3	MSMG	MSMG	+	+	+	+	+	-	MSMG
F36-2019	MSMG	MSMG	+	+	+	+	+	-	MSMG
F37-2019	MG	MSMG	+	-	+	+	+	-	MSMG
F38-2019	MSMG	MSMG	+	+	+	-	-	-	MS
F39-2019	MG	MSMG	+	+	+	+	-	-	MSMG
F40-2020	MG	MSMG	+	+	+	+	+	-	MSMG
F14-2015-2	MSMG	MSMG	+	+	+	-	-	-	MS
F14-2015-3	MSMG	MSMG	+	+	+	+	+	-	MSMG

F14-2015-4	MSMG	MSMG	+	+	+	+	+	-	MSMG
F17-2015-3	MSMG	MSMG	+	+	+	-	-	-	MS
F41-2016-1	MSMG	MSMG	+	-	+	+	+	-	MSMG
F41-2016-2	MSMG	MSMG	+	+	+	+	+	-	MSMG
F41-2016-3	MSMG	MSMG	+	+	+	+	+	-	MSMG
F41-2016-4	MSMG	MSMG	+	+	+	+	+	-	MSMG
F41-2016-5	MSMG	MSMG	+	+	+	+	+	-	MSMG
F41-2016-6	MSMG	MSMG	+	+	+	+	-	-	MSMG
F41-2016-7	MSMG	MSMG	+	+	+	+	+	-	MSMG
F41-2016-8	MSMG	MSMG	+	+	+	+	-	-	MSMG
F41-2016-9	MSMG	MSMG	+	+	+	+	+	-	MSMG
F42-2017	MSMG	MSMG	+	+	+	+	-	-	MSMG
F43-2019-1H1	MG	MSMG	+	+	+	+	+	-	MSMG
F43-2019-2H1	MG	MSMG	+	-	+	+	+	-	MSMG
F43-2019-3H1	MG	MSMG	+	-	+	+	+	-	MSMG
F43-2019-4H2	MG	MSMG	+	+	+	+	+	-	MSMG
F43-2019-5H2	MG	MSMG	+	-	+	+	+	-	MSMG
F44-2020	MG	MSMG	+	+	+	+	-	-	MSMG
F19-2020-2	MG	MSMG	+	+	+	+	+	-	MSMG

Figure 5.1: **Limit of detection for each MG target**. Read counts on a logarithmic scale for each MG target for limit of detection dilutions of MG strain R_{low} from 10^6 to 10^0 copies per reaction.

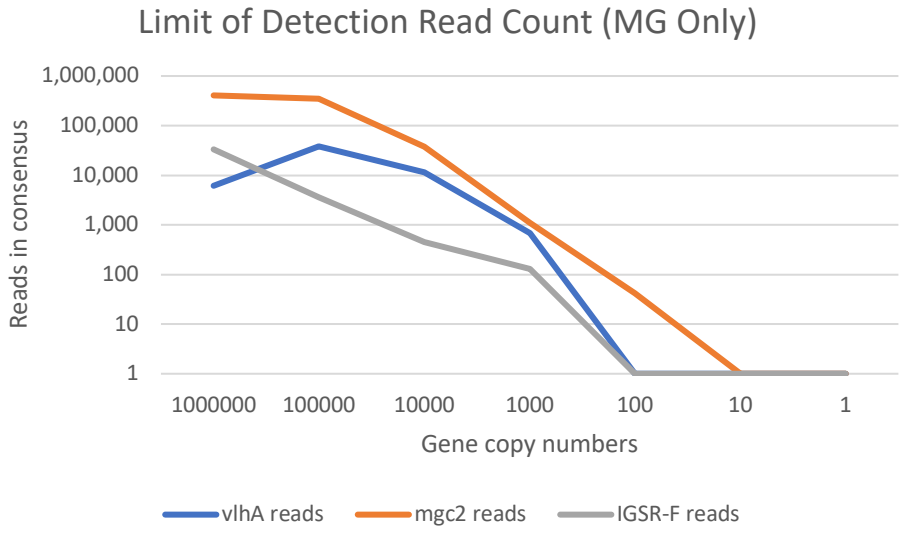


Figure 5.2: **Limit of detection for each MS target**. Read counts on a logarithmic scale for each MS target for limit of detection dilutions of MS strain WVU1853 from 10^6 to 10^0 copies per reaction.

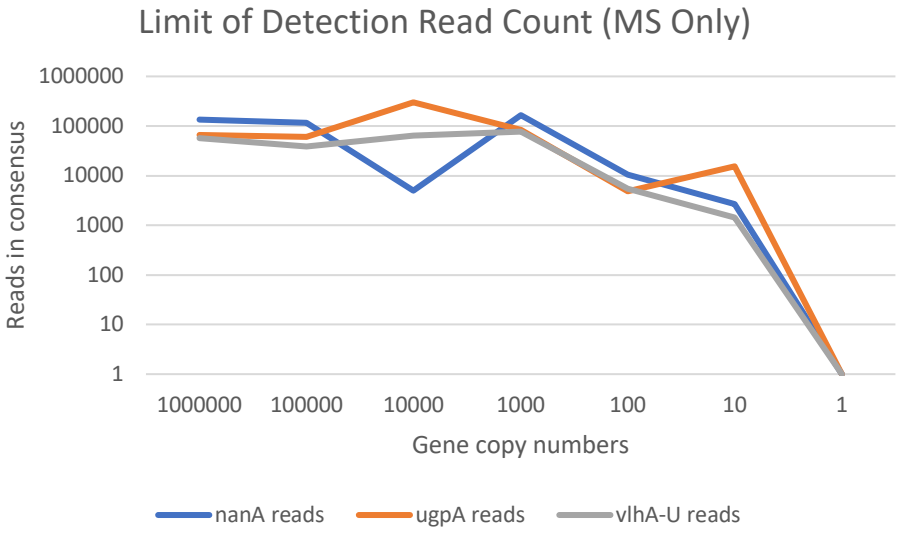


Figure 5.3: **Proportion of reads for each target for diagnostic and limit of detection samples assigning as either MSMG positive, MG positive, or MS positive**. MS targets in blue hues and MG targets in red hues.

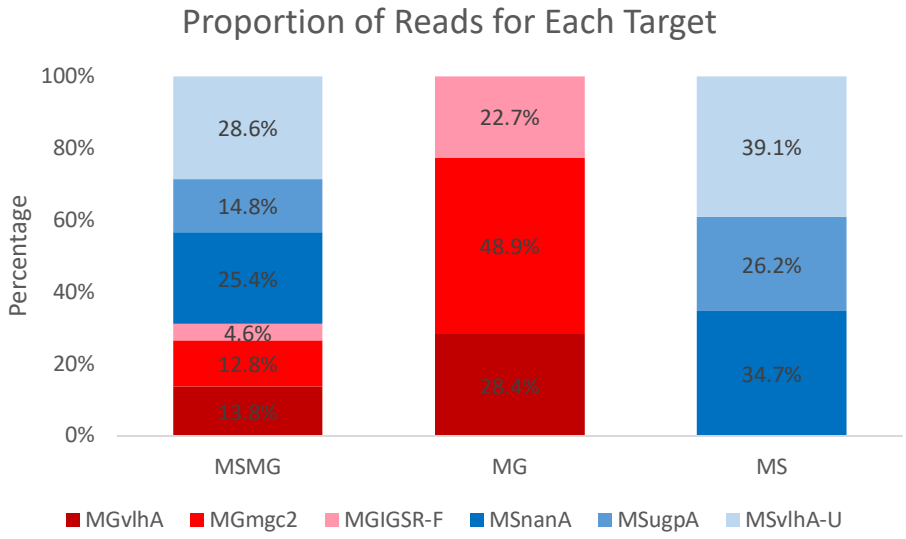


Figure 5.4: **MS *nanA* cladogram**. Cladogram for diagnostic samples returning a positive result for the MS *nanA* target with bootstrap proportion out of 100 and scale bar for number of substitutions per site. Cladogram of consensus alignments (MAFFT) was generated in the Geneious Prime suite using the PhyML plugin and HKY85 substitution model.

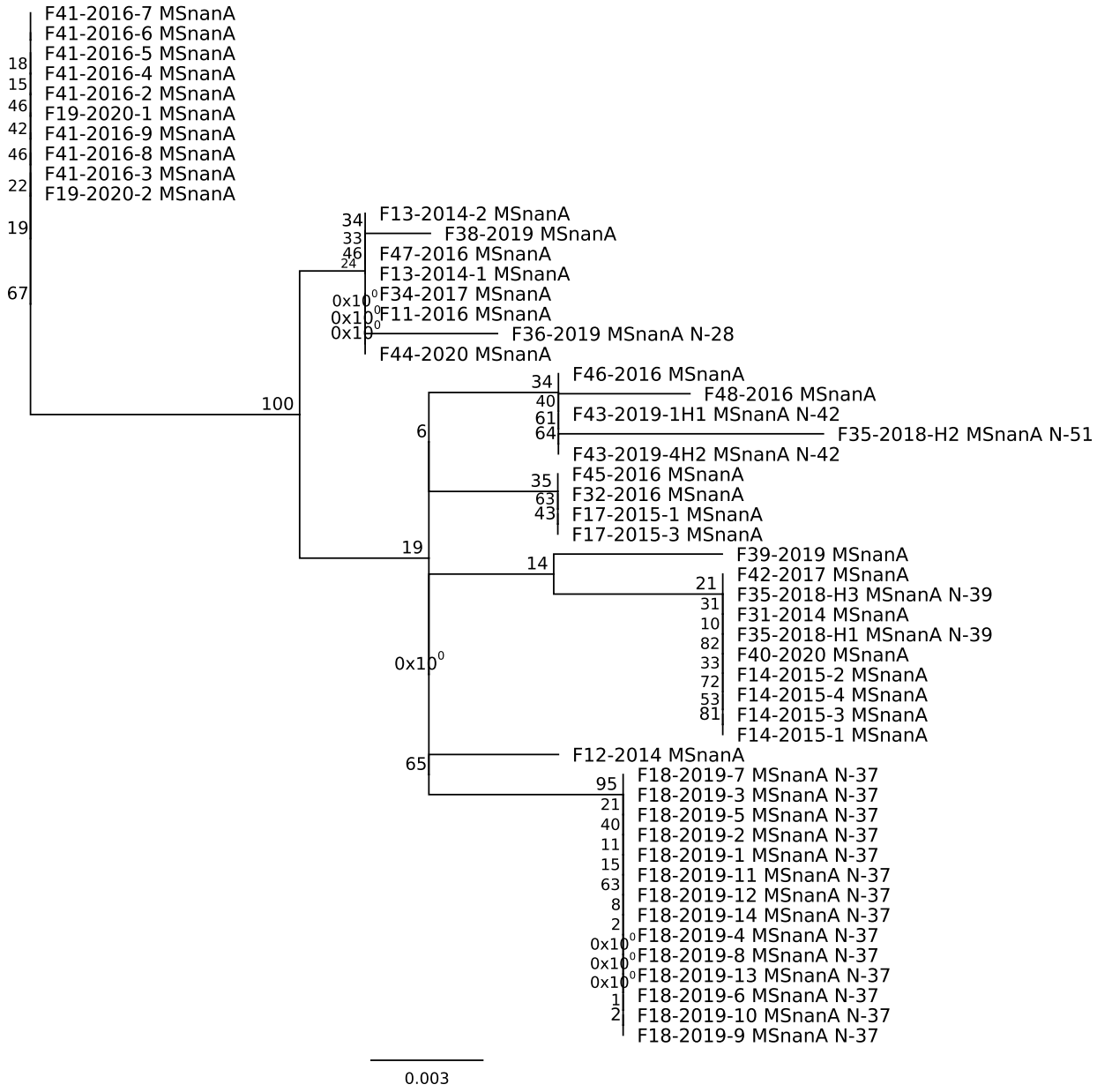


Figure 5.5: **MS *ugpA* cladogram**. Cladogram for diagnostic samples returning a positive result for the MS *ugpA* target with bootstrap proportion out of 100 and scale bar for number of substitutions per site. Cladogram of consensus alignments (MAFFT) was generated in the Geneious Prime suite using the PhyML plugin and HKY85 substitution model.

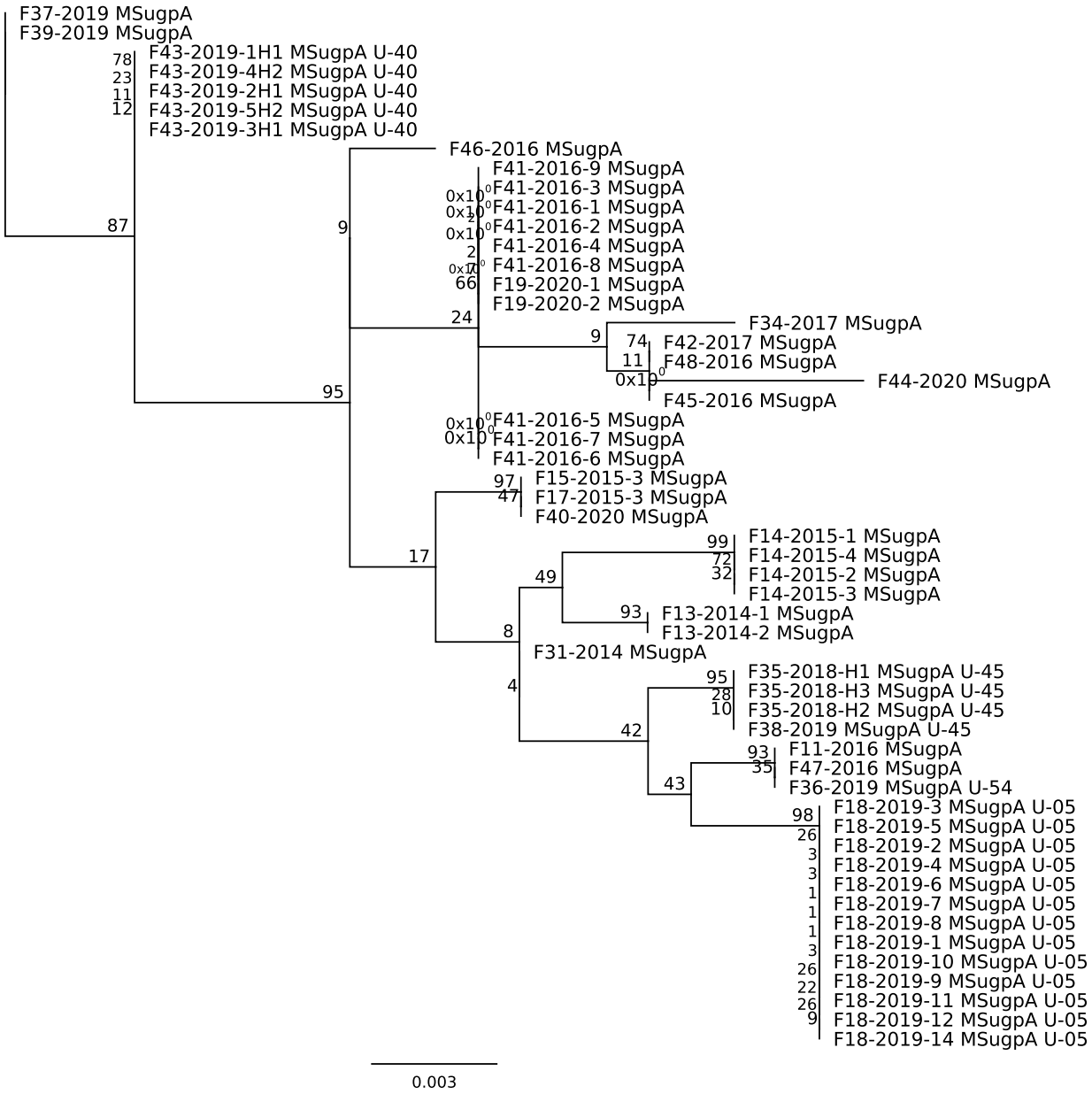


Figure 5.6: **MS *vlhA* cladogram**. Cladogram for diagnostic samples returning a positive result for the MS *vlhA*-U target with bootstrap proportion out of 100 and scale bar for number of substitutions per site. Cladogram of consensus alignments (MAFFT) was generated in the Geneious Prime suite using the PhyML plugin and HKY85 substitution model.

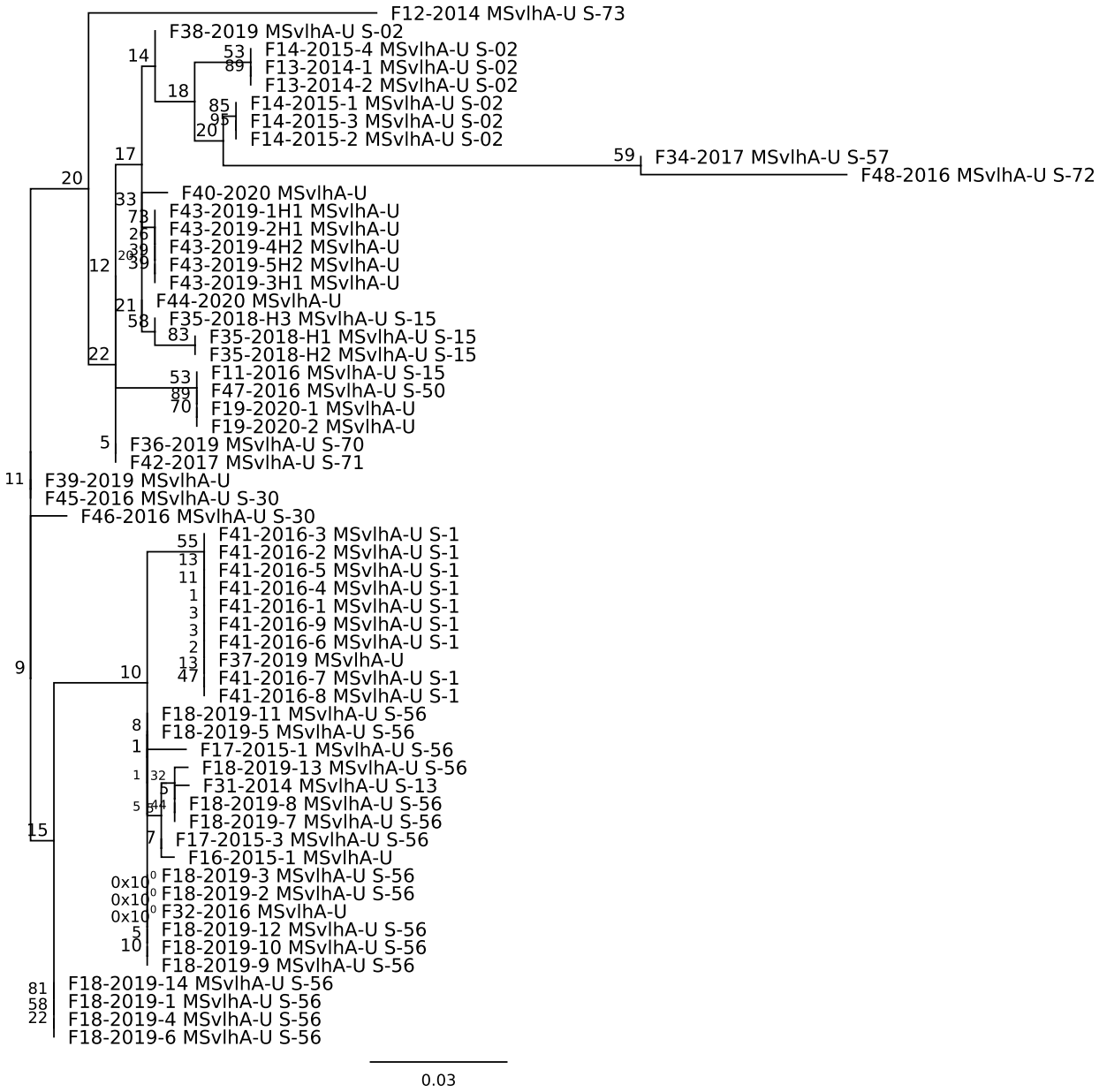


Figure 5.7: **MG *mgc2* cladogram**. Cladogram for diagnostic samples returning a positive result for the MG *mgc2* target with bootstrap proportion out of 100 and scale bar for number of substitutions per site. Cladogram of consensus alignments (MAFFT) was generated in the Geneious Prime suite using the PhyML plugin and HKY85 substitution model.

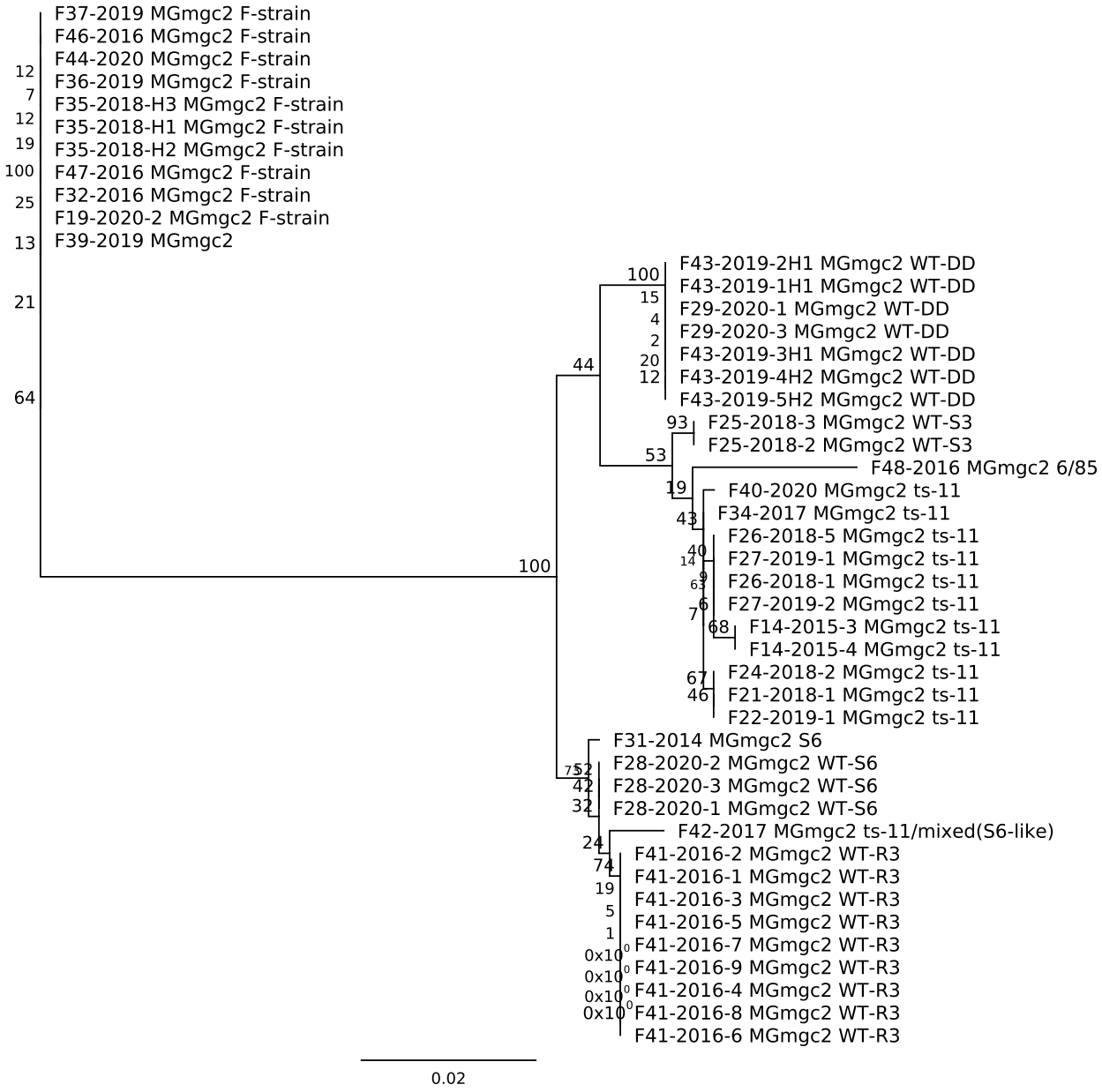
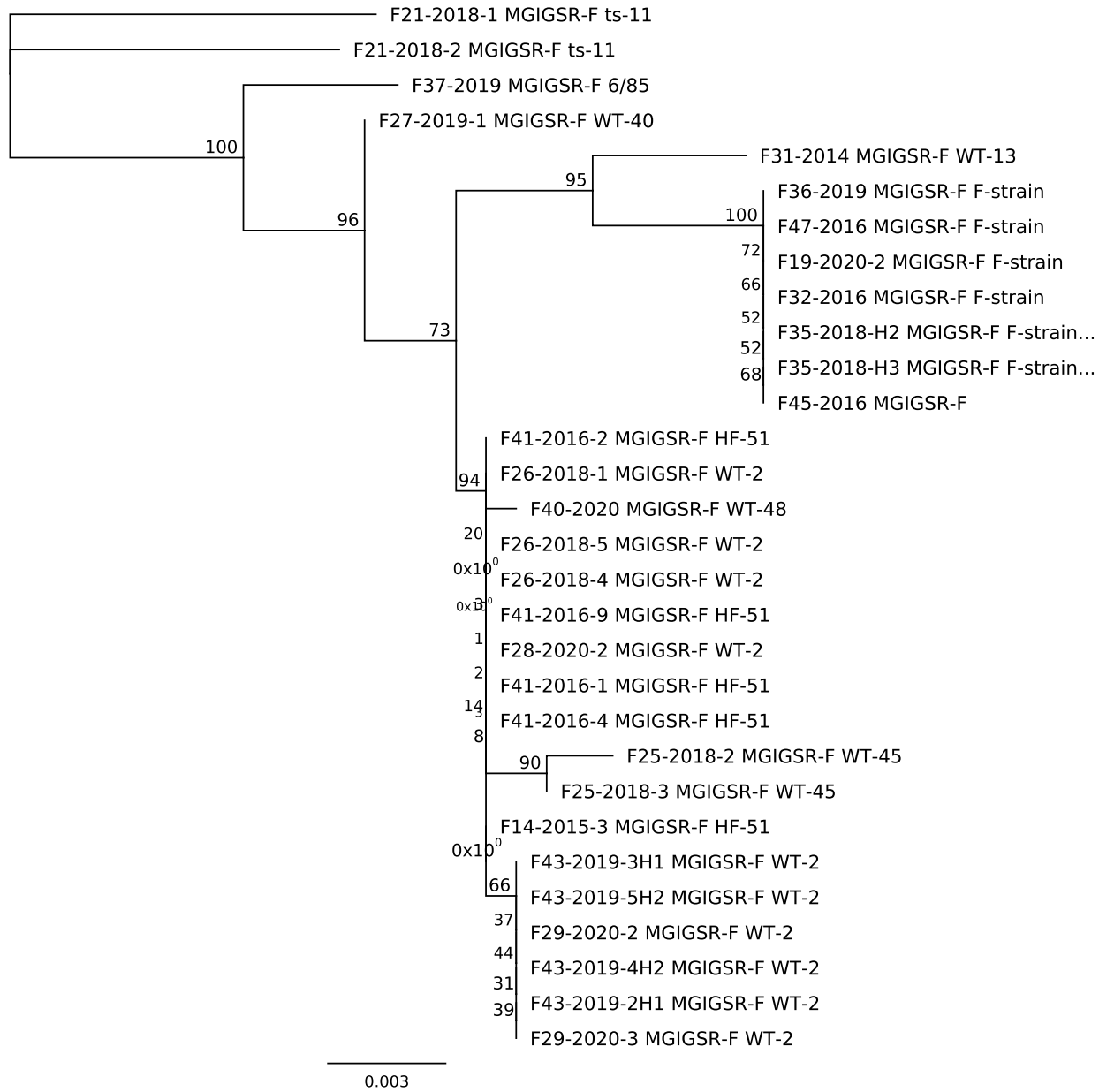


Figure 5.8: **MG 16S-23S rRNA intergenic spacer region cladogram**. Cladogram for diagnostic samples returning a positive result for the MG IGSR-F target with bootstrap proportion out of 100 and scale bar for number of substitutions per site. Cladogram of consensus alignments (MAFFT) was generated in the Geneious Prime suite using the PhyML plugin and HKY85 substitution model.



CHAPTER 6

DISCUSSION AND CONCLUSION

Respiratory disease in poultry, triggered by infections of *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), and many more pathogenic microorganisms, is the cause of severe economic losses worldwide via increased condemnations in broiler processing plants, reduced growth and feed efficacy, and declines in egg production in layer and breeder flocks. Both MG and MS provide more complex issues for control compared to some of the other disease-causing agents important in the global poultry industry, due the fact that MG and MS are capable of vertical (through the eggs) as well as horizontal transmission. Therefore, control of the economically important avian *Mycoplasma* species is imperative not only to protect infected flocks from clinical diseases themselves but also to prevent transfer and clinical effects in the progeny of breeders. Control of MG and MS has primarily been achieved by maintaining breeding stock free of infection, isolation, and biosecurity as well as the introduction of vaccines and when necessary, antibiotic treatment. Implementing robust programs for control of these pathogens relies heavily on sensitive and rapid molecular diagnostics. As quantitative PCR protocols are amenable to high throughput testing, they have the potential to be used not only as confirmatory tests but also as screening tests for the early detection of pathogenic avian respiratory diseases. However, some of the advantages of qPCR may be reduced due to inappropriate collection, handling, and processing of samples. Additionally, turn-around time to final diagnosis of flocks presenting non-specific respiratory signs may be reduced due to

dissimilar sampling and processing recommendations for different pathogens even though the pathogens may be concentrated in the same host location (the upper respiratory tract). Differences in the number of swabs needed, transport media, swab materials, and pooling recommendations hinder the ability to consolidate testing for multiple pathogens in one sampling. Rapid diagnosis may also be achieved by development of tests which can detect multiple pathogens in a single protocol with high sensitivity and specificity as well as being able to use the same samples for multiple tests. Although our overall aim in this research was to improve poultry molecular diagnostics by the development of assays that could detect multiple pathogens, the first part of the research focused on the validation of transport medias and swab sampling sites recommended for other pathogens for the detection of MG and MS.

In Chapter 2, the effect of transport media on the sensitivity and specificity of diagnostic MS and MG qPCR assays was investigated using medias which are commonly used for transport of swabs for detection of several avian respiratory pathogens. Currently, there are no studies establishing a suitable media for submission of respiratory swabs for MS and MG quantitative PCRs. Generally, dry swabs submitted for MS and MG qPCR are prepared for nucleic acid extraction in phosphate buffered saline (PBS) or PCR grade water [1]. However, swabs can be submitted to labs already suspended in several different media, including water, brain heart infusion (BHI) broth, and modified Frey's mycoplasma broth (FMS). The study outlined in Chapter 2 was also designed to be inclusive of media recommended for other respiratory pathogen submissions. For instance, a modified PBS (mPBS) containing calf serum and antibiotic-antimycotic solutions is often used to prepare tracheal swabs for ILTv qPCR [2, 3] and BHI has been determined to be the ideal media for transfer of avian influenza (AI) samples [4].

Pure cultures MS and MG of high titer (approximately 8.0log10CCU) were diluted into the various media to be tested, DNA extracted, and qPCR performed. FMS was quickly eliminated from further study as it appeared to inhibit both of the qPCRs. This may be due to the complexity of components in FMS, such as swine serum, NAD, L-cysteine, and phenol red pH indicator dye. Next, tracheal swabs from infected chickens were prepared in PBS, water, BHI, and mPBS followed by nucleic acid extraction and qPCR. The results from this part of Chapter 2 indicated that tracheal swabs prepared in BHI resulted in significantly higher MCN values as measured by both MS and MG qPCRs ($P< 0.01$). This suggested that either PBS, mPBS, and water were inhibitory to the qPCRs or that BHI had a superior preservative effect to the other medias during the simulated 24-hour transport time.

In concluding this study, BHI was validated as the media of choice in transporting and preparing samples for MS and MG qPCR. Using BHI as the transport medium will reduce diagnostic costs when the poultry industry is sampling for both mycoplasma infections, AI, and infectious bronchitis as it has already been validated for testing some respiratory viruses [4, 5]. Further studies would be necessary to verify that BHI would be an adequate medium in which to transport other relevant respiratory pathogens, such as avian infectious laryngotracheitis (ILT) herpesvirus.

With the appropriate media for submissions selected, next the effect of swab location (trachea, choanal cleft, and oropharynx) on qPCRs for MS, MG, and ILTv was assessed in Chapter 3. Results from this study indicate that choanal cleft swabs consistently returned the highest percent positives during both early and late infection with all three respiratory pathogens. Choanal cleft swabbing is also arguably the easiest and quickest method of the three approaches investigated. However, if a flock is likely in the early stages of MS or MG infection, it may be

more practical to take tracheal swabs, which return higher genome copies at two weeks post infection. The specific circumstances on a case-by-case basis will need to dictate if tracheal swabs will be preferred over choanal cleft swabs. For ILTv detection, nearly all samples were negative for all swab locations. These results can be attributed to the nature of an ILTv infection via vaccination since viral DNA is shown to peak in the trachea between 4 and 6 days post vaccination [6]. It will be necessary to repeat the ILT portion of this study in which samples will be taken at even earlier time points for more realistic and reliable results. This said, given the positive results, choanal cleft swabs appear to be best in terms of both percent positives and MCN log10 at both 1- and 5-weeks post ILTv vaccination. Oropharyngeal swabbing performed poorly across the board and is therefore not recommended for MS, MG, or ILTv detection. While oropharyngeal swabs are preferred for isolation of avian influenza due to concerns of invasiveness and skill needed for tracheal swabs, tracheal swabs were equivalent to oropharyngeal swabs for detection of avian influenza via RT-qPCR [4, 7]. It should be noted that oropharyngeal sampling involves swabbing of the choanal cleft in addition to general swabbing around the oral cavity. So, it is possible that oropharyngeal swabs performed poorly compared to tracheal and choanal cleft swabs if excess mucus buildup had an inhibitory effect on the qPCR or diluted out total pathogen concentration in the samples.

In Chapter 4, two duplex quantitative PCRs were developed for simultaneous detection of ILTv and either MS or MG in the preliminary steps toward creating a triplex qPCR for all three. The duplex qPCRs were validated against previously established individual quantitative PCR methods using (1) plasmids containing the genes of interest and (2) tracheal swabs from broilers experimentally infected with an ILTv vaccine and either MS or MG. The limit of detection for the two duplex assays was evaluated and both were able to detect down to 25 copies per reaction

for each target. While higher than the limits of detection observed in the originally published single target assays [8-10], detection limits down to 25 copies per reaction are generally substantial to validate diagnostic qPCRs. Reproducibility and amplification efficiency of the two duplex assays were largely in the ideal ranges. However, it may be advisable to assess the effect of those targets with amplification efficiencies over 110% on weaker, borderline negative samples to assure that the efficiency is not so high as to give false negative results.

Clinical sensitivity and specificity were 100% for both MS and MG detection for the duplex assays, however swabs of birds confirmed negative for MS, MG, and ILTv will need to be further assessed on this assay for better confidence in the clinical specificity. For ILTv detection, the assays were also 100% sensitive. However, with both duplex assays, there were a few false positives for ILTv that dropped the specificity to undesired levels. It should be noted however that the amplification efficiency measured for the single target ILTv assay was considerably lower than efficiencies for ILTv amplification in both the duplex assays, which were near perfect. This means that the original assay may have a tendency to report false negatives as opposed to the duplex assays reporting false positives. It should therefore be taken into account that the analysis of these duplex assays relies heavily on the notion that the previously published single target assays from which they were derived are impeccable in terms of sensitivity, specificity, reproducibility, and amplification efficiency.

The duplex qPCRs developed in Chapter 4 were designed with distinct fluorescent labels for each of the probes with the end goal in mind to develop a triplex qPCR for MS, MG, and ILTv detection. However, several attempts to develop this triplex failed, highlighting the limitations of multiplexing qPCR assays. While the fluorescent dyes of the probes were selected to be in distinct channels on the ABI 7500 Fast (FAM in channel 1, TAMRA in channel 3, and

Cy5 in channel 5), the master mix used for this assay also has a ROX reporter dye falling in channel 4. Crosstalk can be created when fluorescent signals from reporter dyes bleed over into adjacent channels. While the ROX dye could have been eliminated to create separation between these channels, it is a necessary component used to normalize fluorescent signals for precise quantification. This said, it may be more prudent to develop multiplex qPCRs for greater than two targets once more sophisticated qPCR platforms or reagents are developed.

Given the limitations of qPCR in terms of multiplexing, the potential of multiplexed amplicon sequencing for diagnostic application was investigated in Chapter 5. The sequencing protocol was designed for use on the Oxford Nanopore Technologies MinION sequencer as it would be an accessible low-footprint option for diagnostic laboratories wishing to perform sequencing in-house. While MinION nanopore sequencing is a compelling platform, high error rates necessitate high coverage sequencing [11-14], facilitated in this assay with a pre-library preparation amplification step of select targets. A bioinformatic pipeline was developed to be as "user-friendly" as possible with little need for advanced computational skills; it is believed that this approach will make the assays less intimidating and more accessible to poultry diagnostic labs. The assay was also developed with the intention to offer flexibility for future expansion of its detection abilities toward other relevant respiratory pathogens of chickens.

Targets selected in this assay for MS include *nanA*, *ugpA*, and *vlhA*, while targets for MG include *mgc2*, *vlhA*, and the 16S-23S rRNA intergenic spacer region (IGSR). Aside from MG *vlhA*, all of these targets are used in the gene targeted sequencing (GTS) approach currently used for mycoplasma strain typing at PDRC [15-18]. Analysis of limit of detection of the assay for either MS or MG revealed that while the assay effectively detected MS down to 10^1 copies per PCR reaction, the limit of detection in MG only samples was poor with detection of MG *vlhA*

and *mgc2* targets through 10^3 copies and for MG IGSR only through 10^5 copies. In addition, a bidirectional dilution scheme to show the limit of detection in the event of coinfections revealed that amplification of the MG targets would be greatly suppressed when both MS and MG were present in the sample, except in cases where MG concentration was very high.

The clinical specificity for all diagnostic samples was 100% for both the MS and MG targets. Meanwhile, the assay was 92.2% sensitive for MS targets and only 71.4% sensitive for MG targets. Using this assay purely for identification would result in too many false negatives to be acceptable for use diagnostically. This assay may be more effective if the primer pool was limited to three targets for MS or MG separately, though the MG *vlhA* target would need to be eliminated or redesigned to prevent artifact amplification.

Overall, the designed assay is more useful for strain typing. MG *mgc2*, MS *nanA*, and MS *ugpA* accurately classified field isolates by their known strain types and repeat samples from the same farms. Though MS *vlhA*-U and MG IGSR clustered a few strains together, all repeat farm samples clustered separately from others. The upstream region of the MS *vlhA* gene includes tandem repeats encoding proline-rich repeats (PRR) [19] which can often convolute differentiation between strains. Additional studies have indicated the importance of using multiple genomic targets with MS *vlhA* to strengthen the discriminatory power of strain differentiation [15, 20, 21]. It may be necessary to utilize multiple targets and assign allelic profiles, as has been seen in multilocus sequence typing (MLST) [22-25], with both MS and MG strain differentiation in this assay. Indeed, using the MG *mgc2* target with the MG IGSR target parses out the poor differentiation of MG IGSR alone.

Further, it may be beneficial to obtain the original Illumina sequences for the diagnostic samples to compare with the MinION sequencing results in this study. It is unknown how strains

which grouped together with this assay might have been differentiated using Illumina sequencing. Addition of Illumina sequencing data would also be beneficial in ascertaining the error rate of nanopore sequencing using amplicons in this assay. However, many studies have already illustrated the use of amplicon sequencing to overcome these error rates [26-29].

In conclusion, the effect of transportation/preparation media on quantitative MS and MG PCR was evaluated and BHI determined to be the ideal choice (Chapter 2). The effect of swab location on MS, MG, and ILTv qPCRs was evaluated and choanal cleft swabs were determined to be superior to tracheal and oropharyngeal swabs for recovery (Chapter 3). Two highly sensitive duplex qPCRs for detection of ILTv and either MS or MG were developed (Chapter 4). And, finally, a novel multiplex amplicon nanopore sequencing panel using MS and MG strain typing genes was developed along with a user friendly bioinformatic workflow (Chapter 5). This information will allow the poultry industry to maximize the benefits of costly diagnostic assays, set scientifically based standards in different laboratories for sample collection and handling, and meet demanding turnaround times for conclusive diagnosis of respiratory diseases.

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