AN EVALUATION OF THE MOLECULAR EPIDEMIOLOGY AND TRANSMISSION OF AVIAN *MYCOPLASMA* GENOTYPES IN COMMERCIAL POULTRY

by

NATALIE KATHLEEN ARMOUR

(Under the Direction of Maricarmen García and Naola Ferguson-Noel)

ABSTRACT

*Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS), the most pathogenic mycoplasmal pathogens of poultry, are both horizontally and vertically transmitted. Targeted sequencing of the 16S-23S rRNA intergenic spacer region (IGSR), *mgc2*, MGA_0319 and *gapA* genomic regions was used to develop a database of South African MG sequences, and to elucidate the molecular epidemiology of MG in South Africa. The sequences of the twelve unique MG genotypes identified were highly similar to the available live MG vaccines, necessitating a combination of IGSR and *mgc2* sequencing for differentiation. Targeted genetic sequencing was instrumental in identifying previously reported cases of apparent reversion to virulence and vertical transmission of the live ts-11 vaccine in the state of Georgia. The egg transmission and pathogenicity of ts-11 vaccine and ts-11-like isolates from ts-11 vaccinated breeders (K6222B) and their broiler progeny (K6216D) were evaluated in this research. K6216D transmitted via the egg at an average rate of 4.0% in the third and fourth weeks post infection, while egg transmission of K6222B and ts-11 vaccine was not detected. K6216D and K6222B were

significantly more virulent and invasive than ts-11 vaccine. These results provide the first conclusive evidence of transovarian transmission of an isolate of the ts-11 genotype. A severe outbreak of MS in the state of Arkansas prompted an investigation of the horizontal transmission of two MS isolates genotyped as S-10 and S-17 based on targeted genetic sequencing. MS transmission from infected seeder chickens was detected by real-time PCR at 6 weeks post infection in both the S-10 and S-17 groups; however, the transmission patterns differed, with S-10 transmitting to adjacent contact chickens and S-17 to fomite contact chickens, but not to fomite contacts medicated with tylosin. A novel infection model incorporating mucin was subsequently developed to evaluate the fomite transmission of MS. Low-level MS transmission from S-17-inoculated cotton fabric, and from S-10-inoculated plastic, cotton fabric and feather fomites to sexually mature broiler breeder chickens was detected by real-time PCR, but not by culture or serology, 3 weeks after fomite placement. These studies represent the first experimental evaluations of the horizontal transmission of MS in sexually mature chickens.

INDEX WORDS: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, poultry, sequence database, molecular epidemiology, vertical transmission, horizontal transmission, fomite, virulence, pathogenicity

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2014

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December 2014

# DEDICATION

This dissertation is dedicated to the LORD God - my strength, my song and my salvation. All praise belongs to You.

I also dedicate this dissertation to the family You blessed me with, whose unconditional love and support have made it possible for me to run this race:

To my late father, Stuart, who always encouraged me and helped me to pursue my passions;

To my dear mother, Sheila, whose prayers, support and encouragement have continuously strengthened me;

To my caring sister, Hayley, and brother-in-law, Sean, who have faithfully prayed and cheered me on through many seasons;

And to the Bessette, West and Vaccaro families, whose friendship and prayers have been a source of joy and strength.

# ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to my major professors Dr. Maricarmen García and Dr. Naola Ferguson-Noel for their invaluable mentorship and support that has made this work possible. I was also honored to have Dr. John Glisson, Dr. Stephen Collett, Dr. Robert Hogan and Dr. Eric Lafontaine on my committee, and I am truly grateful for their advice, guidance and support.

The staff and students at the Poultry Diagnostic and Research Center have been key to the successful completion of this work; in particular, I would like to thank Mrs. Ruth Wooten, Mrs. Victoria Laibinis, Mr. Patrick Black, Mrs. Rosie Barber, Ms. Rachel Jude, Ms. Michelle Farrar and Dr. Vijay Durairaj for their assistance.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## The Mycoplasmas

The genus *Mycoplasma* is phylogenetically classified in the kingdom Bacteria, phylum Tenericutes, class Mollicutes, order Mycoplasmatales and family Mycoplasmataceae. Other Mollicute orders include Acholeplasmatales, Anaeroplasmatales, Entomoplasmatales and several unclassified Mollicutes (http://www.ncbi.nlm.nih.gov/). Taxonomic classification of the Mollicutes is based on phylogenetic analysis of 16S ribosomal RNA gene sequences (34, 337).

Their miniscule size, small genomes and complete lack of cell walls make Mollicutes unique among the prokaryotes (89, 290). Because Mollicutes are bound only by plasma membranes, they are resistant to antibiotics that affect cell wall synthesis; this characteristic has been exploited by the inclusion of penicillin in media to retard contaminant growth (89). The lack of a cell wall also renders Mollicutes more susceptible to environmental conditions (89).

Organisms in the genus *Mycoplasma* have the smallest genomes of any free-living organisms (580-1350 kilobases) (89, 291). This small genome size has been attributed to a process of reductive evolution, resulting in the loss of many genes with biosynthetic, metabolic and regulatory functions (291). Intense interest has been generated in

mycoplasma genomes as models to elucidate the minimal set of genes required for life (106, 107, 291).

Mycoplasmas are further characterized by their small size (0.2-0.5 μm), low G+C-content genomes (23-40%) and requirement for cholesterol (89). Mycoplasmas infect humans, animals, insects and plants, but species tend to be host-specific, infecting one or only a few hosts. They are generally non-invasive; human and animal mycoplasmas primarily colonize the respiratory, urogenital and intestinal tracts, eyes, mammary glands and joints (89, 291).

A significant portion of the small genome of mycoplasmas encodes antigens that are involved in the generation of surface variability by phase variation, antigenic variation or size variation (256, 291). The generation of phenotypic diversity is an important survival strategy of the mycoplasmas, allowing them to evade immune detection, and thereby colonize and persist in the host (256) .

A number of avian *Mycoplasma* species have been identified. *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) are the most pathogenic of the mycoplasmas that infect chickens; while MG, MS, *M. meleagridis* and *M. iowae* are pathogens of turkeys (89). MG and MS are OIE-listed, notifiable animal diseases, according to the World Organization for Animal Health (OIE) (http://www.oie.int/). Biochemical characterization of MG and MS is based on their ability to ferment glucose, but not to hydrolyze arginine, and on their lack of phosphatase activity (89, 289).

## *Mycoplasma gallisepticum*

*Mycoplasma gallisepticum* (MG), the most virulent and economically significant of the avian mycoplasmas, is the causative agent of chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys (196, 289). The significant economic losses incurred due to MG infection of flocks are primarily as a result of airsacculitis condemnations at processing, reduced feed efficiency, reduced egg production, and the related costs of control (including medication and vaccination) and prevention (including surveillance, biosecurity and eradication) (196, 289).

Clinical disease induced by MG is characterized by respiratory rales, coughing, nasal discharge, conjunctivitis, infraorbital sinusitis (which is common in infected turkeys) and reduced feed consumption (289). The characteristic airsacculitis of CRD is frequently complicated by a respiratory virus infection (e.g. infectious bronchitis or Newcastle disease) and *Escherichia coli*, resulting in increased mortality (289). Turkeys are more susceptible to MG than chickens, and may display sinusitis, respiratory distress, decreased feed intake and weight loss (289); meningoencephalitis has also been reported with MG infection in turkeys (48). Chronic egg production losses are common with MG infection, and the infected chickens may be asymptomatic. Egg production losses ranging from 5 to 16 eggs per hen housed have been reported in MG-positive flocks (40, 238).

MG is a pathogen of worldwide distribution (289). The implementation of national control programs in several countries (such as the National Poultry Improvement Plan (NPIP) in the United States (1)) has been instrumental in the control MG in primary and multiplier breeding stock, although outbreaks sometimes occur in meat flocks (289). The prevalence of MG in multiple-age commercial egg complexes and backyard poultry

flocks is high, however, and these operations may serve as a reservoir of infection (163, 289).

While MG primarily infects gallinaceous birds, it has also been recognized as a pathogen of house finches since 1994, when it was identified as the cause of an epornitic of conjunctivitis in house finches in the United States (198, 199). There are infrequent reports of natural infections in other bird species, including pheasants, chukar and grey partridge, peafowl, and bobwhite and Japanese quail (21, 55, 246, 289, 293, 333).

The complete genome sequences of a number of MG isolates from poultry and house finches have been published (60, 275, 316, 326). The genome of the virulent strain $R_{low}$ comprises 1,012,800 base pairs (bp), a G+C content of 31% and a total of 682 coding DNA sequences (CDSs); of which 51 genes belong to the *vlhA* gene family (275, 316). Comparative genomic analyses of complete genome sequences have shed light on the molecular basis of MG virulence (316, 326), and have revealed an extraordinarily high point mutation rate for MG genomes (60).

### *Mycoplasma synoviae*

*Mycoplasma synoviae* (MS) typically presents as a subclinical respiratory infection, but may cause a more severe respiratory disease with airsacculitis, which is exacerbated by concurrent respiratory virus or *E. coli* infection. Systemic infection may result in infectious synovitis, characterized by an exudative synovitis or bursitis (91). Economic losses with MS infection are primarily related to airsacculitis condemnations, reduced feed efficiency and costs associated with prevention, control and monitoring.

Clinical disease with the respiratory form of MS is similar to that induced by MG, although it is usually less severe. Birds affected with infectious synovitis typically exhibit a pale comb, lameness, retarded growth and emaciation, with swellings around the affected joints (usually the hock joints and foot pads) and breast blisters (91). In recent years, the respiratory form of MS has been more frequently observed in chickens in the United States; the synovitis form is, however, more common in turkeys (91).

MS has been implicated as a complicating factor in the pathogenesis of *E. coli* peritonitis syndrome in layers (287). Egg production losses are not usually a feature of MS disease, although they have been observed (91). Recently, MS-induced eggshell-apex abnormalities (EAA), characterized by a rough shell surface, thinning and cracks in the apex region of the eggshell, have been reported in several countries (31, 80, 143). The condition results in economic loss due to egg breakages (80). Commercial egg layers are more susceptible to EAA-inducing MS strains than broiler breeder hens (82).

MS is worldwide in distribution (91); the prevalence of MS in most countries is greater that that of MG, due to control and eradication programs targeting MG but not MS (185). While chicken and turkey primary breeding stock is generally free of infection, the decision to slaughter MS-infected multiplier breeder flocks is sometimes made on an economic basis (91). Most multiple-age commercial egg complexes are positive for MS (163).

Chickens and turkeys are the natural hosts of MS, but natural infections have been reported in several other bird species, including ducks, geese, guinea fowl, pigeons, Japanese quail, pheasants and red-legged partridges (20, 23, 24, 29, 91, 278, 281, 294, 324).

The complete genome sequence of a field isolate of MS revealed a genome size of 799,476 bp, with a total G+C content of 28% and a total of 694 CDSs (332). Comparative analysis of MS and MG genomes indicated that multiple horizontal gene transfer events, involving 14 genetic regions, including hemagglutinin genes, have most likely occurred (332).

## Pathogenesis of the Infectious Process

Mycoplasmas, despite their minute size and reduced genomes, are notorious for their ability to survive and persist in complex hosts, inducing chronic disease. While the pathogenesis of mycoplasmal infections has not been fully elucidated (163), several virulence factors are regarded as key to the success of the avian mycoplasmas as pathogens, namely; motility, cytadhesion, phenotypic variation and the acquisition of nutrients (91, 256, 289). Some strains of MG and MS are also capable of host cell invasion (68, 347). No toxins have been identified for MG or MS.

As primarily mucosal pathogens, attachment and colonization is a prerequisite for the pathogenic processes of MG and MS (91, 289). The ability of MG and MS to vary the expression of their surface antigens is thought to be an important mechanism facilitating immune evasion, host adaptation and persistence in the host (256). MG and MS also have the capacity to indirectly inflict damage by modulating the host immune response, which can result in significant immunopathology (291) (reviewed under “Immunity, Immunomodulation and Immunopathology”).

Common sequelae of MG infections of the trachea are the release of mucus granules, followed by death and exfoliation of epithelial cells, ciliostasis and occasional

deciliation, and finally, increased epithelial thickness due to cellular infiltrations and edema (8, 69). Although usually less severe, similar changes have been observed for MS (127, 260). These changes probably play important roles in the pathogenesis of MG and MS infections.

MG and MS-related disease is exacerbated by concurrent respiratory viral or other bacterial infections, immunosuppression, adverse environmental conditions and other stressors (91, 289). Mixed infections of MG or MS with respiratory viruses and E. coli precipitate chronic respiratory disease (CRD) (162). Concurrent infections with Newcastle Disease or Infectious Bronchitis viruses (including vaccine viruses) result in more severe MG- (4, 162, 299) or MS- (132, 133, 173, 331) related disease.

The pathogeneses of egg production drops induced by MG (and occasionally MS) infection are not well understood. Salpingitis, characterized by epithelial hyperplasia and marked lymphoplasmacytic infiltration, has been reported in chickens naturally or experimentally infected with MG (64, 263, 282), and was associated with the presence of MG organisms in the oviduct, and a drop in egg production (263).

## Virulence Factors

***M. gallisepticum* virulence factors**. Different MG strains display wide variations in virulence. Differences in virulence between MG isolates belonging to the same strain may also be seen with variations in culture methods, passage levels and inoculation routes and dosages (289). Marked intra-strain differences in virulence have been reported for the R strain of MG; low-passage isolates ($R_{low}$) display significant virulence,

cytadhesion and cell invasiveness, while high-passage isolates ($R_{high}$) are attenuated in virulence (277, 289, 347).

The ability of MG to adhere to host cells is a prerequisite for its colonization of the respiratory mucosa and subsequent pathogenesis (289). MG attachment to host cells is primarily mediated by a bleb-like unipolar terminal organelle (317). The terminal organelle is associated with a cytoskeletal structure, which is thought to drive the localization of adhesins and gliding motility (28, 291). Gliding motility is considered to be an important aspect of the pathogenesis of mycoplasmas, facilitating access to target tissues, while bypassing host physical barriers, such as mucus and ciliary activity (28, 176, 180, 249, 307).

GapA (previously known as *M. gallisepticum* cytadhesin 1, Mgc1) is the primary cytadhesin, and is encoded by a single gene, *gapA*, which has homology to the *M. pneumoniae* P1 cytadhesin gene (113, 153). GapA is co-expressed with the accessory cytadhesin CrmA (274). MG attachment in *in vitro* assays was significantly inhibited by anti-GapA Fab fragments (113), and in transposon mutants in which either *gapA* or *crmA* were disrupted (244). Co-expression of both GapA and CrmA was necessary to restore the cytadherance and virulence of an attenuated MG strain, $R_{high}$ (274, 277).

The putative cytadhesin phase variable protein A (PvpA) and MG cytadhesin 2 (Mgc2) are both located in the terminal organelle, and bear homology to the *M. pneumoniae* P30 cytadhesin (26, 128, 354). PvpA exhibits size variation between MG strains (26). Both GapA and PvpA undergo high-frequency phase variation, characterized by on-off switching in expression, which contributes to the phenotypic variation of MG

(256). Phase variation is mediated by reversible base substitutions at the 5' end of the *gapA* and *pvpA* genes, resulting in premature termination of translation (26, 256, 346).

GapA, CrmA and Mgc2 were recently demonstrated to be required for the gliding motility of MG (137). Mutants deficient in GapA or CrmA expression displayed a loss of motility, haemadsorption and the characteristic flask-shape of the cells (137). Transposon insertion in *mgc2* resulted in a loss of motility but not haemadsorption, and complementation of the mutant with *mgc2* restored the motile phenotype (137).

The variable lipoprotein haemagglutinin (VlhA, previously pMGA), the MG haemagglutinin, plays a major role in attachment and in the generation of antigenic diversity (220, 222, 223). A sialoreceptor binding motif in the VlhA may facilitate host-cell attachment (231). VlhA is expressed by a multi-gene family, comprising up to 70 *vlhA* genes, depending on the strain examined (14, 256). The expression of *vlhA* genes has been found to oscillate *in vitro* and *in vivo* in response to VlhA-specific antibodies (108, 109, 221). The antigenic variation of VlhA is mediated by variations in length of the GAA trinucleotide repeat upstream of the promoter (108, 109, 207, 208).

The ability of various surface-exposed proteins to bind components of the extracellular matrix (ECM) is thought to contribute to adherence and colonization of MG (47, 95, 141, 233). *Pneumoniae*-like protein A (PlpA) and HMW3-like protein (Hlp3) were identified as fibronectin-binding proteins; these proteins are present in the virulent strain $R_{low}$, but absent in its attenuated derivative $R_{high}$ (233). Plasminogen binding of MG is mediated by α-enolase; MG adherence to chicken fibroblasts was significantly inhibited in the presence of α-enolase-specific antibodies (47). An OsmC-like protein, MGA_1142, was demonstrated to bind heparin (141). MGA_1142 was subsequently

reported to be an organic hydroperoxide resistance protein, and may be involved in the detoxification of endogenous and exogenous peroxides (142).

While MG is primarily regarded as a non-invasive pathogen, systemic infections have been reported (289), including infections of the brain (48, 321). The ability of MG to invade non-phagocytic cells has been demonstrated, and has been postulated to provide a mechanism for immune evasion, antibiotic resistance, chronic infection and systemic invasion (334, 347). The virulent MG strain $R_{low}$, but not the attenuated $R_{high}$, was demonstrated to invade, survive and multiple intracellularly for at least 48 hrs in cultured cell lines (347), and was isolated from the internal organs of inoculated chickens (243). Invasion of erythrocytes was also demonstrated, both *in vitro* and *in vivo*, after experimental infection of chickens (334).

Recently, several other MG virulence factors have been identified, which have putative functions in nutrient acquisition and metabolism. Transposon mutagenesis of pathogenic MG strains has been used to inactivate the genes encoding dihydrolipoamide dehydrogenase (Lpd) (99, 134), the polynucleotide binding protein MslA (230, 315) and the predicted ABC sugar transport permease MalF (325). The resulting mutants all demonstrated attenuated virulence in chickens, suggesting the role of these proteins in the virulence of MG (99, 134, 230, 315, 325).

Sialidase (neuraminidase), was investigated as a potential virulence factor based on the fact that MG and MS bind to host cells via sialic acid receptors, and considering the associations of sialidases with virulence in other bacterial pathogens (25, 234). Marked strain differences in sialidase activity, which appeared to correlate with virulence, were observed for MG (25). The virulence of sialidase knock-out mutants was

attenuated in chickens; however, sialidase complementation did not completely restore virulence (234). A number of genes encoding other proteins and lipoproteins with known or putative roles in cytadhesion, metabolism and transport have been identified as potential virulence factors based on comparative genomic analysis of virulent and attenuated MG strains (275, 316, 326).

***M. synoviae* virulence factors.** Marked variations in pathogenicity exist between different MS strains; while many isolates are relatively avirulent, pathogenic isolates can cause significant clinical disease and even systemic invasion (91, 126, 210, 240, 302). MS has been isolated from the brain and liver in birds with severe synovitis and/or airsacculitis lesions (49, 302). There are strain differences in tissue tropism; some isolates are more likely to cause airsacculitis and others to cause synovitis (169, 210). Strain differences in pathogenicity for chicken embryos have also been reported, although embryo pathogenicity does not correlate with pathogenicity in chickens (91, 209).

Lockaby *et al.* (1999) evaluated MS isolates classified as pathogenic, moderately or mildly pathogenic for their ability to adsorb to and haemagglutinate red blood cells, colonize tracheal ring and tendon cell cultures and induce ciliostasis. Differences in pathogenicity did not correlate with attachment ability, and were only partially explained by differences in colonization (211). In another study, MS isolates with the haemagglutination-positive phenotype were found to induce synovitis more frequently than haemagglutination-negative isolates (250).

The variable lipoprotein haemagglutinin (VlhA) is a major membrane antigen of MS (261). Similar to the VlhA of MG, the MS VlhA is encoded by a multigene family, and functions in cytadhesion and in the generation of phenotypic diversity by antigenic

variation, which is thought to facilitate immune evasion (18, 256). The molecular mechanisms mediating antigenic variation of the MS VlhA are, however, quite different. The MS *vlhA* gene family comprises one functional gene and at least 70 pseudogenes, which undergo unidirectional recombination with the *vlhA* gene (256, 262). The resulting chimeric gene is expressed as a prolipoprotein, which is post-translationally cleaved into two fragments, MSPA and MSPB (22, 261, 262). MSPA, the MS haemagglutinin, is more antigenically variable and plays the primary role in cytadhesion; MSPB is highly immunogenic but of uncertain function (256).

Similar to MG, MS isolates have been demonstrated to invade non-phagocytic cells (35, 68). Invasion of chicken erythrocytes, chicken embryo fibroblasts and human epithelial cells was reported; invasion occurred within 24-48 hrs and differences in invasion frequency were reported for different strains, but not for different passages of the same strain (35, 68).

The MS enolase is capable of binding plasminogen and fibronectin, and may be involved in binding ECM components or other virulence-related functions (12). Sialidase (neuraminidase) activity has been demonstrated for MS, and is suspected of contributing to its virulence (25, 232). MS strains displayed significant differences in sialidase activity, with greater activity in strains associated with virulence than in strains which did not induce clinical disease (25, 232).

The ability of MS to induce apoptosis in chondrocytes was suggested by the upregulation of apoptotic genes, secretion of nitric oxide and development of an apoptotic phenotype in chicken chondrocyte cultures infected with MS (67).

## Immunity, Immunomodulation and Immunopathology

Immunity against MG and MS is mediated primarily by bursal-derived lymphoid cells (5, 178, 183, 330), and mucosal antibodies are important for protection (11, 100, 139, 349, 351). The immune response to mycoplasma infection is, however, characterized by a robust lymphoproliferative response involving accumulations of lymphocytes, plasma cells, macrophages and heterophils (289). The tissue damage or "immunopathology" resulting from this inflammatory cellular infiltrate, rather than a direct effect of the organism itself, is considered to be the primary cause of the pathogenic effects of mycoplasma infection (291).

The chronicity of infection appears to be a result of the ability of mycoplasmas to evade host immunity through phenotypic variation, while concurrently modulating the immune response to infection. Mycoplasmas modulate host cell-mediated immunity by cytokine-mediated suppression or activation of lymphocytes and chemokine-mediated effects on chemotaxis (289). The expression, by both MG and MS, of a cysteine protease capable of cleaving chicken IgG, may be another mechanism by which these pathogens affect the host immune response (51).

***M. gallisepticum* immunity.** Infection with MG elicits a degree of immunity against subsequent exposure, although recovered birds may be indefinitely infected, and can transmit MG both laterally and vertically (289). The degree of protection induced against MG challenge is related to the virulence of the strain used for immunization (202). Vaccination with the live attenuated ts-11 vaccine elicited protection from virulent MG which lasted for at least 40 weeks (340).

The important role of bursal-derived lymphoid cells in mediating defense against MG is well known. Chickens bursectomized *in ovo* or at hatch had lower antibody titers, worse airsac lesions and higher mortality after MG inoculation, compared with intact chickens (5). Lam and Lin (1984) demonstrated that the protect effect of immunization against MG challenge was abolished in bursectomized, but not in thymectomized chickens, indicating that vaccine-induced protection is dependant on bursal-derived lymphoid cells, rather than on cell-mediated immunity (183).

Circulating antibody levels do not, however, correlate well with protection against MG; this has been demonstrated for antibodies induced by vaccination with live attenuated (183, 259) or inactivated vaccines (319). Maternal antibodies also provided very little protection against MG challenge in chicks (206). Maternal antibodies in embryonated eggs were reported to protect the embryo from MG-induced mortality, but did not appear to affect the isolation of MG from the egg (194). Improved survival of infected embryos in the presence of maternal antibodies will increase the probability of hatching infected chicks (194).

Several studies have shown that mucosal antibodies in the respiratory tract play a major role in protection against MG (11, 100, 139, 349, 351). Antibody titers in tracheal washes correlated with a reduction in MG-induced lesions and colonization in the trachea after challenge (349, 351). Tracheal washings from MG-inoculated chickens containing MG-specific antibody provided protection against MG in a tracheal-ring organ culture system; protection was dose-dependent and involved inhibition of attachment of MG to the tracheal mucosa (11). Protection induced by a live attenuated vaccine was correlated with lower numbers of infiltrating heterophils, B cells and $CD4^+$ and $CD8^+$ T cells, and

increased numbers of MG-specific IgG- and IgA- secreting cells in the trachea (139). A response by natural killer and cytotoxic T cells to MG infection was reported, although the efficacy of these cells in controlling infection was not determined (100).

MG infection resulted in upregulated expression of several cytokine and chemokine genes, including lymphotactin, macrophage inflammatory protein-1β, CXCL13 and CCL5; but in downregulated expression of others, including CCL20, IL-8 and IL-12 (179, 181, 182, 239). Stimulation of leukocytes from infected chickens with MG antigen resulted in a lymphoproliferative response, and the production of nitric oxide and interferon (292). The ability of MG to induce immune suppression is suggested by a temporary T cell suppression in infected chickens (96) and by reduced humoral immune responses to turkey rhinotracheitis virus in a dual infection with MG in turkeys (253).

***M. synoviae* immunity.** Considerably less is known about immunity to MS, although many of the responses are probably similar to those described for MG. Chickens vaccinated with live attenuated temperature-sensitive MS vaccines were protected from air sac lesions induced by virulent MS strains for at least 21 weeks (255) or 40 weeks (148) after vaccination. Exposure to MS via the respiratory route protected chickens against subsequent footpad challenge (91).

Immunity to MS has been shown to be cloacal bursa-dependent; bursectomized chickens developed severe synovitis and airsacculitis lesions after MS challenge compared with intact or thymectomized chickens (178, 330). Thymus-dependent lymphocytes are not required for protection, and may in fact be necessary for the development of macroscopic lesions (178).

The MSPB portion of the MS haemagglutinin is highly immunogenic (256), and induces the secretion of nitric oxide, IL-6 and IL-1β in chicken macrophages (189). Nitric oxide production by infected macrophages and chondrocytes was mediated by MSPB-induced expression of TLR15, resulting in increased expression of the transcription factor nuclear factor kappa B (272).

## Survival Outside of the Host

Studies evaluating the survival of MG and MS outside of the host on different substrates have reported survival times that are surprisingly long for bacteria which lack a cell wall and have complex nutritional requirements (3, 43, 50, 229, 248, 280, 306, 309). Published maximum survival times for MG and MS on various substrates are presented in Table 1.1.

These studies have indicated differences in the survival times of different *Mycoplasma* species (306); in general, MG appears to survive for longer outside of the host than MS (50, 306). There are also differences in the survivability of different MG and MS strains (43, 50, 229, 248, 306).

Substrate type is an important determinant of mycoplasma survivability, as is temperature, humidity and pH (289). Mycoplasmas tend to remain viable for longer at low temperatures and high humidity, and do not tolerate low pH conditions (43, 91, 248, 306, 309). Variability in the results of different studies can be attributed to differences in these parameters, and to different strains and suspension media used.

The longest mycoplasma survival times were reported in egg yolk, with viable MG detected for up to 20, 7 and 16 weeks at 37, 20 and 6°C respectively (43). Liquid

suspension media protect mycoplasmas from desiccation; egg yolk components may also provide a nutrient source. MG and MS have been reported to remain viable up to 4-5 and 7 days respectively in water (50, 229, 280, 306); in one of these studies, the water used was reported to have been potable chlorinated city water (50)

Survival times are generally longer on organic substrates than on inorganic substrates; this may be a function of greater surface absorbance/porosity of organic substrate surfaces (50). Christensen *et al*. (1994) demonstrated the longest survival times for MG and MS on feathers (MG, ≤ 4 days; MS, ≤ 3 days) and cotton (MG, ≤4 days; MS, ≤2 days); MG remained viable on hair for 3 days, and MG and MS survived in the nasal cavity for 24 and 12 hours respectively (50). A recent study reported extended MG and MS survival times on synthetic (kanekalon) hair compared with natural hair (3). Marois *et al.* (2002) reported the isolation of MS from feed, soil and water samples for up to 5, 7 and 7 days after inoculation respectively; these are among the longest reported environmental survival times for MS (229).

Nagatomo *et al.* (2001) reported the use of gastric mucin at a 5% inclusion level in broth and agar media for mycoplasma survival experiments, using dry paper discs as a substrate. MG survived for remarkably long periods under these conditions (up to 7 days at 30 or 37°C or outdoor temperature; 14 days at room temperature and 28 days at 4°C) (248). Although the substrate survival of MG in a media without mucin was not reported (248), these results suggest that mucin in respiratory secretions may prolong the survival of mycoplasmas in the environment.

Mycoplasmas can survive in, and extensively contaminate the environment of infected flocks. MS was isolated from feed, drinking water, chicken feces, dust and

feathers in the environment of naturally and experimentally infected chicken and turkey flocks (224, 228, 229), and was isolated for up to 3 days after depopulation of a contaminated isolator (228). In another study, MG was also detected by culture or PCR in feed, drinking water, dust and feather samples from infected chicken and turkey flocks (227).

The production of biofilms, comprised of sessile bacterial communities attached to a surface and surrounded by an extracellular polymeric matrix, is mechanism employed by some bacteria to survive and persist in the host or in the environment (65). The ability of some *Mycoplasma* species, including MG, to produce biofilms has been demonstrated *in vitro* (46, 235); *M. pulmonis* was shown to produce biofilms *ex vivo* and *in vivo* (308). There are marked differences in biofilm-producing ability between different MG strains (46). Biofilm production may facilitate the survival of MG (and possibly MS) in the environment (46).

The ability of MG and MS to survive in the environment, and the relative efficiencies of different materials to act as fomites facilitating the indirect transmission of MG and MS are of key importance for biosecurity programs. MG and MS can potentially be introduced into naïve flocks infected in the hatchery (e.g. egg yolk), placed on contaminated premises (e.g. feathers, feces, wood shavings or timber), fed contaminated feed or water or in contact with contaminated equipment (e.g. plastic) or people (e.g. clothing, hair, skin). Survival of MG and MS outside of the host may be facilitated by favorable environmental conditions, by respiratory mucus, and by their ability to form biofilms.

## Horizontal Transmission

MG and MS can be transmitted both horizontally and vertically, through the egg. Horizontal transmission of MG and MS may occur following direct contact between clinically or sub-clinically infected and susceptible birds or indirectly, by contact between susceptible birds and contaminated fomite materials (91, 289). Direct horizontal transmission of MG and MS occurs via the respiratory route, following contact of the upper respiratory tract and/or conjunctiva with contaminated aerosols or secretions (91, 289).

Infection with MG and MS is permanent; infected birds remain carriers for life (91, 289). Carrier birds are essential for the maintenance of MG and MS, since these organisms do not survive for long periods outside the host. Backyard and commercial egg laying flocks are common reservoirs of MG and MS; in addition, some free-ranging songbird species are potential sources of infection (28, 163, 289).

Reports demonstrating the ability of MG and MS to survive for up to several days outside of the host have highlighted the potential for indirect transmission of these pathogens (3, 43, 50, 229, 248, 280, 306, 309). Contaminated dust, droplets and feathers which become airborne may act as fomites facilitating the indirect horizontal transmission of MG (289).

There are strain differences in the rates of MG and MS transmission. The transmissibility of available live attenuated MG vaccines is low relative to typical field strains (92, 160, 200). Variability in the transmissibility of different MS isolates has also been reported (73, 336). It is possible that differences in tracheal replication efficiency, and thus in shed of infectious organisms account for some of these strain differences in

transmissibility. It appears that strain virulence and transmissibility are sometimes related characteristics (e.g. for the live MG vaccine strains), however, this is not always the case; the transmission rates of some MG (310) and MS (73) strains were similar to or greater than those of more virulent strains.

***M. gallisepticum* horizontal transmission.** There are numerous reports of experimental horizontal transmission of MG to direct contact chickens or turkeys commingled with infected birds (7, 54, 62, 84, 92, 145, 160, 167, 184, 200, 236, 254, 283). Kleven *et al.* (1981) reported that the F strain transmitted from infected chickens to chickens in an adjacent pen, but no transmission occurred if pens were separated by an aisle or empty pen (160). MG has also been demonstrated to transmit from experimentally infected house sparrows (170) and house finches (313) to chickens in the same pen; transmission did not occur across a wire fence or room (313).

McMartin *et al*. (1987) observed four phases in the direct transmission of the S6 strain of MG: a long latent phase before antibody was detected in the MG-inoculated bird (median 15 days); a short period in which 5-10% of in-contact chickens became seropositive (median 1 day); a constant phase characterized by the development of 90-95% seropositivity in the remaining population (median 24 days); and a short terminal phase in which the remainder of the population tested positive (median 4 days) (236). Increasing the population density increased the rate of horizontal transmission of MG (236).

Indirect transmission of MG has less frequently been experimentally demonstrated (54, 61, 84), although it is considered to be an important mode of spread of MG under field conditions. MG of the ts-11 genotype was detected in one chicken in a

pen separated by an aisle from ts-11 vaccinated chickens, but with a common handler (54). Indirect transmission related to the handler or to airborne contaminated dust is likely to have occurred in this case (54). Feberwee *et al*. (2005) reported the airborne transmission of MG in an experimental model designed to evaluate MG transmission dynamics and the effect of interventions (84). The transmission of MG from infected to naïve house finches by bird feeders acting as fomites has also been reported (61).

Much of the field evidence for the indirect transmission of MG between farms/premises is based on the application of molecular strain differentiation techniques, which have facilitated epidemiologic outbreak investigations. Molecular epidemiology was used to investigate outbreaks of MG genotyped as F strain in turkey breeders and meat-type turkeys; the index turkey flock was reported to have been in close proximity to a commercial egg-laying flock from infected with F strain (197). F strain infection was also identified in several unvaccinated Jordanian chicken flocks, and was theorized to have originated from previous vaccination of flocks in the region (103). The use of random amplified polymorphic DNA (RAPD) to investigate MG in turkey flocks in California revealed similar banding patterns for isolates from farms belonging to the same companies (44).

***M. synoviae* horizontal transmission.** The horizontal transmission of MS appears to be similar to that of MG, except that it is more rapid, usually resulting in infection of all birds in a house (91). Reports evaluating the horizontal transmission of MS are relatively scarce, and no transmission studies have been performed on recent MS isolates. An MS field isolate previously considered to be slow-spreading based on low seroreactivity (336) was later found to be similar in transmissibility to a virulent MS

strain by PCR and culture (73). This study highlighted the importance of incorporating DNA detection or culture into MS monitoring programs, which should not be solely dependent on serology (73).

Horizontal transmission by direct contact between experimentally infected and susceptible chickens has been reported; in these studies, infection in the contacts was detected 1-6 weeks after exposure to the principals (73, 168, 217, 267, 336). In one study, MS transmitted readily to direct contacts, but not to chickens separated from the infected chickens by two empty pens (168). Kleven and Fletcher (1983) reported low recovery of MS from experimentally infected house sparrows, and no transmission to chickens housed in the same pen, indicating that house sparrows are not efficient biological carriers of MS (170).

Ewing *et al*. (1998) evaluated the comparative transmission of three MS isolates. MS was first detected in the contact chickens by culture and/or PCR 1-3 weeks after exposure to the inoculated birds, with positive serum plate agglutination (SPA) results 1-3 weeks later. MS antibodies were not detected by the haemagglutination inhibition (HI) test in any of the contact-infected chickens for the duration of the study (73). The rate of MS transmission is related to the strain used for infection, and is likely to be reduced by laboratory attenuation, but appears to be independent of the titer of the inoculum (73).

The ability of MS to survive for up to several days on contaminated materials (3, 50, 224, 306) and to extensively contaminate the environment of infected flocks (224, 229) provided proof of concept for indirect MS transmission. In 2005, Marois *et al.* demonstrated, for the first time, the fomite transmission of MS using day-old SPF chicks in isolators, and showed that detection of indirectly-transmitted MS could be surprisingly

delayed (228). In that study, MS infection was first detected by culture and PCR 13, 33 or 54 days respectively after placement of chicks in isolators contaminated with either MS culture, depopulated MS-infected chicks or material (feed, feathers and dust) collected from a naturally infected layer flock (228).

## Vertical Transmission

Egg transmission plays an important role in the epidemiology of MG and MS. MG and MS are considered to be among the most significant pathogens that can be spread through the international trade in poultry hatching eggs (53).

***M. gallisepticum* vertical transmission.** A number of studies have contributed to our current knowledge of the egg transmission of MG (37, 56, 76, 77, 111, 112, 144, 203, 212, 264, 270, 297, 300, 301). These studies have highlighted the variable and unpredictable nature of MG egg transmission. Peak egg transmission rates ranging from 14 to 53% and occurring between 3 and 8 weeks after challenge with the virulent R strain have been reported (111, 112, 203, 270, 301). Marked variations in egg transmission rates between individuals in the same group are also common (76, 297).

Egg transmission after experimental challenge of chickens with virulent MG is typically preceded by clinical signs and drops in egg production, usually occurring within one week of inoculation (111, 112, 203, 270, 301). The rate of egg transmission is highest in the acute stage of infection, when levels of MG in the respiratory tract peak, and tends to decrease with time as the infection becomes more chronic (76, 111, 196, 203).

The majority of egg isolations have been made during the first 8 weeks after experimental infection of chickens (111, 112, 203, 270, 300, 301), however, MG could

still be isolated from eggs 25 to 32 weeks after inoculation (111, 112). Average egg transmission over periods of up to 22 to 32 weeks after R strain inoculation ranged from 3 to 12% in three studies (111, 112, 203). The rate of egg transmission during chronic infections under field conditions is likely to be lower than with experimental infection (196). Even a low rate of egg transmission may, however, result in infection of the entire flock due to horizontal transmission occurring after hatch, highlighting the importance of interventions that control vertical transmission at the breeder level (289).

The timing of infection, route of inoculation and strain inoculated can influence the rate of egg transmission of MG. Lin and Kleven (1982) reported egg transmission of the F strain of MG when birds in production were inoculated by aerosol but not by eye-drop (203). The rate of egg transmission of the virulent R strain was higher than that of the F strain (203). Egg transmission of strain 80083 (the parent strain of ts-11 vaccine) was detected following intra-abdominal injection, but not after combined intra-nasal and intra-tracheal inoculation (310).

Egg transmission of MG has also been reported with natural infection of ducks and geese kept in close proximity to infected chickens (23, 24). MG was isolated from 26% and 66% of duck and goose eggs respectively; examined eggs were either infertile or contained dead or abnormal embryos (23, 24).

The pathogenesis of egg transmission of MG has not been fully elucidated. The detection of MG in eggs is frequently associated with the presence of air sac lesions and the isolation of MG from the air sacs and/or oviducts, and less commonly from the ovaries (76, 297, 301). Considering the close proximity of the abdominal airsacs to the oviduct, it seems likely that ova are infected in the oviduct following air sac infection

(111, 297). Another explanation could be that the ova are infected as a consequence of bacteremia (111); however, MG was not isolated from the eggs of hens intravenously inoculated with MG (76), and hens shedding MG in their eggs were not found to be bacteremic (297), suggesting that this mechanism is less likely.

Early studies demonstrated marked reductions in egg transmission of MG after challenge in hens exposed to virulent MG as pullets (75, 265, 266). An important objective of the use of currently available attenuated vaccines in pullets prior to egg production is to reduce the egg transmission of MG (13, 111, 339). Medication is another intervention that has been successfully used to reduce MG egg transmission, either by medicating infected hens (270) or their eggs, by egg inoculation or egg dipping (114, 117, 177).

Fabricant *et al.* (1959) reported the greatest success in MG isolation from, in descending order, the egg yolk, embryo and allanto-amnionic fluid (76). Glisson *et al*. (1984) cultured the largest percentage of MG isolates from viable 18-day-old embryos, indicating the importance of culturing viable embryos, and not only eggs that fail to hatch, in order to more accurately determine the egg transmission rate (111). The importance of culturing viable embryos was underscored by the finding that embryo mortality due to MG was completely blocked in the presence of maternal antibody, which did not influence the isolation of MG from infected eggs (194). In another study, MG was isolated at a significantly higher rate from the vitelline membranes of eggs tested within 2 days of oviposition than from 18-day-old embryos (300). While testing of fresh eggs may prove a higher rate of MG egg infection, because viable embryos are not tested, this

method may less accurately depict the rate of vertical transmission of MG to the offspring.

***M. synoviae* vertical transmission.** Vertical transmission is known to play an important role in the spread of MS in chickens and turkeys, and is a potential contamination risk for live virus vaccines cultivated in chicken eggs (91). However, few studies have evaluated the egg transmission of MS (39, 214, 328, 329). The mechanism of egg transmission of MS is not completely understood; it probably involves contamination of the ova following air sac infection or bacteremia (214).

Rates of egg transmission in naturally and experimentally infected chickens, based on MS isolation from infertile eggs, dead embryos or day-old progeny, have ranged from 0.4% to 10.4% (39, 214, 328, 329). Carnaghan (1961) reported the detection of MS in 3% of eggs laid by experimentally infected hens over a 32 week period; all infected eggs were laid between 14 and 24 weeks after infection (39). Vardaman (1976) detected MS in 6-10% of eggs laid by MS-inoculated hens within the first month after infection, but did not detect MS in eggs laid from 1-7 months after inoculation (328).

Hens experimentally infected as pullets transmitted MS through the egg at a rate of 0.4%, indicating that egg transmission of MS was not prevented by infection during the rearing period (214). In general, the egg transmission rate of MS in commercial breeder flocks tends to peak in the first 4 to 6 weeks after infection, followed by a reduction or cessation in transmission, although infected flocks may transmit MS through the egg at any time (91).

MS has also been isolated from embryonated duck and goose eggs, following natural MS infections of ducks and geese with epidemiological links to infected chickens

(23, 24). MS was isolated from 24% and 72% of the dead, abnormal or infertile duck and goose eggs examined respectively (23, 24).

## Diagnosis

**Serology.** Antibody detection is the primary diagnostic tool used to screen flocks for mycoplasma infections. The serum plate agglutination (SPA), hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) tests are used for the detection of MG and MS antibodies (164). In order to confirm mycoplasma infection, positive serologic reactions on a combination of these tests (e.g. SPA and HI or ELISA) may require confirmation by PCR and/or culture.

Serum plate agglutination (SPA) is widely used as a flock screening test for MG and MS (10, 164). SPA is rapid, inexpensive and highly sensitive (164). Early detection of infection (7-10 days after infection) is facilitated by the detection of IgM antibodies (164, 296). The SPA test is, however, subject to low specificity, resulting in false positives, and may be insensitive for MS detection in turkeys (164). False SPA positives have been attributed to the adsorption of serum proteins to the mycoplasma surface during cultivation for antigen preparation (10), previous vaccination with oil emulsion vaccines against other pathogens, especially those containing serum components (110), or other unexplained factors (164). The occurrence of false positive reactors with the SPA test necessitates the use of a follow-up test to confirm positive reactors; confirmatory tests include the HI or ELISA serologic tests, culture or PCR (164).

The ability of MG and MS to hemagglutinate chicken or turkey erythrocytes facilitates the use of the hemagglutination inhibition (HI) test for serologic analysis (89).

The HI test is commonly used as a confirmatory serologic test for SPA reactors; it is very specific, but less sensitive than the SPA test (164). IgG antibodies are predominantly detected, and infected flocks test positive 2-3 weeks or longer after infection (10, 296). Improved MG and MS antibody detection with the HI test has been reported with the use of homologous test antigen prepared from the same strain as the strain used for challenge; in some cases, antibody was not adequately detected by heterologous antigen (168, 174, 202).

Enzyme-linked immunosorbent assay (ELISA) tests are available as commercial kits, and are used for the detection of MG and MS antibody in serum (10, 59, 124, 269, 320), egg yolk (116, 155) and respiratory secretions (11, 351). Multiplex ELISAs facilitating the simultaneous detection of MG and MS have also been developed (17, 269). The sensitivity and specificity of ELISA tends to be intermediate between those of the SPA and HI tests (289). IgG is predominantly detected, and infected flocks test positive within 2-3 weeks of infection (10, 164).

**Isolation and identification.** Mycoplasma isolation by culture is considered the gold standard for avian mycoplasma diagnosis. Mycoplasmas are fastidious organisms, with complex nutritional requirements, and are relatively slow growing (89, 163). Modifications of the media described by Frey *et al.* (1968) and Bradbury *et al.* (1977) are commonly used to culture avian mycoplasmas (27, 94, 164). Mycoplasma media are protein-rich, comprising 10-15% animal (usually swine) serum and often incorporating yeast components (89). MS requires nicotine adenine dinucleotide (NAD) for its propagation (89). MG and MS ferment glucose, which frequently added to culture media (164). Mycoplasmas are resistant to penicillin and thallium acetate; these components are

added to retard contaminant growth (89). Media pH is adjusted to 7.8 with sodium hydroxide, and phenol red is often added to broth media as a pH indicator (164).

Clinical samples are usually inoculated directly into broth media, incubated aerobically at 37°C, and passed into fresh broth media and plated onto agar when the phenol red pH indicator changes color to orange or yellow (164). MS is highly sensitive to low pH, and may no longer be viable if the phenol red indicator has turned yellow (indicating a pH < 6.8) (91). Tiny "fried-egg" colonies (0.1-1mm diameter) with dense elevated centers form after 3-5 days of incubation (89, 91, 164, 289). *M. gallinarum* and *M. gallinaceum* are rapidly growing, non-pathogenic avian mycoplasmas which frequently contaminate cultures (89). MG isolation can also be performed in embryonated chicken eggs (289).

Several methods have been used to identify mycoplasma colonies. Direct or indirect immunofluorescence techniques involve the identification of colonies or colony imprints using fluorescein-conjugated specific antiserum (164, 318). Direct immunofluorescence is a commonly used technique, which is accurate, rapid and can be used for mixed cultures (164). Immunoperoxidase may also be used alone or in combination with immunofluorescence (19). Positive identification by growth inhibition is based on the inhibition of colony growth by specific hyperimmune sera (52, 164).

*In vivo* bioassays have proven effective for the isolation of MG from flocks in which infection was suspected but could not be confirmed by culture. Mycoplasma-free chickens are inoculated with potentially infected material from the flock and monitored by culture and/or PCR (215). Enhanced multiplication after *in vivo* passage may facilitate MG detection.

**Molecular techniques.** A number of molecular techniques have been developed for the detection of MG and MS genetic material. Oligonucleotide (DNA or RNA) probes are a rapid and simple method for the direct detection of MG and MS DNA (16, 63, 93, 98, 136, 357), and have also been used for strain differentiation (157, 247, 353) but have reduced sensitivity, and have largely been replaced by polymerase chain reaction (PCR) (91).

PCR techniques have several advantages over culture; they are rapid, not susceptible to contamination, do not rely on the presence of viable organisms, and have comparable (or sometimes superior) sensitivity to isolation and identification (91, 289). Sensitivity may, however, be reduced in the presence of PCR inhibitors (345).

PCR with restriction fragment length polymorphism (PCR-RFLP) followed by gel electrophoresis of the cut PCR product has been successfully used for both *Mycoplasma* species identification (79, 187) and for MG (166) and MS (242) strain differentiation. An advantage of this technique is that it does not require mycoplasma isolation.

Species-specific PCR methods targeting the 16S rRNA genes of MG (154) and MS (188) have been developed. PCRs targeting genes which can be used for both species identification and strain differentiation (after sequencing of the PCR amplicon) have been developed more recently; these include PCRs targeting the *mgc2, gapA* and LP (MGA_0319) genes (88, 97, 252) and the 16S-23S rRNA intergenic spacer region (IGSR) of MG (286) and the *vlhA* gene of MS (129). Multiplex PCRs targeting MG, MS and other avian pathogens have also been described (216, 273).

Real-time PCR has several advantages over conventional PCR, including speed, sensitivity and ability to quantify DNA copy numbers (quantitative PCR). A number of

real-time PCR techniques have been reported for the detection of MG, MS or both MG and MS in duplex assays (36, 38, 138, 237, 288, 312).

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting method based on selective amplification of restriction fragments (175). AFLP is a reproducible technique, which has been used for both species and strain differentiation (130, 131, 175), however it is fairly complex and requires sequencing technology (164).

The application of silver nanorod array-surface-enhanced Raman spectroscopy (NA-SERS) for the species identification of MG and MS was recently reported (123). Detection sensitivities were comparable to real-time PCR, and specificity was generally high, except for MS detection (123). NA-SERS does not require DNA extraction or sequencing technology (123).

## Molecular Epidemiology and Strain Differentiation

Molecular epidemiology has been defined as the use of molecular techniques to identify microorganisms responsible for infectious diseases and their genes responsible for virulence, immunogenicity and drug resistance; and to determine the physical sources, phylogenetic relationships, and routes of transmission of these microorganisms and/or their genes (191)

Molecular strain differentiation techniques are valuable epidemiologic tools, enabling the tracing of disease sources and the differentiation of field and vaccine strains. Techniques facilitating strain differentiation have become increasingly important since the widespread use of live MG and MS vaccines. Understanding the molecular epidemiology of MG and MS facilitates the design and monitoring of improved control

strategies (88). Genotyping methods should be reproducible, rapid to perform, easy to interpret, and have sufficient discriminatory power to differentiate related and unrelated strains (88).

Discriminatory power is the ability of a typing method to distinguish different strains or genotypes (135). Discriminatory power can be calculated using Simpson's index of diversity or the discrimination index (*D*), which is a measure of the probability that two unrelated strains will be placed into different typing groups (135). A *D* index > 0.90 is considered adequate, and a *D* index > 0.95 is considered good typing discrimination (88, 135).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mycoplasmal proteins (158) and Southern blot hybridization with DNA and ribosomal RNA gene probes (157, 247, 353) have been used for MG and MS strain differentiation, but are costly, complex and time-consuming and are no longer in common use (289). PCR with restriction fragment length polymorphism (PCR-RFLP) is a relatively inexpensive technique, which has been successfully used for MG (166) and MS (242) strain differentiation. Although largely replaced by more rapid and discriminatory genotyping methods, PCR-RFLP is still used when sequencing technology is not available.

The DNA fingerprinting method arbitrarily-primed PCR (AP-PCR) or random amplified polymorphic DNA (RAPD), which has been widely used to type MG and MS strains (45, 78, 101), involves the use of short, arbitrary primers to generate banding patterns on agarose gels (164). This technique facilitates accurate strain differentiation, but is difficult to reproduce and standardize, hampering interpretation of results and

interlaboratory comparisons (88, 289). Amplified fragment length polymorphism (AFLP) is a discriminatory method for MG and MS strain differentiation, and is more reproducible than RAPD; however, it is time-consuming and sequencing technology is required (81, 131, 164). Pulse-field gel electrophoresis (PFGE) has also been used for MG and MS typing (225, 226). PFGE is more reproducible but less discriminatory than RAPD; this method is also expensive and time-consuming (225, 226, 289). A disadvantage of all of these DNA fingerprinting methods is the requirement for mycoplasma isolation in pure culture.

Genotyping by PCR and sequencing has replaced many of the older strain identification techniques due to its speed, accuracy and reproducibility, facilitating the development of sequence databases for interlaboratory comparison (88). An advantage of PCR and sequencing over RAPD is that is can be performed directly on clinical samples, without the requirement for culture. Mixed strain infections, cannot, however be identified, and sequencing technology is required (88).

Gene-targeted sequencing (GTS) of multiple surface protein-encoding genes of MG (*mgc2, gapA, pvpA*, MGA_0319) facilitated better strain discrimination than RAPD (*D* index, 0.965, compared with 0.958 for RAPD), and the results correlated with epidemiological links (88, 171). The discriminatory indices of the genetic targets evaluated in that study was as follows: *pvpA,* 0.920; *mgc2*, 0.915; MGA_0319, 0.874 and *gapA, 0.713*. Sequencing more targets increased discriminatory power (88). The *mgc2* (131) and *pvpA* (146, 279, 311) genes have also been targeted individually for genotyping. PCR and sequencing of the 16S-23S intergenic spacer region (IGSR) facilitates very good differentiation of MG strains (estimated *D* index, 0.950) (285), and

is frequently used in combination with the *mgc2* target for strain differentiation (103, 156). All of these genomic targets have proven sequence stability (88, 286).

Fewer genetic targets have been described for MS strain differentiation. PCR and sequencing of the conserved 5' end of the *vlhA* gene is most commonly used to identify MS strains (119, 122, 129, 338). The 16S-23S intergenic spacer region (ISR) may also be used for MS strain differentiation, preferably in combination with other mutation detection techniques (284).

Recently, PCR and high-resolution melting curve analysis (PCR-HRM) has been introduced as a rapid and cost-effective alternative for MG and MS strain differentiation, without the requirement for sequencing (105, 140). PCR-HRM involves the separation of DNA strands of PCR amplicons along a precisely controlled temperature gradient; strain differentiation is based on slight, sequence-specific differences in the resulting melting curves (115, 348). The technique has successfully been used for differentiation of MS (140, 303) and MG (104, 105) strains.

## Intervention Strategies

Preventing infection with the pathogenic avian mycoplasmas is the gold standard for mycoplasma control, since there is no effective way to reliably eliminate an established infection. In cases where it is considered difficult or impossible to maintain flocks free of MG or MS infection, medication may be considered as a short-term intervention for the alleviation of clinical disease and production losses. Vaccination with inactivated bacterins or live vaccines is a medium- to long-term intervention (163).

## Prevention of Infection

In order to maintain flocks free of MG and MS, it is imperative that replacement stock originates from mycoplasma-free sources, and that biosecurity is adequate to prevent the introduction of mycoplasmas (91, 163, 289, 339). Effective mycoplasma monitoring and surveillance systems should be in place to ensure that the MG- and MS-clean status of breeder flocks is maintained (163, 289).

Since MG and MS are vertically transmitted, replacement stock must originate from MG-and MS-clean parent flocks. While most breeding companies have eliminated MG and MS at the primary breeder level (163), less stringent prevention measures at the secondary / multiplier breeder level has resulted in increased MG and particularly MS prevalence in multiplier breeders and their broiler progeny. In the United States, it is common practice for integrators to cull breeder flocks that become infected with MG. The decision to slaughter breeders infected with MS, however, may be subject to economic considerations (91). In recent years, less virulent strains of MS have been more readily tolerated at the breeder level, resulting in an increased MS prevalence in multiplier breeders and their offspring (185).

Adequate biosecurity measures are necessary to prevent mycoplasma introduction onto clean premises. The level of biosecurity which is required to prevent mycoplasma introduction is generally increasing worldwide, with increasing poultry population densities, frequently comprising multiple commercial poultry types, as well as backyard flocks (163, 196, 289). Multiple-age production systems for table egg, breeder and meat production, have become more common worldwide, contributing to the increasing challenge of mycoplasma control (196, 289).

Effective monitoring and surveillance systems must be in place to ensure that mycoplasma infections are detected early, facilitating rapid biocontainment and preventing spread to other flocks (163). In the United States, the National Poultry Improvement Plan (NPIP) co-ordinates programs for the monitoring and control of MG, MS and MM in primary and multiplier chicken and turkey breeding flocks and hatcheries (1). NPIP-certified flocks must be regularly tested by serology and culture or PCR, using approved methods.

## Medication

The main objectives of antibiotic medication are to reduce clinical signs, lesions and egg transmission of MG or MS (15, 114, 118, 163, 251, 268, 270). Antibiotic injection or dipping of hatching eggs has been successfully used to reduce or eliminate egg-transmitted MG or MS (114, 117, 177). Medication has also been used to reduce the severity of egg production drops associated with MG infection, and of eggshell pathology caused by MG or MS infection (41, 80, 271). Antibiotic treatment was shown to reduce tracheal colonization by MG (58), which may result in reduced shed and horizontal transmission. Treatment of infected flocks is a short-term intervention to reduce production losses associated with virulent MG or MS infection, and is not a reliable strategy to eradicate mycoplasmas (163, 295, 314).

The lack of a cell wall renders mycoplasmas resistant to β-lactam antibiotics like penicillin or cephalosporins, which act by inhibiting cell wall synthesis (163, 289). MG and MS have demonstrated sensitivity to several antibiotic groups, including macrolides, tetracyclines and fluoroquinolones (30, 42, 102, 121, 150-152, 165, 186, 192, 201, 314,

323, 335, 341). MS isolates appear to be more resistant to erythromycin than MG isolates (30, 341). In the United States, macrolides and tetracyclines are most commonly used to control MG and MS infections (163), and tylosin is the most commonly used macrolide antibiotic. The development of antibiotic resistance has been demonstrated for MG and MS isolates, and several procedures for antibiotic sensitivity testing have been described (30, 120).

## Vaccination

MG and MS vaccines are used to provide protection against respiratory disease, synovitis (for MS), egg production drops and egg transmission (74, 339). The use of live MG vaccines to displace virulent field strains as part of MG eradication programs has also been demonstrated (167, 195, 327). Live vaccines and inactivated oil-emulsion bacterins are available for the control of MG and MS; a fowlpox-vectored MG recombinant vaccine is also registered; and several experimental live attenuated MG vaccines have been developed. Antigenic variability of different MG and MS strains does not appear to necessitate the use of strain-specific or multivalent vaccines (339).

***M. gallisepticum* bacterin vaccines.** MG bacterin vaccines comprise inactivated MG organisms suspended usually in oil emulsion or aluminum hydroxide adjuvants, and are administered by subcutaneous or intramuscular injection (125, 339, 350). A significant advantage of the use of bacterins over live vaccines is that bacterins do not introduce live organisms onto the premises; there is therefore no risk of vaccinal transmission or reversion to virulence. Mycoplasma bacterins can, however, cause severe injection site reactions, resulting in losses at processing (66).

MG bacterins have demonstrated efficacy at, and are primarily used for reducing ovarian regression, egg production losses and vertical transmission associated with MG (90, 111, 112, 125, 301). In other studies, bacterins were less effective at protecting against egg production drops (159, 339). Variability in bacterin efficacy has been attributed to differences in bacterins used, challenge doses and strains, and intervals between vaccination and challenge in different studies (339). Bacterins are generally less effective than live MG vaccines at protecting against respiratory tract colonization and lesions induced by MG (2, 161, 319), and bacterin vaccination did not reduce the horizontal transmission of the virulent R strain (86).

***M. synoviae* bacterin vaccines.** There is little evidence for the efficacy of MS bacterin vaccines, and it appears that they are not in common use (339).

**Live attenuated *M. gallisepticum* vaccines.** There are currently three live MG vaccines which are commonly used in various countries worldwide; F strain (213), ts-11 (343, 344) and 6/85 (71). K-strain (92) is another live MG vaccine, which has recently been used on a limited scale. These vaccines are characterized by differences in virulence and immunogenicity. For the live mycoplasma vaccines, immunogenicity and protection induced are correlated with virulence or reactivity; more reactive vaccine strains generally induce better protection against challenge than less reactive strains (2, 193, 202, 339). In the United States, vaccination is primarily used to control MG in multiple-age commercial layer farms.

Although commercially available F strain vaccines are relatively mild, the original F strain was described by Yamamoto and Adler (1956) as a virulent MG strain (289, 352). F strain vaccines have since been used extensively worldwide for the

immunization of long-lived chickens; most commonly commercial layers in multiple-age complexes (289). F strain vaccines are sold in lyophilized form, and are registered for application by coarse spray (Poulvac®Myco F, Zoetis Animal Health) or in the drinking water (Avipro®MG F, Lohmann Animal Health) to chickens 9 and 12 weeks of age or older respectively, prior to the onset of egg production.

F strain vaccines have demonstrated very good efficacy at the prevention of tracheal and airsac lesions (2, 90, 202, 205, 298), ovarian regression (90), egg production drops (40, 111, 238) and egg transmission (111) induced by virulent MG. F-strain persists in the upper respiratory tract for the life of the flock (160), and its ability to reduce MG tracheal colonization (57) and to displace virulent MG strains (167, 172, 195) has been demonstrated.

The F strain is mildly virulent to chickens; it is capable of inducing airsac lesions in chicks (299) and drops in egg production (2, 32), and has been demonstrated to transmit vertically through the egg if aerolized during lay (203). F strain vaccines are too pathogenic for use in turkeys, and should not be used in turkey-producing areas (197, 204). Horizontal transmission of F strain occurs to non-vaccinated chickens in the same house (160), and there is evidence of F strain transmission from vaccinated chickens to neighboring houses or farms in the area (103, 197, 339). There is no published report of reversion to virulence of F strain vaccines.

The ts-11 vaccine was developed by N-methyl-N-nitro-N-nitrosoguanidine (NTG) mutagenesis of an Australian field isolate, and selected for its temperature sensitive ($ts^+$) phenotype (344). The $ts^+$ phenotype of ts-11 is characterized by viable counts greater than $10^2$ higher at 33°C (the permissive temperature), compared with 39.5°C (the restrictive

temperature) (255, 342). The parent strain of ts-11, 80083, is a virulent field isolate, and was shown to cause air sac lesions, egg production losses (65.7%) and egg transmission (32.8%) after intra-abdominal inoculation (310). In the United States, ts-11 is sold as a frozen vaccine, for eye-drop application in chickens 9 weeks of age or older (*Mycoplasma gallisepticum* vaccine®, Merial Select).

The genetic basis for the attenuation of ts-11 has not been fully elucidated (339). The back-mutation rate of ts-11 from a $ts^+$ to a $ts^-$ phenotype is $10^{-4}$, however $ts^-$ clones derived from ts-11 vaccine and $ts^-$ isolates made from vaccinated chickens are avirulent, indicating that the attenuation of ts-11 is not contingent on the $ts^+$ phenotype (339). The majority of cells in the ts-11 vaccine do not express the primary cytadhesin GapA (244, 304). GapA expression has, however, been observed in ts-11 re-isolates from vaccinated chickens, and a $GapA^+$ ts-11 vaccine was avirulent (304).

ts-11 has been demonstrated to provide protection against airsac lesions and respiratory disease induced by virulent MG infection (2, 259, 339, 343), although its efficacy was lower than that of F strain (2). The ability of ts-11 to protect against MG-induced egg production drops and to reduce vertical transmission of MG field strains has also been reported (13, 339).

The duration of protection induced by ts-11 vaccine was reported to be at least 40 weeks (340). Chickens vaccinated with ts-11 were protected from challenge despite a weak systemic antibody response (259). Following repeated placements of ts-11 vaccinated pullets, ts-11 displaced circulating F strain on a commercial layer farm, highlighting its potential use in MG eradication programs (327). However, ts-11 is less

effective at displacing virulent MG strains than F strain (167). The efficacy of ts-11 appears to be highly dose-dependent (257, 343, 344).

ts-11 is regarded as a strain of low or no virulence; there is little or no respiratory reaction post-vaccination (2, 200, 344) and no negative impact on egg production or eggshell quality (33). The ts-11 vaccine is apathogenic for turkeys (344), and appears to be incapable of infecting turkeys (87). The risk of horizontal transmission of ts-11 from vaccinated flocks is probably low; ts-11 was detected in 40% of non-vaccinated chickens commingled with ts-11 vaccinates within 9 weeks of vaccination, but there was no evidence of transmission to birds in an adjacent pen (200). In another study, however, ts-11 was detected in 50% of non-vaccinated direct contact chickens 3 weeks after vaccination, and from 1/15 chickens separated by a corridor from ts-11 vaccinates, but with a common handler (54). There have also been field reports of the detection of ts-11-like isolates in flocks which may have been inadvertently vaccinated, with subsequent spread to a neighboring broiler breeder flock occurring in one case (289).

There is one report providing evidence for reversion to virulence and vertical transmission of ts-11 vaccine (70). This report followed the isolation of ts-11-like MG from the clinically ill broiler progeny of breeders which had previously been vaccinated with ts-11 vaccine. The ts-11-like isolates were indistinguishable from ts-11 vaccine by several genotyping methods; one of these isolates was reported to be significantly more pathogenic than ts-11 vaccine (70).

Reversion to virulence and egg transmission of ts-11 is probably a rare event. Whithear (1996) reported that no MG was isolated from 3,750 eggs laid by hens vaccinated with ts-11 or in direct contact with ts-11 vaccinates (339). MG was, however,

detected in the eggs of broiler breeders vaccinated by eye-drop at 15 and 37 weeks of age, although the possibility that the semen used for artificial insemination was contaminated could not be ruled out (339). Whithear *et al*. (1990) also reported the isolation of MG from previously vaccinated layers in Australia, which had an identical restriction endonuclease analysis (REA) banding pattern to ts-11 and its parent strain 80083, and which was intermediate in virulence between ts-11 and 80083 (342).

Although details of the development of 6/85 vaccine have not been published, this strain is known to have originated in the United States and to share DNA and protein banding patterns similarities with the S6 strain of MG (72, 289, 339). 6/85 is manufactured as a lyophilized vaccine, for fine spray application to chickens 6 weeks of age or older (Mycovac-L, Merck Animal Health). 6/85 has also been used to a limited extent in turkeys in the field (163).

The least reactive of the live MG vaccines, 6/85 is of no or minimal virulence to chickens and turkeys (2, 71), and stimulates a low or undetectable serological response in vaccinated chickens (2, 200). 6/85 vaccinated chickens were significantly protected against virulent MG (71), but protection was inferior to that induced by F strain vaccine (2). Isolation of 6/85 from vaccinated chickens may be inconsistent, and 6/85 was not able to displace virulent R strain infection (167, 200). Vaccination with 6/85 reduced, but did not prevent the horizontal transmission of the R strain (83).

The 6/85 vaccine is poorly transmissible, and was not isolated from unvaccinated chickens commingled with 6/85 vaccinates over a 15 week period (200). Repeated back-passage of 6/85 resulted in no significant increase in virulence (355). There are reports of isolations of MG genotyped as 6/85 from unvaccinated, clinically ill commercial layers

and turkeys; however, the possibility that these were field isolates resembling 6/85 vaccine could not be ruled out (171, 322).

K-strain is a naturally attenuated MG vaccine, which was originally isolated from layer chickens in Colorado in 1984 (92). Chickens vaccinated with K-strain were significantly protected against tracheal and airsac lesions induced by virulent MG challenge (92). K-strain is avirulent and did not revert to virulence after back-passage. The rate of horizontal transmission is low; K-strain transmitted to direct contacts from 4 weeks post infection, but not to birds separated from the vaccinates by a chain-link fence or by an empty pen (92). No vertical transmission was detected. All inoculated chickens were colonized, and K-strain persisted in the upper respiratory tract for at least 5 months (92).

**Live attenuated *M. synoviae* vaccines.** MS-H is currently the only commercially-available live MS vaccine. MS-H is registered for eyedrop application between 3 and 6 weeks of age (Vaxsafe® MS, Bioproperties, Australia). Similar to ts-11 vaccine, MS-H was produced by NTG mutagenesis of an Australian MS field isolate, and selected for its $ts^+$ phenotype (241). The parent strain of MS-H, 89079/7/NS, was isolated from a commercial layer chicken with respiratory disease (241). The genetic basis for the attenuation of MS-H is not known; it is not contingent on the $ts^+$ phenotype (260).

Vaccination with MS-H resulted in significant protection from air sac lesions and colonization induced by virulent MS (147, 218), and significant reductions in eggshell apex abnormality (EAA) lesions in the eggs of hens challenged with an EAA-inducing MS strain (85). Protective immunity was induced within 4 weeks of vaccination, lasted for at least 40 weeks, and was dose-dependent (147-149). Turkeys vaccinated with MS-H

by aerosol but not by eye-drop were protected from tracheal lesions and colonization by virulent MS (258).

MS-H is avirulent in chickens and turkeys and did not revert to virulence after back-passage (217, 258). Horizontal transmission of MS-H from vaccinated chickens to non-vaccinated commingled chickens and to chickens in different houses on the same site has been reported (217, 219). Egg transmission of MS-H was not detected (219).

**Recombinant *M. gallisepticum* vaccines.** There is one commercially-available MG recombinant vaccine, which comprises the fowlpox virus as a vector for MG gene inserts (40k and mgc genes) (356). This recombinant FPV-vectored MG (rFPV-MG) vaccine is registered for wing-web application in chickens older than 8 weeks (Vectormune®FP MG, Ceva Animal Health LLC, Lenexa, KS). The advantage of a rFPV-MG vaccine over live MG vaccines is that no live MG organisms are introduced; the safety of this vaccine and its ability to protect against FPV challenge have been established (190, 356). There are, however, few reports on the efficacy of the rFPV-MG vaccine; in one study, vaccinated chickens were not protected against virulent R strain challenge (90).

**Experimental *M. gallisepticum* vaccines.** An experimental MG vaccine designated GT5 was engineered by complementation of the attenuated, cytadherence-defective strain MG strain $R_{high}$ with the gene encoding the primary cytadhesin GapA from the virulent, cytadherence-competent strain $R_{low}$ (276, 277). GT5 was significantly less virulent than $R_{low}$, and induced significant protection against tracheal lesions and colonization induced by $R_{low}$ (276).

Mg 7, another experimental MG vaccine, was developed by transposon mutagenesis of the dihydrolipoamide dehydrogenase (*lpd*) gene of the virulent MG strain $R_{low}$ (134). Mg 7 was significantly attenuated compared with $R_{low}$, and was reported to induce comparable protection from tracheal and air sac lesions after $R_{low}$ challenge to that of ts-11, F strain and GT5, but with reduced recovery of the challenge strain (99, 134).

Recently, recombinant MG-vectored vaccines have been developed, comprising strain ts-11 as a vector for the expression of chicken interferon-gamma (IFN-γ) (245), the primary cytadhesin GapA (304) or GapA, infectious bronchitis virus (IBV) S1 glycoprotein and interleukin-6 (IL-6) (305). The recombinant ts-11-IFN-γ vaccine stimulated an enhanced cellular immune response compared with ts-11 alone (245). Complementation of ts-11 with *gapA* increased the immunogenicity of ts-11 (304), while ts-11 expressing GapA, IBV S1 and IL-6 was reported to provide partial protection from IBV challenge (305).

## Background to the Research

### Molecular epidemiology and the need for a model MG sequence database

Large concentrations of poultry in restricted geographic areas, multiple-age poultry production complexes and local reservoirs of infection present a continual risk of MG and MS introduction onto clean premises (163, 289). In cases where the maintenance of mycoplasma-free flocks is not regarded as feasible or possible, vaccination may be a viable option (163). The increased use of live vaccines to control MG and MS has necessitated the use of strain differentiation techniques to discriminate vaccine and field strains (88).

Targeted PCR and sequencing of certain genetic regions, including multiple surface protein-encoding genes (*mgc2, gapA, pvpA*, MGA_0319) and the 16S-23S rRNA intergenic spacer region (IGSR) have been demonstrated to facilitate very good strain differentiation of MG isolates, with increased discriminatory power achieved by sequencing of multiple genetic regions (88, 286). Targeted genetic sequencing is highly reproducible, and DNA sequence data can be used to develop reference MG sequence databases, allowing global interlaboratory comparisons and effective identification and tracking of MG strains, without the requirement for mycoplasma culture (88). Understanding the epidemiology of specific MG strains facilitates the design of rational and effective MG control strategies.

MG is one of the most economically significant poultry pathogens worldwide (289). In South Africa, as in many other countries, MG has been implicated as the cause of severe respiratory disease and economic loss. Farmers depend on vaccination (with live and / or killed oil emulsion vaccines) and antibiotic treatment to control this disease in infected flocks. Of the live vaccines available for MG control, only strains ts-11 (343, 344) and 6/85 (71) are commercially available in South Africa; F strain vaccines (6, 213) are not currently registered for use.

At the time of the study, little was known about the epidemiology of MG in South Africa; to our knowledge, there was no available sequence database or published strain information. A pilot study was performed using targeted genetic sequencing of the IGSR and *mgc2* regions to genotype several South African MG DNA samples. South African genotypes were found to be highly unique, and different from all other sequences in a database comprising MG sequences from multiple countries.

There is a lack of available information on the molecular epidemiology of MG in many countries. It was thought that the development of a South African MG sequence database could be used as a model to guide the development of MG sequence databases internationally, which would have application in elucidating the epidemiology of MG and refining diagnostic techniques for different countries.

Cases of apparent reversion to virulence and vertical transmission of ts-11 vaccine

The ts-11 vaccine is one of the most widely used live MG vaccines worldwide. It is recognized as a strain of low or no virulence (344), and has been demonstrated to provide protection against respiratory disease, egg production drops and vertical transmission associated with virulent MG infection (2, 13, 339, 343).

ts-11 was produced by chemical mutagenesis of a virulent Australian *M. gallisepticum* field isolate and selected for its temperature sensitive ($ts^+$) phenotype (growth at 33°C) (310, 344). The ts-11 vaccine's lack of virulence is not contingent on the $ts^+$ phenotype (339); the genetic basis of its attenuation has not been fully elucidated.

In 2007, a decision was taken to vaccinate a number of broiler breeder flocks in northeastern Georgia with ts-11 vaccine, to control an MG epidemic that began in the area in 2006. Between 2008 and 2011, MG was found to be the cause of severe respiratory disease in the broiler progeny of several ts-11-vaccinated breeder flocks from four companies. MG isolates from these broiler flocks and their parents were indistinguishable from ts-11 vaccine strain by all genotyping methods used, and were therefore termed "ts-11-like" (70). Spikes in mortality and rising MG antibody titers in the breeder flocks were reported to precede clinical disease in the progeny by

approximately 2 months (70). A pathogenicity study confirmed that K6216D, a ts-11-like MG isolate from one of the infected broiler flocks, was significantly more virulent than ts-11 vaccine. The epidemiology of the outbreaks, the genotyping results and the pathogenicity study findings indicated that reversion to virulence and vertical transmission of the ts-11 vaccine had occurred (70).

## Outbreaks of MS in the state of Arkansas

MS was identified as the cause of outbreaks of severe respiratory disease in broiler and broiler breeder chickens in the state of Arkansas between 2008 and 2009. The respiratory disease was associated with decreased feed efficiency, increased mortality and airsacculitis condemnations in affected broilers, and drops in egg production and increased mortality in broiler breeders. The severity of this outbreak was unusual for MS, which typically causes a subclinical upper respiratory disease (91). This apparent atypical virulence was accompanied by evidence of extensive vertical and horizontal transmission.

The pathogenicity two MS isolates from this outbreak, K6191C and K6315D, belonging to the genotypes designated S-10 and S-17 respectively, was previously investigated in broiler breeder pullets (9). The S-10 isolate was significantly more pathogenic than the S-17 isolate ($P \leq 0.05$), and similar in pathogenicity to the pathogenic reference strain K1968 (9). The rate of transmission of S-10 MS to direct, adjacent, downwind and fomite contact chickens was found to be similar to, but slightly slower than that of K1968 (9).

## Specific Aims and Objectives of the Research

Specific Aim 1. Develop and apply an MG sequence database

**Objective 1.1. Develop a South African MG sequence database.** The first objective of this study was to develop an MG sequence database by targeted genetic sequencing of extracted MG DNA from a range of commercial poultry types and geographic locations within South Africa. Four previously characterized genetic regions were selected for analysis: two genes encoding surface proteins involved in cytadhesion (*mgc2* and *gapA*), one gene encoding a predicted conserved surface lipoprotein (MGA_0319) and the 16S-23S rRNA intergenic spacer region (IGSR) (88, 286).

**Objective 1.2. Use the MG sequence database to elucidate the molecular epidemiology of MG in South Africa.** The second objective was to use this sequence data to characterize South African MG wild-type genotypes, determine their distribution within the country and their identity to MG vaccines and wild-types from other countries.

**Objective 1.3. Determine which genetic target(s) facilitate optimum differentiation of South African MG strains.** The final objective was to assess the relative ability of the selected targets and combinations of targets to differentiate South African wild-type genotypes from each other and from the live MG vaccine strains (ts-11, 6/85 and F strain).

Specific Aim 2. Evaluate the egg transmission and pathogenicity of MG isolates of the ts-11 genotype

**Objective 2.1. Evaluate the egg-transmission potential of the ts-11-like isolates.** The first objective was to investigate the ability of ts-11 vaccine and ts-11-like MG isolates

from a broiler flock (K6216D) and their ts-11-vaccinated parent flock (K6222B) to transmit via the egg.

**Objective 2.2. Evaluate the pathogenicity of the ts-11-like isolates and their effect on egg production.** The second objective was to evaluate the pathogenicity of these isolates in sexually mature chickens, and the effect of infection on egg production.

Specific Aim 3. Comparatively evaluate the transmissibility of two MS genotypes.

**Objective 3.1. Evaluate the transmissibility of the S-10 and S-17 genotypes of MS in sexually mature broiler breeders.** The first objective was to evaluate and compare the transmissibility of the S-10 and S-17 genotypes of MS in sexually mature broiler breeders. The transmission of MS to birds associated with infected seeder groups by proximity (adjacent contacts), airflow (downwind contacts) and fomite placement (fomite contacts) was to be evaluated.

**Objective 3.2. Evaluate the effect of prophylactic tylosin medication on MS transmission.** The second objective was to investigate the effect of prophylactic tylosin medication on MS transmission to fomite contact birds, by comparing transmission to medicated fomite contacts and to non-medicated fomite contacts.

Specific Aim 4. Develop an infection model to evaluate the fomite transmission of MS

**Objective 4.1. Develop an infection model to evaluate MS fomite transmission.** The first objective was to develop an infection model to facilitate the comparative evaluation of MS transmission to chickens from various fomite materials commonly found in the poultry house environment (feathers, cotton fabric and plastic bootcovers). This objective

included investigating the feasibility of adding mucin to MS infection cultures, to simulate the consistency and composition of avian respiratory secretions.

**Objective 4.2. Evaluate the fomite transmission of isolates of the S-10 and S-17 genotypes of MS in sexually mature broiler breeder chickens.** The second objective was to evaluate and compare the fomite transmission potential of MS isolates of the S-10 and S-17 genotypes from directly-inoculated materials commonly found in the poultry house environment (feathers, cotton fabric and plastic) to naïve sexually mature broiler breeder chickens in pen trials.

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Table 1.1. Maximum reported survival times for MG and MS on various substrates

| Substrate | Survival time[ab] | |
|---|---|---|
| | MG | MS |
| Water | 0d [37°C] or 2d [20°C] (309); 4d (50); 5d (280, 306) | 2d (306); 7d (229) |
| Rubber | 2d (50) | 8h (50) |
| Plastic | 1d (50) | NT[d] |
| Paper[c] | ≤ 7d [30°C, 37°C or outdoor temp.], 14d [room temp.] or 28d [4°C] (248) | NT |
| Cotton | 1d [37°C], 3d [20°C] or 7 d [6°C] (43); 4d (50) | 2d (50) |
| Straw | 2d (50) | 12h (50) |
| Shavings | 8h (50) | 4h (50) |
| Timber | 1d (50) | 12h (50) |
| Soil | 3d (306) | 2d (306); 7d (229) |
| Feed | 4h (50) | 0h (50); 5d (229) |
| Chicken feces | 1d [37°C], 3d [20°C] or 7d [6°C] (43) | NT |
| Egg yolk | 16d [20°C] or 10d [37°C] (309); 20w [37°C], 7w [20°C] or 16w [6°C] (43) | NT |
| Feathers | 4d (50) | 3d (50) |
| Feather meal | 6h [37°C], 1d [20°C] or 4d [6°C] (43) | NT |
| Hair (natural) | 4h (3); 3d (50) | 4h (3); 8h (50) |
| Hair (synthetic) | > 4d (3) | > 4d (3) |
| Ear | 4h (50) | 4h (50) |
| Nose | 1d (50) | 12h (50) |
| Skin | 0h (50) | 0h (50) |

[a] Survival times in hours (h), days (d) or weeks (w).
[b] References are in parentheses.
[c] Paper discs inoculated with broth medium infused with mucin.
[d] NT = not tested.

# CHAPTER 2

# THE DEVELOPMENT AND APPLICATION OF A *MYCOPLASMA GALLISEPTICUM* SEQUENCE DATABASE[1]

[1] N.K. Armour, V.A. Laibinis, S.R. Collett and N. Ferguson-Noel. 2013. *Avian Pathology.* 42,5:408-415.

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Key words: *Mycoplasma gallisepticum*, South Africa, PCR, targeted sequencing, genotypes, IGSR, *mgc2*, MGA_0319, *gapA*

Abbreviations: bp = base pairs; IGSR = 16S–23S rRNA intergenic spacer region; *mgc*2 = *Mycoplasma gallisepticum* cytadhesin 2; PCR = polymerase chain reaction; PDRC = Poultry Diagnostic and Research Center; RAPD = random amplified polymorphic DNA; SNP = single-nucleotide polymorphism

## Summary

Molecular analysis was conducted on 36 *Mycoplasma gallisepticum* DNA extracts from tracheal swab samples of commercial poultry in seven South African provinces between 2009 and 2012. Twelve unique *M. gallisepticum* genotypes were identified by PCR and sequence analysis of the 16S-23S rRNA intergenic spacer region (IGSR), *M. gallisepticum* cytadhesin 2 (*mgc2*), MGA_0319 and *gapA* genetic regions. The DNA sequences of these genotypes were distinct from those of *M. gallisepticum* isolates in a database composed of sequences from other countries, vaccine and reference strains. The most prevalent genotype (SA-WT#7) was detected in samples from commercial broilers, broiler breeders and layers in five provinces. South African *M. gallisepticum* sequences were more similar to those of the live vaccines commercially available in South Africa, but were distinct from that of F strain vaccine, which is not registered for use in South Africa. The IGSR, *mgc2* or MGA_0319 sequences of three South African genotypes were identical to those of ts-11 vaccine strain, necessitating a combination of *mgc2* and IGSR targeted sequencing to differentiate South African wild-type genotypes from ts-11 vaccine. To identify and differentiate all twelve wild-types, *mgc2*, IGSR and MGA_0319 sequencing was required. Sequencing of *gapA* was least effective at strain differentiation. This research serves as a model for the development of an *M. gallisepticum* sequence database, and illustrates its application to characterize *M. gallisepticum* genotypes, select diagnostic tests and better understand the epidemiology of *M. gallisepticum*.

## Introduction

*Mycoplasma gallisepticum*, the etiologic agent of chronic respiratory disease in chickens and infectious sinusitis in turkeys, is the most pathogenic mycoplasmal pathogen of poultry (16). *M. gallisepticum* has a considerable economic impact on the poultry industry worldwide, through production losses, airsacculitis condemnations and mortality, and the associated costs of control and surveillance testing. In South Africa, *M. gallisepticum* has been implicated as the cause of severe respiratory disease. Farmers depend on vaccination (with live and / or killed oil emulsion vaccines) and antibiotic treatment to control this disease in infected flocks. Of the live vaccines available for *M. gallisepticum* control, only strains ts-11 (24, 25) and 6/85 (3) are commercially available in South Africa; F strain vaccines (1, 18) are not currently registered for use.

The genome of *M. gallisepticum* evolves extremely rapidly (2), resulting in genetic variants (strains) with marked differences in antigenicity, pathogenicity and transmissibility (6, 13-17, 21, 26). These strain differences potentially have implications for both the success of vaccination, and *M. gallisepticum* detection by diagnostic assays (6, 13, 14, 16, 22). With the worldwide increase in live vaccine usage to control *M. gallisepticum*, techniques that allow differentiation of wild-type (field strain) *M. gallisepticum* from the vaccine strains are particularly important. In addition, strain differentiation techniques may be utilized as valuable tools for the control of *M. gallisepticum*, facilitating rapid recognition of outbreaks, epidemiological traceability and targeted control (5).

A number of molecular techniques have been described for *M. gallisepticum* strain differentiation, including restriction fragment length polymorphism (RFLP) (14),

amplified fragment length polymorphism (AFLP) (11), random amplified polymorphic DNA (RAPD) (4, 8), and recently, PCR and high-resolution melting curve analysis (10). While RAPD has been widely and successfully used for *M. gallisepticum* strain differentiation, it has intrinsic problems with reproducibility, hampering inter-laboratory comparisons and long-term epidemiological studies (5, 23).

Targeted sequencing of selected *M. gallisepticum* genomic regions has been demonstrated to facilitate very good strain differentiation, with sequencing of multiple genetic targets allowing better discriminatory power than RAPD analysis (5). Despite the extraordinary point substitution rate of *M. gallisepticum* (2), selected genomic targets have proven sequence stability (5, 20). Targeted genetic sequencing is also a highly reproducible strain differentiation method, allowing the development of a reference database and global comparisons between laboratories (5). A significant advantage of targeted sequencing over RAPD is that is can be performed directly from clinical samples, without the requirement for mycoplasma isolation in pure culture (5, 20).

The first objective of this study was to develop an *M. gallisepticum* sequence database by targeted genetic sequencing of extracted *M. gallisepticum* DNA from a range of commercial poultry types and geographic locations within South Africa. The next objective was to use this sequence data to characterize South African *M. gallisepticum* wild-type genotypes (hereafter referred to as "wild-types"), determine their distribution within the country and their identity to *M. gallisepticum* vaccines and wild-types from other countries, and finally to assess the ability of the selected targets to differentiate South African wild-types. Four previously characterized genetic regions were selected for analysis: two genes encoding surface proteins involved in cytadhesion (*M. gallisepticum*

cytadhesin 2 [*mgc2*] and *gapA*), one gene encoding a predicted conserved surface lipoprotein (MGA_0319) and the 16S-23S rRNA intergenic spacer region (IGSR) (5, 20). This research demonstrates the value of an *M. gallisepticum* sequence database, and is the first report on the diversity and distribution of *M. gallisepticum* wild-types in South Africa.

## Materials and Methods

***M. gallisepticum* DNA.** Tracheal swab samples were obtained from commercial broiler, broiler breeder and layer flocks from seven of the nine South African provinces between 2009 and 2012. An opportunity sampling approach was employed, which depended on voluntary sample submission from flocks known or suspected of being infected with *M. gallisepticum*. In addition, *M. gallisepticum* DNA extracts contributed by five South African diagnostic laboratories were incorporated into the study. All submitters were requested to complete a questionnaire detailing province of origin, flock *M. gallisepticum* vaccination history, and poultry type from which samples were collected. The *M. gallisepticum* vaccination history of the 36 flocks from which samples were collected was as follows (numbers of flocks bracketed): non-vaccinated (15 flocks); ts-11 vaccinated (three flocks); recombinant fowlpox-*M. gallisepticum* vaccinated (two flocks); *M. gallisepticum* bacterin vaccinated (three flocks); vaccination history unknown or not reported (13 flocks). DNA extraction from swab samples was performed at Allerton Provincial Veterinary Laboratory in South Africa (KwaZulu-Natal Department of Agriculture and Environmental Affairs, Pietermaritzburg, KwaZulu-Natal) using the Qiagen DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the

manufacturer's instructions. Extracted DNA was submitted to the Poultry Diagnostic and Research Center (PDRC, University of Georgia, Athens, GA) in 1.5 ml Eppendorf Safe-Lock tubes™ (Eppendorf AG, Hamburg, Germany) or on Whatman™ FTA™ cards (Piscataway, NJ). DNA was extracted from FTA™ cards using the Qiagen QIAmp® DNA Mini Kit (QIAGEN, Valencia, CA). Then 200 µl Whatman™ FTA™ Purification Reagent (Piscataway, NJ) was added to five 3-mm disks from each card, followed by vortexing for 15 sec and incubation on ice for 10 min with intermittent vortexing. The rest of the extraction procedure was performed according to the manufacturer's instructions.

**PCR amplification and sequencing of targeted genes.** The four genetic regions selected for analysis, with expected PCR product sizes and discrimination indices for targeted DNA sequencing are presented in Table 2.1. Primer sequences, thermocycler programs and PCR amplification methods used were as previously described (5, 20). Amplified DNA was purified using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and submitted to the Georgia Genomics Facility (University of Georgia) for sequencing. Purified amplicons were sequenced twice in both directions with forward and reverse amplification primers, using the ABI 3730xl DNA Analyzer capillary sequencer (Applied Biosystems, Foster City, CA). Raw sequence data were analyzed with the EditSeq™ program (in Lasergene®; DNASTAR, Inc.), and the complete overlapping of complementary sequences, editing and consensus construction were produced with the SeqMan™ program (in Lasergene®; DNASTAR, Inc.).

**Sequence analysis.** DNA sequences of genome targets for the live *M. gallisepticum* vaccine strains (F-strain, ts-11 and 6/85) and for *M. gallisepticum* isolates

from different countries were obtained by GenBank internet search (http://www.ncbi.nlm.nih.gov/genbank) and from the database of the Mycoplasma laboratory at the PDRC. The PDRC database contains sequences of the IGSR and a 236 to 302 base pair (bp) fragment of the *mgc2* gene (7), with a total of 1380 sequences from 25 countries worldwide. South African wild-type *mgc2* sequences were edited to start and end at equivalent sequence positions to the *mgc2* gene fragment to facilitate comparison with all IGSR and *mgc2* (fragment) sequences in the database. *M. gallisepticum* vaccine and reference strains and 25 *M. gallisepticum* isolates from 12 countries, for which both IGSR and *mgc2* (fragment) sequences were available, were selected for more detailed comparison with the South African wild-types (Table 2.2). To conduct targeted sequence analysis, all consensus sequences were edited to start and end at equivalent sequence coding positions using the EditSeq™ program (in Lasergene®; DNASTAR, Inc.). Alignments of the sequences of individual and multiple genetic regions were constructed by the Clustal-W method using the MegAlign™ program (DNASTAR Lasergene®, Madison, WI). Dendrograms were constructed from Clustal-W alignments by the neighbor joining method with 1000 bootstrap replicate analysis using Molecular Evolutionary Genetics Analysis (MEGA) version 5.05 software (http://www.megasoftware.net). Amino acid translations and protein sequence alignments were performed using the EditSeq™ and MegAlign™ programs (DNASTAR Lasergene®, Madison, WI) respectively.

**Designation of sequence types and wild-types.** Sequences were named with a letter to designate the genetic region analyzed (IGSR = "R", *mgc2* = "C", MGA_0319 = "P" and *gapA* = "G"), followed by a number, depending on their sequence similarity to

the live *M. gallisepticum* vaccine strains. Sequences identical to those of the live vaccine strains were designated as follows: ts-11, "0"; 6/85, "1"; F strain, "2". Sequences not identical to the vaccine sequences were assigned numbers greater than 2. If the sequence of one or more genetic regions was not identical to that of the live vaccine sequences, the sample was considered to be wild-type *M. gallisepticum* DNA, and was assigned a wild-type number (eg. SA-WT#1).

**Discrimination index of targeted genetic sequence analysis.** The discriminatory power of a typing method depends on the number and relative frequency of the genotypes distinguished (12). Discriminatory power can be calculated using Simpson's index of diversity or the discrimination index (*D*), which is a measure of the probability that two unrelated strains will be placed into different typing groups (12). A *D* index > 0.90 is considered adequate, and a *D* index > 0.95 is considered good typing discrimination (5, 12). Discrimination indices were calculated for targeted sequence analysis of individual and multiple genetic regions.

**GenBank accession numbers.** Accession numbers for IGSR and *mgc2* (fragment) sequences of *M. gallisepticum* reference and vaccine strains and isolates from other countries selected for comparison with the South African wild-types are shown in Table 2.2. Accession numbers for the IGSR, *mgc2*, MGA_0319 and *gapA* sequences of South African *M. gallisepticum* genotypes and the ts-11 genotype are shown in Table 2.3. *mgc2*, MGA_0319 and *gapA* sequences of the vaccine strains 6/85 and F were previously submitted to GenBank under the following accession numbers: AY556231, AY556075 and AY556153 (*mgc2,* MGA_0319 and *gapA* respectively of strain 6/85) and AY556230, AY556074 and AY556152 (*mgc2,* MGA_0319 and *gapA* respectively of F strain) (5).

## Results

**Genotyping of South African *M. gallisepticum* samples.** Sequence data of adequate quality were obtained from 36 *M. gallisepticum* DNA samples. Thirty-five of the 36 *M. gallisepticum* positive samples were identified as wild-types, because the sequences of one or more genetic targets were not identical to those of the live vaccine strains (Table 2.3 and Supplementary Figures 1 to 5, Appendix A). One sample, which originated from a commercial layer flock vaccinated with ts-11, was identical to ts-11 vaccine strain in all four genetic regions, and was therefore designated "ts-11-like". Twelve distinct wild-types (SA-WT#1 to #12) were identified based on combined sequence analysis (Table 2.3 and Supplementary Figures 1 to 5). Complete sequences of the IGSR or *mgc2* targets could not be obtained for SA-WT#5, SA-WT#11 and SA-WT#12; nonetheless these samples could be identified as unique wild-types based on sequencing of the other three genetic targets.

Eight different sequence types could be distinguished by *mgc2* sequencing, four types by IGSR or MGA_0319 sequencing, and only two types by *gapA* sequencing (Table 2.3 and Supplementary Figures 1 to 5). The *gapA* sequences of ts-11 (Type 0) and 6/85 (Type 1) were identical, and were thus designated G0/1. SA-WT#7 was the most prevalent wild-type (42.9% of samples); the prevalence of the other wild-types ranged from 2.9 to 8.6%. SA-WT#1 and SA-WT#9 were identical to ts-11 in the *mgc2* or IGSR regions respectively, while SA-WT#4 was identical to ts-11 in the IGSR, MGA_0319 and *gapA* regions. The *mgc2* sequence of SA-WT#4 was 99.8% similar to that of ts-11, differing only by a single-nucleotide polymorphism (SNP) at position 345. This change represents a non-synonymous polymorphism, since the resulting codon encodes lysine

(AAA) in SA-WT#4 and asparagine (AAC) in ts-11. Repeated amplification and sequence analysis of the *mgc2* targets of both samples identified as SA-WT#4 yielded inconclusive results, with sequences either 99.8% or 100.0% similar to ts-11 (A or C at position 345 respectively). SA-WT#4 was isolated from broilers and layers in KwaZulu-Natal, which were not vaccinated with live *M. gallisepticum* vaccines. Respiratory disease was reported in both cases, although it is unclear whether other causes of respiratory infection were excluded. None of the South African sequences matched the F strain (Type 2) sequence in any genetic region analyzed.

**Discrimination indices of genetic targets.** To evaluate and compare the capability of targeted genetic sequencing to discriminate between *M. gallisepticum* isolates, *D* index values were estimated for individual genetic targets and for multiple targets (Table 2.4). The *D* index values of 0.745 (IGSR), 0.894 (*mgc2*), 0.615 (MGA_0319) and 0.295 (*gapA*) were estimated for sequencing of individual targets. Sequencing of more than one genetic target increased discriminatory power, with discrimination indices of 1.000 (IGSR/*mgc2*), 0.955 (*mgc2*/MGA_0319) and 0.927 (IGSR/MGA_0319) achieved by sequencing of multiple targets.

**Origin of South African *M. gallisepticum* wild-types.** The majority (40.0%) of samples submitted originated from KwaZulu-Natal, while 42.9% of samples originated from the six other provinces sampled (17.1% from the Eastern Cape, 11.4% from the Western Cape, 5.7% from the Free State and 2.9% each from the North-West Province, Gauteng and Mpumalanga), and 17.1% of samples were of unknown provincial origin. Seven of the 12 wild-types identified were detected in samples from KwaZulu-Natal, four from the Eastern Cape, three from the Western Cape, and one each from North-West

Province, Gauteng, Mpumalanga and the Free State. SA-WT#7 was the most widespread *M. gallisepticum* wild-type, and was detected in five of the seven provinces sampled. The other wild-types were detected in one or at most two provinces. SA-WT#7 was detected in samples from broilers, broiler breeders and layers, while SA-WT#1, SA-WT#4, SA-WT#6 and SA-WT#8 were detected in two bird types, and SA-WT#2, SA-WT#3, SA-WT#5, SA-WT#9, SA-WT#10 and SA-WT#11 were detected in only one bird type.

**IGSR/*mgc2*/MGA_0319/*gapA* dendrogram.** Dendrograms were constructed of multiple genetic sequence alignments to allow sample comparison based on two or more genetic regions. The dendrogram of the IGSR/*mgc2*/MGA_0319/*gapA* alignment was used to compare South African wild-types with each other and the vaccine strains (Figure 2.1). In general, South African wild-type sequences were highly similar to each other, with sequence similarity ranging from 97.6 to 100%. (Note that although sequence similarity for SA-WT#6, SA-WT #7 and SA-WT#8 and SA-WT#4 and ts-11 was estimated to be 100% by the alignment software, these sequences were not identical.) The *mgc2* sequence of SA-WT#6 differed from that of SA-WT#7 by a SNP, while SA-WT#8 differed from SA-WT#7 by a single nucleotide deletion in the IGSR region. A SNP of the *mgc2* gene differentiated SA-WT#4 and ts-11, as previously discussed. The South African wild-type sequences were more similar to ts-11 (sequence distance range: 97.8 - 100%) and 6/85 (sequence distance range: 97.9 – 98.6%), but grouped distinctly from the F strain (sequence distance range: 96.0 – 96.5%).

**Individual IGSR and *mgc2* (fragment) alignments.** To facilitate comparison of South African wild-types with all *M. gallisepticum* isolates in the PDRC database (including those isolates for which the sequence of only one genetic target was available),

individual IGSR and *mgc2* (fragment) alignments were also constructed. The South African wild-type IGSR sequences were unique, differing from all other IGSR sequences in our database. SA-WT#6, SA-WT#7, SA-WT#8, SA-WT#9, SA-WT#10 and SA-WT#11 had 3 characteristic deletions in the *mgc2* gene fragment (49 to 51 bp, 100 to 123 bp and 163 to 165 bp), resulting in these sequences being 30 bp shorter than the other South African wild-type sequences. These deletions were absent in the vaccine strains and in all other *mgc2* (fragment) sequences examined, except for one 2008 isolate from a peafowl in the USA (USA/65099/PF08), whose *mgc2* (fragment) sequence was identical to those of SA-WT#7, SA-WT#8, SA-WT#9 and SA-WT#10. The *mgc2* (fragment) sequence of SA-WT#3 was identical to those of two US isolates (USA/74235/CK09 and USA/94473/CK12) and one Australian isolate (AUS/019/CK97; GenBank accession no. AY556302). The SA-WT#5 *mgc2* (fragment) sequence was identical to that of the reference strain USA/A5969/CK55, and several wild-types from the USA, Colombia, Spain, Malaysia and Thailand.

**IGSR/*mgc2* (fragment) dendrogram.** The construction of an IGSR/*mgc2* (fragment) dendrogram allowed South African wild-types to be compared with 25 wild-type sequences from 12 countries based on both the IGSR and *mgc2* (fragment) sequences (Figure 2.2; origins of foreign *M. gallisepticum* DNA samples are listed in Table 2.2; sequence alignments in Supplementary Figures 1 and 2). The South African wild-type IGSR/*mgc2* (fragment) sequences were unique and different from all other wild-types analyzed. They were more similar to sequences from Egypt, Jordan, Israel, Thailand, and certain sequences from the United States and Colombia, with sequence distances ranging from 96.4 to 99.8%. In particular, a high degree of similarity was found

between SA-WT#1 and SA-WT#2 and USA/92439/CK12 (sequence distances: 99.8%), and between SA-WT#3 and COL/68938/CK08 (sequence distance: 99.4%). In contrast, South African wild-types were most different from wild-type sequences from Ecuador, Venezuela, Panama, Guatemala, India, Spain, and other US and Colombian sequences, with sequence distances ranging from 96.0% to 98.5%. In general, IGSR/*mgc2* (fragment) sequences formed distinct geographical clusters, represented by sequences from Asia and the Middle East, South Africa, the USA and Central and South America.

## Discussion

Of the four targets analyzed, *mgc2* sequencing had the best discriminatory power for differentiation of South African genotypes, followed by IGSR and MGA_0319, with *gapA* having poor discriminatory power. The discrimination (*D*) index calculated for *mgc2* in this study was 0.894, which is similar to the *D* index of 0.915 previously described for targeted genetic sequencing of isolates from the USA, Israel and Australia (5). However, *D* indices for MGA_0319, *gapA* and IGSR were considerably lower in this study than those previously reported (5, 20). In previous studies, *D* indices were estimated based on typing of larger numbers of *M. gallisepticum* isolates and laboratory strains from the USA (20), as well as from other countries (5). Strain diversity in a restricted geographical area such as South Africa would be expected to be significantly lower than diversity across broader geographical regions, including multiple countries. Our estimates of discriminatory power are therefore conservative, and indicate the ability of sequencing of these targets to distinguish South African genotypes only.

Raviv *et al.* (2007) estimated the discriminatory power of IGSR sequencing to be higher than that of previous estimates for *mgc2*, and reported that strains with ts-11-like *mgc2* sequences were correctly identified as wild-types by IGSR sequencing (5, 20). In this study, *mgc2* sequencing gave better discriminatory power ($D = 0.894$) than IGSR sequencing ($D = 0.745$). This superior discriminatory power may be as a result of increased variability of the South African isolates in the *mgc2* region. Some of this variability was attributed to three deletions of the *mgc2* gene, resulting in the loss of a total of 30 nucleotides in certain South African wild-types (SA-WT#6, SA-WT#7, SA-WT#8, SA-WT#9, SA-WT#10 and SA-WT#11). These deletions were only observed in one other isolate in our sequence database (USA/65099/PF08). Size variation of the *mgc2* gene was first described by Ferguson *et al*. (2005), who concluded that this characteristic was not a reliable strain differentiation tool due to the relatively short insertions/deletions observed and the limited number of strains that could be differentiated. This study supports this conclusion, as only two genotypes could be differentiated based on *mgc2* size polymorphism, and the difference in length was not sufficient to enable differentiation on ethidium bromide-stained agarose gels.

As previously reported (5), we found that sequencing more genetic targets increased the overall discrimination index, with maximum discriminatory power achieved by sequencing both IGSR and *mgc2*. In addition, sequencing of both of these targets was necessary to differentiate three wild-types (SA-WT#1, SA-WT#4 and SA-WT#9) from ts-11. In one case, MGA_0319 sequencing allowed the differentiation of an additional wild-type (SA-WT#12) for which the *mgc2* sequence could not be obtained, probably as a result of sub-optimal DNA quality. Sequencing of MGA_0319 (a shorter region than

*mgc2* and IGSR) may thus be a useful adjunct to strain differentiation in cases where complete IGSR or *mgc2* sequences cannot be obtained.

SA-WT#6 and SA-WT#7, and SA-WT#4 and ts-11 were only distinguishable by SNPs of the *mgc2* gene, while SA-WT#7 and SA-WT#8 differed only by a single nucleotide deletion of the IGSR. The use of single nucleotide changes to differentiate strains is supported by the correlation of these differences with RAPD results, and by the demonstrated sequence stability of the genetic targets (5, 9, 20). It is possible, however, that certain single nucleotide changes of particular targets do not result in strain differences. We recently sequenced the IGSR and *mgc2* (fragment) genetic regions of seven US *M. gallisepticum* samples from ts-11 vaccinated broiler breeders and layers, and found that these sequences could only be distinguished from those of ts-11 by a single nucleotide change in the *mgc2* (fragment) sequence at position 18 (corresponding to position 345 in the *mgc2* sequence) (data not shown). Since the SNP in the *mgc2* sequence which differentiated SA-WT#4 from ts-11 occurred at the same position, we suspect that SA-WT#4 may actually be derived from ts-11. This is an interesting observation, considering that SA-WT#4 was isolated from broilers and layers that were not vaccinated with ts-11. Alternatively, one could speculate that SA-WT#4 may be a field strain that closely resembles ts-11 in all of the particular genomic regions sequenced. Since targeted sequencing cannot differentiate mixed infections of *M. gallisepticum* strains, it is also theoretically possible that these non-vaccinated flocks were co-infected with ts-11 and a field strain. Complete genome sequencing of SA-WT#4 may be useful in determining the relationship of this genotype to ts-11.

Three of the 12 South African wild-types were identical to ts-11 in two or three of the four genetic regions analyzed, and wild-types clustered consistently with ts-11 or 6/85 in the analysis of the combined data. In contrast, South African wild-types demonstrated low sequence similarity to the F strain. As both ts-11 and 6/85 vaccines have been used in South Africa for more than a decade while F strain vaccines are still not available, it may be theorized that vaccine usage has affected the wild-type sequences through horizontal gene transfer or homologous recombination with existing field strains. To further investigate this possibility, it would be necessary to evaluate *M. gallisepticum* DNA pre-dating the introduction of ts-11 and 6/85. Unfortunately, we did not have access to historical MG isolates for this study. Our results indicate that South African wild-types are readily differentiated from F strain by targeted sequencing; a fact which would facilitate differentiation of vaccinated and infected flocks should F strain vaccines be introduced into the country in the future. In contrast, the sequence similarity of South African *M. gallisepticum* wild-types to the commercially available live vaccines, particularly ts-11, highlights the importance of sequencing both the IGSR and *mgc2* targets to allow differentiation of wild-type *M. gallisepticum* from the vaccine strains. Other genetic targets (eg. *pvpA* or *vlhA*) could also be investigated for their ability to facilitate this strain differentiation (5, 10, 19). *pvpA* analysis was performed in a pilot study of South African *M. gallisepticum* wild-types (data not shown), but due to difficulty in amplification and alignment this target was not included in the present study.

The South African wild-type combined IGSR/*mgc2* (fragment) sequences were unique, differing from those of wild-types from other countries in our database. Clustering of isolates according to geographical origin in the IGSR/*mgc2* (fragment)

dendrogram is consistent with the correlation of epidemiologically linked isolates previously reported for sequencing of multiple genetic targets (5). Individual analysis of IGSR and *mgc2* (fragment) targets revealed that while South African wild-type IGSR sequences were unique, three different wild-type *mgc2* (fragment) sequences were identical to certain sequences from other countries. One match of particular interest was that of the *mgc2* (fragment) sequences of SA-WT#7, SA-WT#8, SA-WT#9 and SA-WT#10 and a peacock isolate from the USA (USA/65099/PF08). USA/65099/PF08 was the only isolate outside of South Africa to have the characteristic 30 bp deletion of the *mgc2* gene fragment observed in SA-WT#7, SA-WT#8, SA-WT#9 and SA-WT#10. This phenomenon could theoretically result from a horizontal gene transfer or homologous recombination event, although there is no known epidemiological link between USA/65099/PF08 and these South African wild-types. It is also possible that the *mgc2* deletions in USA/65099/PF08 and SA-WT#7, SA-WT#8, SA-WT#9 and SA-WT#10 represent random mutational events, which occurred independently in unrelated genotypes.

Wild-type prevalence could not be determined in this study because an opportunity sampling approach was used, in which sample numbers, location and bird type sampled were not dictated, but were dependent on voluntary sample submission. However, our results do indicate that one wild-type (SA-WT#7) was the most widespread *M. gallisepticum* wild-type in South Africa between 2009 and 2012, since it was present in 42.9% of samples analyzed, and was detected in samples from broilers, broiler breeders and layers in five of the seven provinces sampled. The majority of wild-types (7 of 12) were detected in samples from KwaZulu-Natal, with fewer wild-types from other

provinces. It should be noted that 40.0% of all samples tested originated from KwaZulu-Natal, and it is likely that more wild-types would have been detected in other provinces had more samples from these provinces been tested.

In conclusion, targeted sequencing of South African *M. gallisepticum* DNA extracts from samples collected between 2009 and 2012 has been used to develop an *M. gallisepticum* sequence database. This database was applied to characterize twelve novel *M. gallisepticum* genotypes and select genetic targets to optimize differentiation of South African wild-types from each other and from the live vaccine strains. We are currently using the sequence data to develop cost-effective and broadly applicable molecular techniques, which will facilitate the differentiation of South African *M. gallisepticum* wild-types from live *M. gallisepticum* vaccine strains. In addition, this study has improved our understanding of the diversity and distribution of *M. gallisepticum* in South Africa, and has provided a foundation for future epidemiological investigation. Analysis of *M. gallisepticum* samples from South Africa in the event of the introduction or removal of live vaccines will allow further study of the effects of vaccination on the genetic variability in field strains. Internationally, this model could be used to guide the development of other *M. gallisepticum* sequence databases, which would have application in elucidating the epidemiology of *M. gallisepticum* and in refining diagnostic techniques for different countries.

**Acknowledgements**

The authors are grateful to Ms. Tracy Schmidt of Allerton Provincial Veterinary Laboratory in South Africa for performing the DNA extraction. They would also like to acknowledge all those who kindly submitted samples for this project, and thank Dr. Stanley H. Kleven for reviewing the manuscript.

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Table 2.1. Genetic regions selected for analysis, with expected PCR product sizes and discrimination (*D*) indices for targeted genetic sequencing.

| **Genetic region analyzed** | **PCR product size (bp)** | ***D* index[a]** |
|---|---|---|
| IGSR | 812 | 0.950 |
| *mgc2* | 824 | 0.915 |
| MGA_0319 | 590 | 0.874 |
| *gapA* | 332 | 0.713 |

[a] As described in Ferguson *et al*. 2005, and Raviv *et al*., 2007.

Table 2.2. *M. gallisepticum* reference and vaccine strains and DNA samples from other countries used to generate the IGSR/*mgc2* dendrogram for comparison with South African wild-type genotypes

| Isolate name[a] | GenBank numbers (IGSR; *mgc2*) | Country of origin | Strain type |
|---|---|---|---|
| COL/68938/CK08 | KC247840; KC247873 | Colombia | Field strain |
| COL/79663/CK09 | KC247841; KC247874 | Colombia | Field strain |
| ECU/77025/CK09 | KC247842; KC247875 | Ecuador | Field strain |
| ECU/77029/CK09 | KC247843; KC247876 | Ecuador | Field strain |
| GUA/79672/CK09 | KC247844; KC247877 | Guatemala | Field strain |
| PAN/68630/CK08 | KC247845; KC247878 | Panama | Field strain |
| PAN/69016/CK08 | KC247846; KC247879 | Panama | Field strain |
| VEN/69993/CK08 | KC247847; KC247880 | Venezuela | Field strain |
| VEN/69994/CK08 | KC247848; KC247881 | Venezuela | Field strain |
| USA/K6001B/CK07 | KC247849; KC247882 | USA (Georgia) | Field strain |
| USA/65099/PF08 | KC247850; KC247883 | USA (Georgia) | Field strain |
| USA/68614/CK08 | KC247851; KC247884 | USA (Alabama) | Field strain |
| USA/75695/CK09 | KC247852; KC247885 | USA (Georgia) | Field strain |
| USA/82065/CK10 | KC247853; KC247886 | USA (Georgia) | Field strain |
| USA/87387/CK11 | KC247854; KC247887 | USA (Florida) | Field strain |
| USA/92439/CK12 | KC247855; KC247888 | USA (Georgia) | Field strain |
| EGY/67240/CK08 | HQ143380[b]; HQ143372[b] | Egypt | Field strain |
| EGY/75651/CK09 | HQ143381[b]; HQ143373[b] | Egypt | Field strain |
| EGY/82133/CK10 | KC247856; KC247889 | Egypt | Field strain |

| | | | |
|---|---|---|---|
| ISR/K3868/CK95 | HQ143384[b]; HQ143377[b] | Israel | Field strain |
| JOR/111/CK09 | KC247857; KC247890 | Jordan | Field strain |
| SPA/5742-3/CK09 | KC247858; KC247891 | Spain | Field strain |
| IND/38825/CK05 | KC247859; KC247892 | India | Field strain |
| THA/3/CK08 | KC247860; KC247893 | Thailand | Field strain |
| THA/5/CK08 | KC247861; KC247894 | Thailand | Field strain |
| USA/A5969/CK55 | KC247862; KC247895 | USA | Reference strain |
| USA/R/CK60 | KC247863; KC247896 | USA (Georgia) | Reference strain |
| USA/F/CK58 | HQ143383[b]; KC247897 | USA (California) | Reference F strain |
| ts-11 | KC247864; KC247898 | Australia | Vaccine strain |
| 6/85 | KC247865; KC247899 | USA | Vaccine strain |

[a]CK = chicken; PF = peafowl; last 2 digits in field strain names indicates year of isolation.
[b]Sequences published by Gharaibeh *et al.* (2011).

Table 2.3. South African *M. gallisepticum* genotypes identified, with corresponding sequence types and genotype prevalence.

| Genotype | IGSR (R) | *mgc2* (C) | MGA_0319 (P) | *gapA* (G) | No. of isolates | % of wild-types |
|---|---|---|---|---|---|---|
| **SA-WT#1** | R4 (KC247830)[a] | C0[b] (KC247866) | P5 (KC247834) | G0/1 (KC247838) | 2 | 5.7% |
| **SA-WT#2** | R4 | C3 (KC247867) | P5 | G3 (KC247839) | 3 | 8.6% |
| **SA-WT#3** | R3 (KC247831) | C4 (KC247868) | P4 (KC247835) | G0/1 | 1 | 2.9% |
| **SA-WT#4** | R0 (KC247832) | C5[c] | P0 (KC247836) | G0/1 | 2 | 5.7% |
| **SA-WT#5** | - | C6 (KC247869) | P5 | G3 | 1 | 2.9% |
| **SA-WT#6** | R4 | C7 (KC247870) | P5 | G0/1 | 3 | 8.6% |
| **SA-WT#7** | R4 | C8 (KC247871) | P5 | G0/1 | 15 | 42.9% |
| **SA-WT#8** | R5 (KC247833) | C8 | P5 | G0/1 | 3 | 8.6% |
| **SA-WT#9** | R0 | C8 | P3 (KC247837) | G0/1 | 1 | 2.9% |
| **SA-WT#10** | R3 | C8 | P5 | G0/1 | 1 | 2.9% |
| **SA-WT#11** | - | C9 (KC247872) | P5 | G0/1 | 2 | 5.7% |
| **SA-WT#12** | R4 | - | P4 | G0/1 | 1 | 2.9% |
| **ts-11** | R0 | C0 | P0 | G0/1 | 1 | N/A |

[a] GenBank accession number of the sequence of a representative sample

[b] Sequences identical to those of the live vaccine strains were designated as: ts-11, "0"; 6/85, "1"; F strain, "2". Sequences not identical to the vaccine sequences were assigned numbers greater than 2.

[c] Note that this sequence differed from that of ts-11 by a SNP. Repeated amplification and sequencing to determine whether this sequence was in fact wild-type (sequence type C5) or ts-11-like (sequence type C0) yielded inconclusive results.

Table 2.4. Comparison of targeted genetic sequencing methods for discrimination of South African *M. gallisepticum* genotypes.

| **Genotyping target(s)** | **Number of sequence types identified (*s*)** | **Total number of genotypes (*N*)[a]** | ***D* index[b]** |
|---|---|---|---|
| IGSR | 4 | 11 | 0.745 |
| *mgc2* | 8 | 12 | 0.894 |
| MGA_0319 | 4 | 13 | 0.615 |
| *gapA* | 2 | 13 | 0.295 |
| IGSR/*mgc2* | 10 | 10 | 1.000 |
| *mgc2*/MGA_0319 | 10 | 12 | 0.955 |
| IGSR/MGA_0319 | 7 | 11 | 0.927 |
| IGSR/*mgc2*/MGA_0319 | 10 | 10 | 1.000 |

[a] N, Total number of genotypes considered for each genetic target (excluding genotypes for which target sequences were not available).

[b] $D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$; *N* = total number of genotypes described, *s* = number of sequence types identified, *n* = number of genotypes belonging to *j*th sequence type.

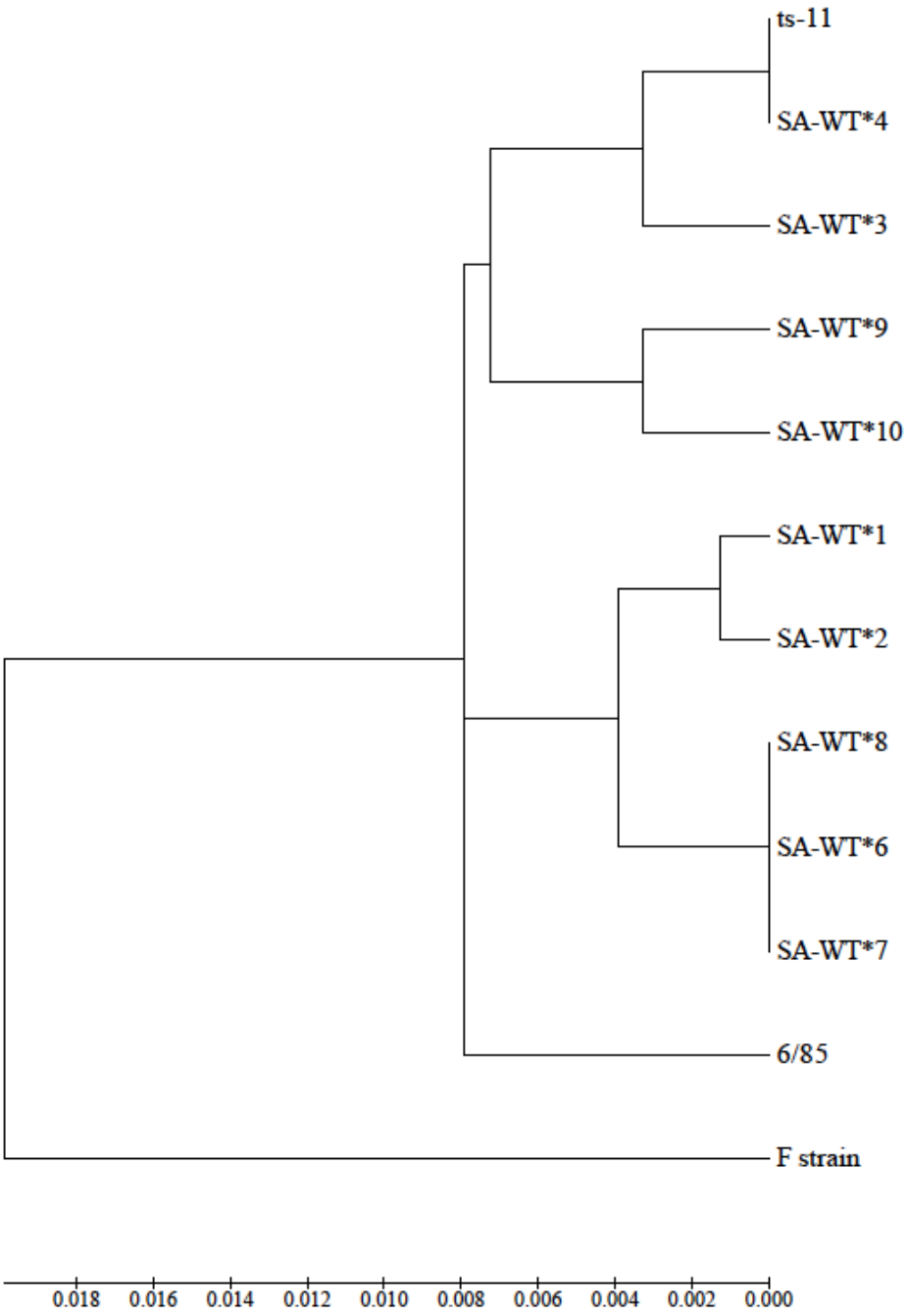


Figure 2.1. Dendrogram of South African *M. gallisepticum* wild-types and live vaccine strains, constructed by Clustal-W alignment of adjoined IGSR, *mgc2*, MGA_0319 and *gapA* sequences by the neighbor-joining method with 1000-bootstrap replicates using MEGA 5.05 (http://www.megasoftware.net).

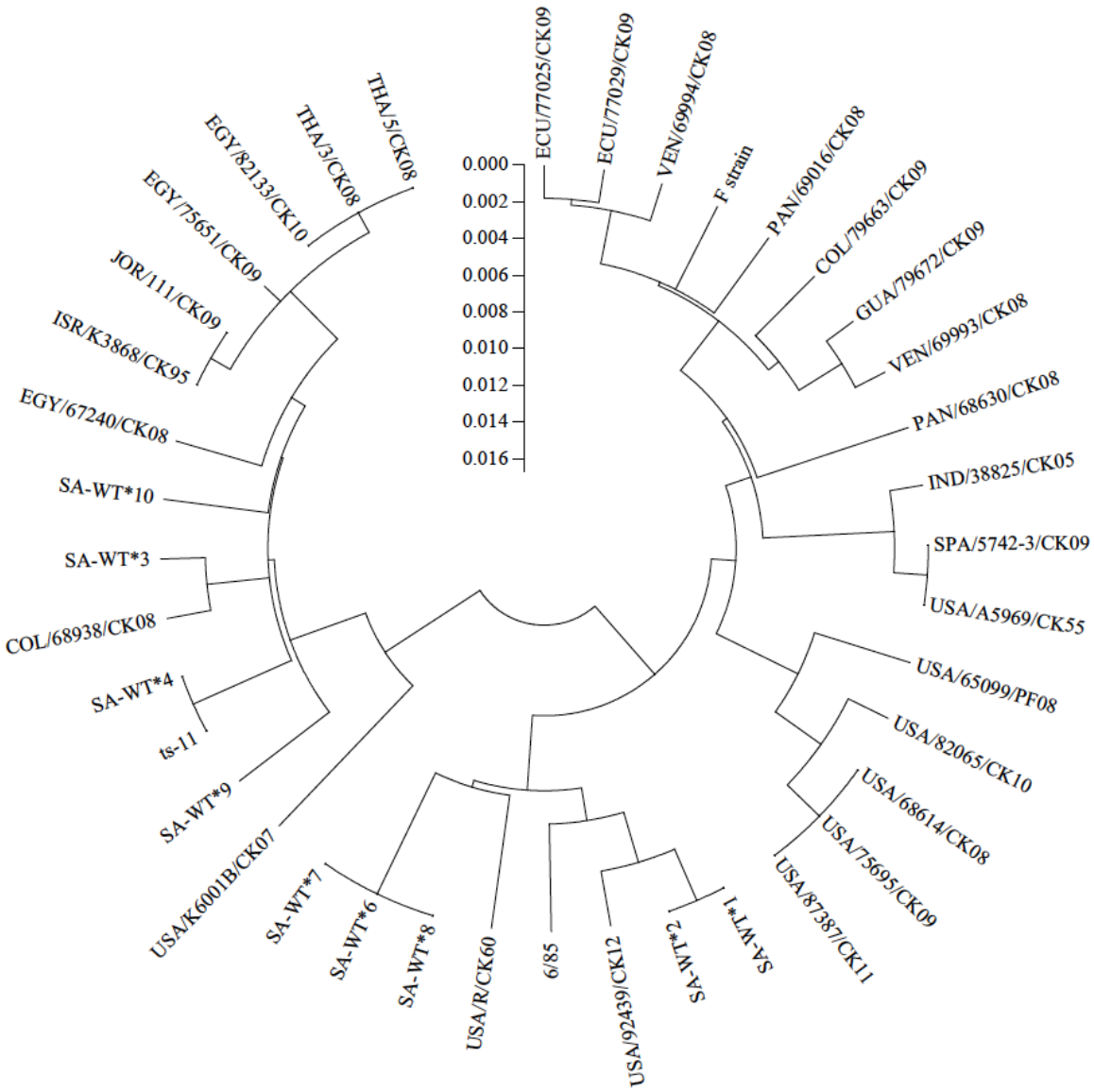


Figure 2.2. Dendrogram of *M. gallisepticum* wild-types from South Africa and 12 other countries, with vaccine and reference strains, constructed by Clustal-W alignment of adjoined IGSR and *mgc2* (fragment) sequences by the neighbor-joining method with 1000-bootstrap replicates using MEGA 5.05 (http://www.megasoftware.net). Origin of foreign *M. gallisepticum* isolates is listed in Table 2.2.

# CHAPTER 3

## AN EVALUATION OF THE EGG TRANSMISSION AND PATHOGENICITY OF *MYCOPLASMA GALLISEPTICUM* ISOLATES OF THE TS-11 GENOTYPE[2]

[2] N.K. Armour and N. Ferguson-Noel. To be submitted to *Avian Pathology.*

Key words: *Mycoplasma gallisepticum*, ts-11 vaccine, ts-11-like isolates, egg transmission, pathogenicity, egg production

Abbreviations: ELISA = enzyme-linked immunosorbent assay; HI = haemagglutination inhibition; IGSR = 16S–23S rRNA intergenic spacer region; PCR = polymerase chain reaction; SPA = serum plate agglutination; $ts^{+}$= temperature sensitive; WPI = weeks post infection

## Summary

Live *Mycoplasma gallisepticum* vaccines are used for the control of respiratory disease, egg production losses and egg transmission associated with *M. gallisepticum* infection in long-lived poultry. The first field case of apparent reversion to virulence and vertical transmission of ts-11, a live *M. gallisepticum* vaccine, was previously reported; in that study, an *M. gallisepticum* isolate from the broiler progeny of ts-11-vaccinated breeders, which was indistinguishable from ts-11 by the genotyping methods used, was found to be significantly more pathogenic than ts-11 vaccine. The objective of the current study was to evaluate the rate of egg transmission and pathogenicity of ts-11 vaccine and ts-11-like isolates from ts-11 vaccinated breeders (K6222B) and their broiler progeny (K6216D). *M. gallisepticum* was isolated from the air sacs, ovaries and oviducts of hens infected with K6216D and K6222B, but not from those infected with ts-11 vaccine. K6216D transmitted via the egg at an average rate of 4.0% in the third and fourth weeks post infection, while egg transmission of K6222B and ts-11 vaccine was not detected. K6216D and K6222B both induced respiratory signs, and significantly more tracheal colonization and more severe tracheal and air sac lesions than ts-11 vaccine ($P \leq 0.05$). There were no substantial differences in the egg production of ts-11, K6216D and K6222B infected groups. These results provide the first conclusive evidence of transovarian transmission of an isolate of the ts-11 genotype, and indicate that ts-11-like isolates vary in their virulence and ability to transmit via the egg.

## Introduction

*Mycoplasma gallisepticum*, the causative agent of chronic respiratory disease in chickens and the most pathogenic of the avian mycoplasmas, is both horizontally and vertically transmitted (26). The reported rates of transovarian transmission of *M. gallisepticum* after experimental infection vary, with peak egg transmission rates ranging from 14 to 53% between 3 and 8 weeks after infection reported in different studies with the R strain of *M. gallisepticum* (13, 14, 19, 21, 31). When hens are experimentally infected with virulent *M. gallisepticum* strains, egg transmission is typically preceded by clinical signs and drops in egg production, usually occurring within 1 week of inoculation (13, 14, 19, 21, 31).

The pathogeneses of egg transmission and egg production drops induced by *M. gallisepticum* infection have not been fully elucidated. Egg transmission probably involves infection of the ova following air sac infection and/or bacteremia (13, 27), while the presence of *M. gallisepticum* organisms in the oviduct has been associated with ovarian and oviduct pathology and egg production drops (9, 20, 22).

In order to maintain flocks free of *M. gallisepticum*, replacement stock should originate from an *M. gallisepticum*-free source; *M. gallisepticum* eradication at the breeder level is therefore of paramount importance (15). Vaccination has been used as an alternative control approach in situations where prevention of infection is not considered feasible (15), or as a component in control programs whose ultimate objective is *M. gallisepticum* eradication. In the United States, vaccination is primarily used to control *M. gallisepticum* in multiple-age commercial layer operations.

The ts-11 vaccine is a live attenuated *M. gallisepticum* vaccine, which was developed by chemical mutagenesis of an Australian *M. gallisepticum* field isolate and selected for its temperature sensitive ($ts^+$) phenotype (growth at 33°C) (32, 35). The ts-11 strain is regarded to be of low or no virulence, and has been demonstrated to provide protection against respiratory disease and egg production drops induced by virulent *M. gallisepticum* infection (1, 33, 34), and to reduce vertical transmission of *M. gallisepticum* field strains (3, 33). The ts-11 vaccine's lack of virulence is not contingent on the $ts^+$ phenotype (33); the genetic basis of its attenuation remains to be determined.

In 2007, a decision was taken to vaccinate a number of broiler breeder flocks in northeastern Georgia with ts-11 vaccine, to control an *M. gallisepticum* epidemic that began in the area in 2006. Between 2008 and 2011, *M. gallisepticum* was found to be the cause of severe respiratory disease in the broiler progeny of several ts-11-vaccinated breeder flocks from four companies. *M. gallisepticum* isolates from these broiler flocks and their parents were indistinguishable from ts-11 vaccine strain by all genotyping methods used, and were therefore termed "ts-11-like" (10). Spikes in mortality and rising *M. gallisepticum* antibody titers in the breeder flocks were reported to precede clinical disease in the progeny by approximately 2 months (10). A pathogenicity study confirmed that K6216D, a ts-11-like *M. gallisepticum* isolate from one of the infected broiler flocks, was significantly more virulent than ts-11 vaccine. The epidemiology of the outbreaks, the genotyping results and the pathogenicity study findings indicated that reversion to virulence and vertical transmission of ts-11 vaccine had occurred (10).

The objectives of the present study were to investigate the ability of ts-11 vaccine and ts-11-like *M. gallisepticum* isolates from a broiler flock (K6216D) and their ts-11-

vaccinated parent flock (K6222B) to transmit via the egg. Concurrently, we sought to determine the pathogenicity of these isolates in sexually mature chickens, and the effect of infection on egg production. Our results provide the first conclusive evidence of transovarian transmission of an isolate genotyped as ts-11, and indicate the existence of a spectrum of egg transmission potential and virulence for isolates of the ts-11 genotype.

## Materials and Methods

**MG strains and isolates.** The ts-11 vaccine used in this trial (Mycoplasma Gallisepticum Vaccine, Serial no. MA649, Merial Select, Gainesville, Georgia, USA) was stored at -80°C until shortly before use, when it was thawed according to the manufacturer's recommendations. K6216D was isolated at the Poultry Diagnostic and Research Center (PDRC), University of Georgia, Athens, GA USA, in July 2008 from a 7.4-wk-old broiler flock with severe respiratory signs, and was previously characterized as significantly more virulent than ts-11 vaccine (10). K6222B was isolated in August 2008 from the 57-wk-old parent flock of the affected broilers, which had been vaccinated with ts-11 vaccine as pullets. The affected breeder flock had experienced an increase in mortality at 36 weeks of age, followed by a steady increase in antibody titers to *M. gallisepticum* (10). Both K6216D and K6222B were indistinguishable from ts-11 vaccine by all genotyping methods used; they did not, however, exhibit the temperature sensitive phenotype (10). R strain is a virulent *M. gallisepticum* strain, which has been well characterized (28). The titer of each inoculum (expressed in color changing units

(CCU)/ml) was determined as previously described (29). The origins, characteristics and inoculation titers of the isolates used in this study are presented in Table 3.1.

**Serology.** All sera were analyzed for *M. gallisepticum* antibodies using the serum plate agglutination (SPA), haemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) tests. The SPA and HI tests were performed as previously described (16), using commercial antigen for the SPA test (Charles River Laboratories, Wilmington, MA) and antigen prepared from the A5969 strain and chicken erythrocytes for the HI test. ELISA tests were performed using a commercially available kit (IDEXX, Westbrook, ME). An SPA score ≥ 1 was considered positive. An HI titer of 1:20 was considered suspect, and ≥ 1:40 was considered positive. A geometric mean sample/positive (S/P) ratio > 0.5 on the ELISA test was considered positive.

**Isolation and identification of mycoplasma.** Cotton swabs used to swab the air sacs, ovaries and oviducts of hens at necropsy, and samples of yolk sac membrane and yolk from eggs were inoculated into 2.5 ml of Frey's modified broth medium and incubated at 37°C. Eggs were opened from the small end to expose the yolk sac; the yolk sac membrane (section approximately 2 mm in diameter) was sampled using sterile instruments, and sterile disposable plastic loops were used to sample the yolk. Inoculation of Frey's modified broth and agar and examination of cultures was performed as previously described (13). Mycoplasma isolates were identified using direct immunofluorescence (16).

**DNA extraction.** DNA extraction from 200 µl aliquots of tracheal washes and choanal cleft swab samples prepared as previously described (24) was performed using

the Mag-Bind® Viral DNA/RNA 96 Kit (Omega Bio-Tek, Norcross, GA) on the MagMAX™ Express-96 Magnetic Particle Processors (Applied Biosystems by Life Technologies, Foster City, CA), according to the manufacturers instructions. Genomic DNA was also extracted from 200 µl of selected *M. gallisepticum* isolates in Frey's modified broth using the QIAGEN DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. Two *M. gallisepticum* isolates from the airsacs, ovaries and oviducts of hens sampled at necropsy and two isolates from eggs were selected from each positive group for PCR and sequence analysis, to confirm that the genotype isolated was the same genotype used to infect the particular group.

**PCR and sequence analysis.** PCR amplification, sequencing and sequence analysis of the 16S-23S rRNA intergenic spacer region (IGSR) genetic target was performed on selected *M. gallisepticum* isolates using previously described primer sequences, thermocycler programs, PCR amplification methods and sequence analysis software (2, 23).

**Real-time (Quantitative) PCR.** Quantitative *M. gallisepticum* real-time PCR (qPCR) was performed on DNA extracted from tracheal washes and choanal cleft swab samples using a previously described protocol (6, 25). The assays are quantitative, using plasmids containing the genome targets as standard DNA controls (11).

**Evaluation of clinical signs.** Birds were observed daily for clinical signs associated with *M. gallisepticum* infection; including respiratory rales, coughing, nasal discharge, conjunctivitis and facial swelling (26).

**Evaluation of lesions.** Air sac lesions were grossly evaluated and scored on a scale of 0 to 4, as previously described (17). For histopathologic evaluation, a section of

the upper third of the trachea (approximately 3 cm distal to the larynx) was fixed in 10% neutral buffered formalin. Measurements of tracheal mucosal thickness were made at four equidistant points on histologic slides of tracheal cross sections (33).

**Chickens and experimental design.** All animal procedures in these experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia, Athens, GA. The bird welfare was adequate and in accordance with the IACUC, and all birds were provided with feed and water *ad libitum*. Chickens were euthanized by cervical dislocation or carbon dioxide inhalation.

One hundred and sixty specific pathogen free (SPF) chickens (140 females and 20 males) were acquired at 4 weeks of age (Charles River Avian Vaccine Services, North Franklin, CT). The chickens were housed in 5 floor pens (1.5 x 4 $m^2$) with pine shavings litter, in a naturally ventilated curtain-sided poultry house. At 18 weeks of age, all chickens were moved to the laying facility, where they were housed in 10 floor pens (1.5 x 3 $m^2$) with nest boxes and pine shavings litter, in a naturally ventilated curtain-sided poultry house. A commercial layer lighting program was followed throughout the rearing and laying periods. In the laying facility, 160 chickens were randomly divided into 5 groups of 28 females and 4 males each. Each group was divided into two replicates (14 females and 2 males), which were housed in adjacent pens.

One week after placement, 20 chickens were randomly selected and screened by serology (SPA, HI and ELISA), culture and real-time PCR to confirm that they were negative for *M. gallisepticum* and *M. synoviae*. At 28 weeks of age (87% average weekly egg production), chickens were inoculated with sterile broth media (negative controls),

ts-11 vaccine, or with log-phase cultures of K6222B, K6216D or R strain (positive controls) via eye-drop (30 µl per bird) and aerosol (approximately 1000 µl per bird). The aerosol inoculation was delivered using a commercial paint sprayer (Preval® Sprayer Division, Precision Valve Corporation, Yonkers, NY).

Egg production data were recorded from the onset of lay (19 weeks) until the termination of the trial (33 weeks). All settable eggs collected from 1 week before until 6 weeks after infection were cultured for *Mycoplasma*. Eggs were collected daily, and cleaned, disinfected and air-dried prior to storage at 18°C for 0 to 4 days. Batches of eggs were set for incubation twice weekly. All eggs were candled at 5, 8, 11 and 15 days of incubation, and infertile eggs and those containing dead embryos were removed for testing by culture. At 18 days of incubation, all remaining eggs were cultured. A total of 5,175 eggs was cultured.

Two weeks after infection, 10 chickens from each group (5 per replicate) were randomly selected for sampling by swabbing the choanal cleft for real-time PCR analysis. Six weeks after infection, all chickens were euthanized, evaluated by air sac lesion scoring, and sampled for real-time PCR, mycoplasma culture and histopathology.

**Statistical analysis.** The Kruskal-Wallis rank sums test was used to analyze air sac lesion scores and *M. gallisepticum* isolations from chickens. Mean tracheal mucosal thickness, MG DNA copy numbers $\log_{10}$, percent egg production and *M. gallisepticum* isolations from eggs were analyzed using the Tukey-Kramer highly significant difference test (JMP® Pro 11.0.0, SAS Institute Inc., Cary, NC). A $P$-value $\leq 0.05$ was considered significant.

## Results

**Clinical signs and mortality.** No clinical signs were noted in the negative control and ts-11-inoculated groups. Birds infected with K6216D and K6222B had moderate to marked respiratory signs, which were similar in severity for both groups; while birds inoculated with the R strain had signs of marked to severe respiratory disease. Clinical signs included depression, conjunctivitis, nasal discharge, snicking and respiratory rales, with periorbital facial swelling and dyspnea in more severely affected birds (usually males). Signs appeared approximately 5 days post infection, with recovery within 7-10 days for birds inoculated with K6216D or K6222B, and up to 14 days for birds inoculated with the R strain. Two chickens in the R strain group were euthanized due to uterine prolapse in the last week of the study; there was no post infection mortality in any other group.

**Mycoplasma isolation from eggs**. Egg culture results for the trial are presented in Figure 3.1 and Table 3.2. Eighteen-day-old embryos in the set of eggs laid between 4.5 and 5 weeks post infection (WPI) were not cultured for *Mycoplasma* because the egg incubator overheated between 15 and 18 days of incubation, resulting in the death of all 297 remaining embryos in this set. *Mycoplasma* spp. were not isolated from any eggs laid in the week prior to infection, or from the eggs of chickens in the negative control, ts-11 or K6222B groups for 6 weeks after infection (Figure 3.1).

*M. gallisepticum* was isolated from 0.6% (1/167) and 7.2% (13/180) of eggs from K6216D-infected chickens in the 3$^{rd}$ and 4$^{th}$ WPI respectively, but was not isolated from eggs laid after 4 WPI (Figure 3.1). Egg transmission of the R strain was first detected in

the 2nd WPI (7.4%; 8/108), after which it peaked in the 3rd WPI (25.4%; 32/126), and remained relatively high until the end of the trial at 6 WPI (19.3%; 23/119). In contrast to the R strain, for which the rate and pattern of egg transmission was similar in both replicates, all K6216D positive eggs were detected in one K6216D-inoculated replicate; no *M. gallisepticum* positive eggs were detected from the other K6216D replicate.

Transovarian transmission of the R strain was significantly greater than that of all other isolates between 1-3 and 4-6 WPI ($P \leq 0.05$); it was not significantly different from K6216D transmission at 3-4 WPI ($P > 0.05$). Egg isolation results for the K6216D group were not significantly different from those of the negative control, ts-11 vaccine and K6222B groups for the duration of the study ($P > 0.05$). On average, K6216D was isolated from 4.0% (14/347) of eggs in the 3rd and 4th WPI; and from 1.5% (14/964) of eggs in the first 6 WPI. Average R strain transmission in the 6 WPI was 15.2% (112/739).

K6216D was only isolated from eggs in the early (5 days) and late (18 days) stages of incubation, with 4.3% (2/46) and 1.3% (12/904) of eggs testing positive at these time points respectively (Table 3.2). In contrast, R strain was isolated from eggs at each stage of incubation, with the highest percentage of isolations from 11-day-old embryos (9/11; 81.8%); and the lowest percentage (4/34; 11.8%) from eggs tested at 5 days of incubation. The overall percentage of R strain isolations from eggs collected post infection was significantly greater than that of all other groups ($P \leq 0.05$). For both K6216D and R strain, the majority of all isolations (85.7% and 77.7% respectively) were made from 18-day-old embryos; the remaining isolations were from infertile eggs or dead

embryos tested prior to 18 days of incubation. 100.0% (12/12) of K6216D-infected and 96.6% (84/87) of R strain-infected embryos were alive at 18 days of age.

**Egg production.** Egg production results are presented in Figure 3.2. One week after R strain infection, the egg production of chickens in that group decreased from 87.2% to 60.7% (a 26.5% reduction); which was significantly lower than that of all other groups ($P \leq 0.05$). Egg production in the R strain group gradually improved after 2 WPI; however it remained considerably lower than pre-infection levels for the duration of the trial.

There were no significant differences in the egg production of the negative control, ts-11, K6222B and K6216D groups for the duration of the study ($P > 0.05$), except at 31 weeks, when egg production of the negative control birds was significantly lower than that of the K6216D group ($P \leq 0.05$), and not significantly different from that of the R strain group ($P > 0.05$). For undetermined reasons, egg production of the negative control group was below standard before and after infection, peaking at only 83.7% at 28 weeks of age. Although egg production differences were only statistically significant for the R strain group, the average weekly egg production after infection (28-33 weeks) was higher than pre-infection (27 weeks) egg production in the negative control (+2.3%), ts-11 (+0.5%) and K6222B groups (+3.6%); but was lower than pre-infection production in groups inoculated with K6216D (-1.1%) and R strain (-17.7%).

**Serology.** Twenty birds (20/30) sampled pre-infection were weakly positive for *M. gallisepticum* antibodies by the SPA test, 2 birds (2/30) were *M. gallisepticum* ELISA positive and 1 bird (1/30) was *M. synoviae* ELISA positive. All birds sampled before

infection (30/30) were, however, negative for *M. gallisepticum* and *M. synoviae* antibodies by the HI test.

**Real-time PCR.** All birds tested pre-infection (30/30) were negative for *M. gallisepticum* and *M. synoviae* by real-time PCR. The quantitative real-time PCR results of the trial are presented in Table 3.3. Choanal cleft swabs from all chickens tested in each of the ts-11, K6222B, K6216D and R strain inoculated groups were PCR positive for *M. gallisepticum* at 2 weeks post infection (WPI), while all negative control chickens were negative for *M. gallisepticum*. The percentage of real-time PCR positives from tracheas collected at necropsy (6 WPI) was as follows: Negative control, 0% (0/32); ts-11, 31% (10/32); K6222B, 69% (22/32); K6216D, 69% (22/32); R strain, 60% (18/30). Mean tracheal *M. gallisepticum* DNA copy numbers for groups inoculated with K6222B, K6216D and R strain were significantly greater than the negative control group at 2 and 6 WPI, while copy numbers in the ts-11 inoculated group were significantly greater than those of the negative control group at 2 WPI, but not at 6 WPI ($P \leq 0.05$). R strain DNA copy numbers were numerically lower than K6222B and K6216D copy numbers at both 2 and 6 WPI, although these differences were not statistically significant ($P > 0.05$). Mean *M. gallisepticum* DNA copy numbers of all inoculated groups were lower at 6 WPI, compared with those at 2 WPI.

**Evaluation of lesions.** Mean tracheal mucosal thickness measurements and air sac lesion scores are presented in Table 3.4. The mean tracheal mucosal thickness measurements of the negative control, ts-11 and K6222B groups were not significantly different from each other; however tracheal mucosal thickness of the K6222B group was numerically greater than that of the negative control and ts-11 groups, and was not

significantly different from that of the K6216D group ($P > 0.05$). The K6216D group had a significantly higher mean tracheal mucosal thickness than that of the negative control and ts-11 groups, while the tracheal mucosal thickness of the R strain-inoculated chickens was significantly greater than that of all the other groups ($P \leq 0.05$).

No birds in the negative control and ts-11 inoculated groups had air sac lesions at 6 WPI. Air sac lesion scores in the K6222B and K6216D groups were statistically similar ($P > 0.05$), although more birds in the K6216D group were affected compared with the K6222B group (9/32 vs 5/32). The air sac lesion scores of R strain-inoculated chickens were significantly higher than those of all other groups ($P \leq 0.05$).

**Mycoplasma isolation from chickens.** *Mycoplasma* spp. was not isolated from the choanal cleft swabs of any chickens tested before infection (30/30). Culture results for air sac, ovary and oviduct swab samples collected from the hens are presented in Table 3.4. *M. gallisepticum* was not isolated from pooled air sac, ovary and oviduct swabs from any of the negative control or ts-11 inoculated hens. *M. gallisepticum* was re-isolated from 32% (9/28) and 25% (7/28) of hens inoculated with isolates K6222B and K6216D respectively, and from 92% (24/26) of hens inoculated with the R strain, which was significantly more than from any other group ($P \leq 0.05$).

**PCR and sequencing.** Two *M. gallisepticum* isolates per group from the air sacs, ovaries and oviducts of hens inoculated with K6222B, K6216D and R strain, and from the eggs of chickens inoculated with K6216D and R strain were randomly selected for IGSR PCR and sequencing. Sequencing of IGSR PCR amplicons confirmed that the sequences of hen and egg isolates from the K6222B and K6216D inoculated groups were

of the ts-11 genotype ("ts-11-like"), while the IGSR sequences of hen and egg isolates from the R strain inoculated group were genotyped as R strain.

## Discussion

The ts-11 vaccine has been widely used as a tool for the control of *M. gallisepticum* since its registration in Australia almost 20 years ago (4). It is recognized as a strain of low or no virulence (35), and has been demonstrated to provide protection against respiratory disease, egg production drops and vertical transmission associated with virulent *M. gallisepticum* infection (1, 3, 33, 34).

In 2007, a number of broiler breeder flocks in northeastern Georgia were vaccinated with ts-11 vaccine in an effort to control an ongoing *M. gallisepticum* epidemic. Although the vaccine appeared to control the epidemic, several ts-11-like isolates were detected in the progeny of vaccinated broiler breeders between 2008 and 2011. A previous study confirmed that a ts-11-like *M. gallisepticum* isolate from one of the infected broiler flocks was significantly more pathogenic than ts-11 vaccine (10). The epidemiology of the outbreaks, the genotyping results and the pathogenicity trial findings indicated that reversion to virulence and vertical transmission of ts-11 vaccine had occurred (10). In the present study, a ts-11-like isolate from broilers (K6216D) was shown to transmit to 1.5% of the eggs tested for 6 WPI, although no egg transmission of ts-11 vaccine or K6222B (a ts-11-like isolate from broiler breeders) was detected. R-strain was detected in 15.2% of eggs tested after infection.

Egg transmission of the R strain peaked at 25.4% in the third week after infection. Glisson and Kleven (1984) detected a similar rate of egg transmission within 4 weeks of R strain infection (13). Other studies, however, have reported peak egg transmission rates for the R strain ranging from 14 to 53% between 3 and 8 weeks after infection, highlighting the variable nature of *M. gallisepticum* vertical transmission (14, 19, 21, 31). K6216D egg transmission peaked at 7.2% in the 4$^{th}$ week post infection and was only detected in the eggs of one replicate; in contrast with the R strain, which transmitted at a similar rate from both replicates. Although eggs were not traced to specific hens in this study, it is theoretically possible that all K6216D positive eggs were laid by as few as two infected hens.

The mechanism of egg transmission of *M. gallisepticum* has not been fully elucidated, but probably involves infection of the ova following air sac and oviduct/ovarian infection or possibly bacteremia (13, 27). In this study, in addition to attempting to isolate *M. gallisepticum* from the eggs of infected hens, the air sacs and surface of the ovaries (which are in close proximity to the abdominal airsacs), as well as the mucosal surface of the oviduct were swabbed for *Mycoplasma* culture. The findings for K6216D and the R strain indicate a good correlation between the isolations of *M. gallisepticum* from the eggs and from the air sacs and reproductive tract; in agreement with other reports (31). However, although *M. gallisepticum* was isolated from a comparable percentage of the air sacs/ovaries/oviducts of the groups infected with K6222B and K6216D (32% and 25%), a similar level of infection of these organs did not result in egg transmission of K6222B. Since all of these regions were swabbed with a single swab, the results do not allow specific localization of the *M. gallisepticum* isolated.

It is therefore possible, for example, that K6222B was present on the air sacs and ovaries of culture-positive birds; whereas K6216D (which was detected in eggs) may have additionally infected the oviduct. More research is required to elucidate the mechanism of egg transmission of *M. gallisepticum*, and to investigate the genotypic and phenotypic characteristics of isolates which facilitate their egg transmission.

*M. gallisepticum* was not detected in the air sacs, ovaries and oviducts of ts-11 vaccine inoculated hens or their eggs during the course of this study. The lack of egg transmission following direct application of ts-11 vaccine is consistent with a report in which vertical transmission was not detected after administration of ts-11 into the abdominal air sacs of hens, or after eyedrop inoculation of ts-11 or direct contact with ts-11 vaccinated birds (33). It is likely that if ts-11 vaccine is vertically transmitted, changes in the vaccine (e.g. mutations, selection of sub-populations) are necessary for this to occur. It would have been ideal to utilize the particular lots of vaccine administered to birds in northeastern Georgia between 2008 and 2011 in this study. The reversion to virulence and vertical transmission of ts-11 may have been precipitated by vaccine characteristics (eg. virulent sub-populations present in the vaccine) that were specific to those vaccine lots. Unfortunately, those lots were not available for inclusion in this study.

Although the 6-week testing period was chosen based on previous studies that reported that this is the period during which egg transmission of *M. gallisepticum* is highest (13, 14, 19), it could be argued that egg transmission may have been detected if the study had been extended. In the field case reported previously, vertical transmission of *M. gallisepticum* likely occurred approximately 6 months after the breeders were vaccinated with ts-11 vaccine (10). A “latent” period may be necessary for the reversion

to virulence and vertical transmission to commence; this time may allow for processes such as bird-to-bird transmission, back passage, selection of sub-populations and genetic mutations to occur.

Vaccine application route and timing of infection may influence egg transmission. Lin and Kleven (1982) reported egg transmission of the F strain of *M. gallisepticum* when birds in production were inoculated by aerosol but not by eyedrop (19). The manufacturer recommends the use of ts-11 in pullets, by eyedrop application of 30 µl doses. In the current study, to improve the likelihood of detecting egg transmission, birds were inoculated in peak egg production (87%) by eyedrop at the recommended dose, as well as by aerosol with approximately 1000 µl/bird. While infection doses were uniformly inoculated in this study, uniformity of vaccine application under field conditions may be lacking, particularly if the cold chain is not maintained. It may be speculated that uneven coverage could facilitate bird-to-bird passage of vaccine, possibly providing a mechanism for reversion to virulence.

Egg transmission of the ts-11-like breeder isolate (K6222B) was not detected, although it appeared to be similar in virulence and invasiveness to K6216D, and it was isolated from the parents of the broilers infected with K6216D. The results indicate that, while the egg transmission of *M. gallisepticum* isolates appears to be related to their virulence and ability to invade systemically, additional factors may be necessary to facilitate infection of the embryonated egg. There may be multiple sub-populations of ts-11 present in a breeder flock after vaccination; with variable levels of virulence and transmissibility among the derivatives of the vaccine strain.

Other factors that could potentially impact *M. gallisepticum* egg transmission in the field include concurrent respiratory infection and management and environmental stressors. A limiting factor in evaluating the vertical transmission of *M. gallisepticum* isolates which are suspected of having low and erratic rates of transovarian transmission is the number of birds that can be inoculated and eggs that can practically be tested. In this study every settable egg laid by 5 groups of 28 hens for 6 weeks after infection (excluding the eggs lost due to the incubator overheating) was cultured; however the total number of eggs tested per group amounts to less than one tenth of the daily peak egg production of a single average 12,500 hen broiler breeder house in the United States.

There was an apparent correlation between the pathogenicity of the infection isolate in chickens and embryo mortality in this study: R strain, which was significantly more virulent in chickens than K6216D, also induced more embryo mortality. However, Levisohn *et al.* (1985) found no direct correlation between the *in ovo* pathogenicity of *M. gallisepticum* strains inoculated into the yolk sac of chicken embryos and their reported *in vivo* virulence (18). In contrast with the direct assessment of *in ovo* pathogenicity by Levisohn, *et al.* (1985), embryo lethality in our study was dependent on the ability of the isolate to infect the reproductive tract and the ova; the "infection dose" received by each embryo is unknown. Interestingly, for both K6216D and the R strain, the majority of infected 18-day-old embryos were alive, indicating that they may have survived to hatch as infected chicks.

While R strain infection induced a marked 26.5% egg production drop within a week of infection, the effect of infection with ts-11, K6222B and K6216D on egg production was not clear. Earlier studies have reported egg production drops of up to

~50-75% after R strain infection; these dramatic production drops are probably related to the more invasive infection methods employed, involving various combinations of aerosol inoculation and infraorbital sinus and air sac injection (13, 14, 21, 30, 31).

The fact that only in the K6216D and R strain groups was average post-infection egg production reduced compared with pre-infection production indicates that K6216D, in contrast to K6222B or ts-11 vaccine, may have negatively impacted egg production; however, these differences were not statistically significant ($P > 0.05$). It is likely that egg production differences would be more marked under commercial production conditions, with considerably larger groups of birds.

The sub-optimal egg production of the negative control group throughout this study may be related to the higher incidence of egg yolk peritonitis in this group (*data not shown*), although the reason for this increased incidence is unclear. The sera that were weakly SPA and ELISA positive pre-inoculation likely represent false positive reactions, which are relatively common with these tests (5, 12).

Based on clinical signs and air sac and tracheal lesions, K6216D and K6222B were significantly more virulent that ts-11 vaccine ($P \leq 0.05$), which was avirulent to birds in this study. Tracheal and air sac lesions induced by K6216D were numerically worse than those induced by K6222B, although these differences were not statistically different ($P > 0.05$). Tracheal colonization (evidenced by *M. gallisepticum* DNA copy numbers) by both K6216D and K6222B was also significantly greater than that of ts-11 vaccine ($P \leq 0.05$). These results corroborate those of the previous study in 5-week-old broilers in which K6216D was found to be significantly more pathogenic than ts-11 vaccine (10).

Considering its extensive use and the paucity of reports indicating the possibility of vertical transmission of ts-11, reversion to virulence and vertical transmission of ts-11 is likely to be a rare event. However, the detection of ts-11 and its differentiation from *M. gallisepticum* field strains is dependent on the availability and application of effective strain differentiation techniques. Whithear *et al*. (1996) reported that *M. gallisepticum* was detected in the eggs of broiler breeders between 44 and 51 weeks of age, which had been vaccinated twice by eye drop with ts-11 vaccine at 15 and 37 weeks of age (33). Whether egg transmission had indeed occurred was unclear, however, because the possibility existed that the semen used for artificial insemination was contaminated (33).

In a field study performed to investigate the prevalence of ts-11-like *M. gallisepticum* in the progeny of ts-11 vaccinated breeders from one company in Georgia, 83 broiler flocks derived from ts-11 vaccinated breeders and 10 broiler flocks from non-vaccinated breeders were sampled at processing. While no *M. gallisepticum* was detected in the progeny of non-vaccinates, one broiler flock (representing 1.2% of the progeny flocks from ts-11 vaccinates) was PCR positive for *M. gallisepticum* of the ts-11 genotype (7, 8).

The parent strain of ts-11 has some degree of virulence; it has been demonstrated to induce respiratory disease and significant egg production drops, and to transmit via the egg following intra-abdominal inoculation (32, 35). N-methyl-N-nitro-N-nitrosoguanidine, the chemical mutagen used to create ts-11, induces multiple linked genetic mutations (33, 35). The ts-11 vaccine strain's lack of virulence is not solely contingent on the temperature sensitive phenotype; the genetic basis for its attenuation remains to be fully determined (33).

The analysis of full genome sequences of the ts-11 vaccine and ts-11-like isolates of varying virulence is ongoing, however preliminary data indicates that there are few genomic differences between ts-11 vaccine and the ts-11-like isolates, supporting the hypothesis that the ts-11-like isolates originated from ts-11 vaccine (*unpublished data*).

In conclusion, our results provide the first conclusive evidence of transovarian transmission of an *M. gallisepticum* isolate of the ts-11 genotype, and indicate the existence of a spectrum of virulence and egg transmission potential for ts-11-like isolates. Research is underway to further confirm that the genetic relatedness of ts-11 vaccine and the ts-11-like isolates supports their epidemiological links; and to identify the genetic changes which may have precipitated reversion to virulence and vertical transmission of ts-11 vaccine.

## Acknowledgements

The authors would like to thank Ms. Rachel Jude, Mrs. Ruth Wooten, Mr. Patrick Black and Dr. Vijay Durairaj for their assistance with this study.

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Table 3.1. The origins, characteristics and inoculation titers of *M. gallisepticum* isolates used in this trial.

| Isolate | Origin | Virulence | *ts*[a] | Genotype | Reference | Inoculation titer (CCU/ml)[b] |
|---|---|---|---|---|---|---|
| ts-11 | Vaccine strain. Chemically mutagenized Australian field isolate. | Avirulent | + | ts-11 | Whithear *et al.*, 1990b | 33°C: 1.0 x $10^{10}$ 37.5°C: 3.9 x $10^{8}$ |
| K6222B | ts-11-vaccinated broiler breeders with increased mortality in Georgia, USA. | Virulent? | - | ts-11 | El Gazzar *et al.*, 2011 | 2.4 x $10^{9}$ |
| K6216D | Broilers (progeny of ts-11-vaccinated breeders above) with respiratory disease in Georgia, USA. | Virulent | - | ts-11 | El Gazzar *et al.*, 2011 | 1.2 x $10^{9}$ |
| R strain | Reference pathogenic strain. | Virulent | - | R strain | Rodriguez & Kleven, 1980 | 3.0 x $10^{9}$ |

[a] *ts* = temperature sensitivity
[b] CCU/ml = color changing units/ml

Table 3.2. Isolation of *M. gallisepticum* from eggs collected for 6 WPI at 5, 8, 11, 15 and 18 days of incubation.[a]

| Treatment group | Days of incubation | | | | | Total eggs |
|---|---|---|---|---|---|---|
| | 5 | 8 | 11 | 15 | 18 | |
| Negative control | 0/39 (0.0%)$^{A}$ | 0/14 (0.0%)$^{A}$ | 0/4 (0.0%)$^{A}$ | 0/3 (0.0%)$^{A}$ | 0/820 (0.0%)$^{A}$ | 0/880 (0.0%)$^{A}$ |
| ts-11 vaccine | 0/42 (0.0%)$^{A}$ | 0/3 (0.0%)$^{A}$ | 0/5 (0.0%)$^{A}$ | 0/2 (0.0%)$^{A}$ | 0/864 (0.0%)$^{A}$ | 0/916 (0.0%)$^{A}$ |
| K6222B | 0/57 (0.0%)$^{A}$ | 0/8 (0.0%)$^{A}$ | 0/4 (0.0%)$^{A}$ | 0/1 (0.0%)$^{A}$ | 0/860 (0.0%)$^{A}$ | 0/930 (0.0%)$^{A}$ |
| K6216D | 2/46 (4.3%)$^{A}$ | 0/6 (0.0%)$^{A}$ | 0/5 (0.0%)$^{A}$ | 0/3 (0.0%)$^{A}$ | 12/904 (1.3%)$^{A}$ | 14/964 (1.5%)$^{A}$ |
| R strain | 4/34 (11.8%)$^{A}$ | 5/8 (62.5%)$^{B}$ | 9/11 (81.8%)$^{B}$ | 7/10 (70.0%)$^{A}$ | 87/676 (12.9%)$^{B}$ | 112/739 (15.2%)$^{B}$ |

[a] Values within a column with a different uppercase, superscripted letter are significantly different ($P \leq 0.05$).

Table 3.3. Detection of *M. gallisepticum* DNA in choanal cleft and tracheal samples at 2 and 6 WPI with ts-11 vaccine, K6222B, K6216D and R strain.

| Treatment group | Real-time PCR results[a] | |
|---|---|---|
| | 2 WPI[b] | 6 WPI[c] |
| Negative control | 0/10[d] (0.0)$^{eA}$ | 0/32 (0.0)$^{A}$ |
| ts-11 vaccine | 10/10 (2.2 ± 0.7)$^{B}$ | 10/32 (0.6 ± 1.0)$^{AB}$ |
| K6222B | 10/10 (4.0 ± 0.4)$^{C}$ | 22/32 (1.2 ± 1.1)$^{BC}$ |
| K6216D | 10/10 (3.9 ± 0.6)$^{C}$ | 22/32 (1.2 ± 1.1)$^{C}$ |
| R strain | 10/10 (3.4 ± 0.4)$^{C}$ | 18/30 (0.9 ± 1.0)$^{BC}$ |

[a] Values within a column with a different uppercase, superscripted letter are significantly different ($P \leq 0.05$).
[b] Choanal cleft swabs tested.
[c] Tracheal washes tested.
[d] No. of positive samples/No. of tested samples.
[e] Mean DNA copy number $\log_{10}$ ± SD.

Table 3.4. Tracheal mucosal thickness, air sac lesion scores and *M. gallisepticum* isolation at 6 WPI with ts-11 vaccine, K6222B, K6216D and R strain[a].

| Treatment group | Tracheal mucosal thickness[b] | Air sac lesion score[cd] | *M. gallisepticum* isolation (air sacs, ovaries, oviduct)[ce] |
|---|---|---|---|
| Negative control | 451.9 ± 95.7$^{A}$ | 0/32 (0.0)$^{A}$ | 0/28$^{A}$ |
| ts-11 vaccine | 436.3 ± 48.3$^{A}$ | 0/32 (0.0)$^{A}$ | 0/28$^{A}$ |
| K6222B | 544.3 ± 133.7$^{AB}$ | 5/32 (0.2)$^{B}$ | 9/28$^{B}$ |
| K6216D | 660.7 ± 175.5$^{B}$ | 9/32 (0.3)$^{B}$ | 7/28$^{B}$ |
| R strain | 917.7 ± 386.0$^{C}$ | 24/30 (1.4)$^{C}$ | 24/26$^{C}$ |

[a] Values within a column with a different uppercase, superscripted letter are significantly different ($P \leq 0.05$).
[b] Mean thickness in micrometers ± SD.
[c] No. of positive samples/No. of tested samples.
[d] Mean air sac lesion score (macroscopically scored from 0 to 4).
[e] A single swab was used to sample these regions from each hen.

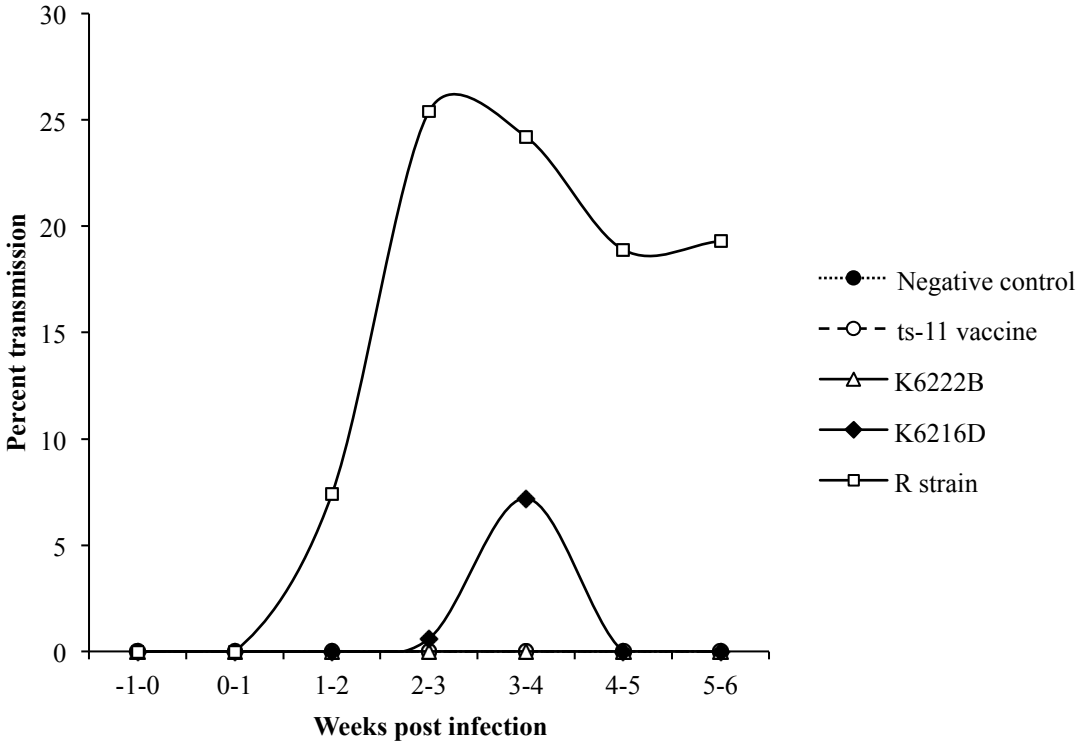


Figure 3.1. Egg transmission of ts-11 vaccine, K6222B, K6216D and R strain from -1 to 6 WPI.

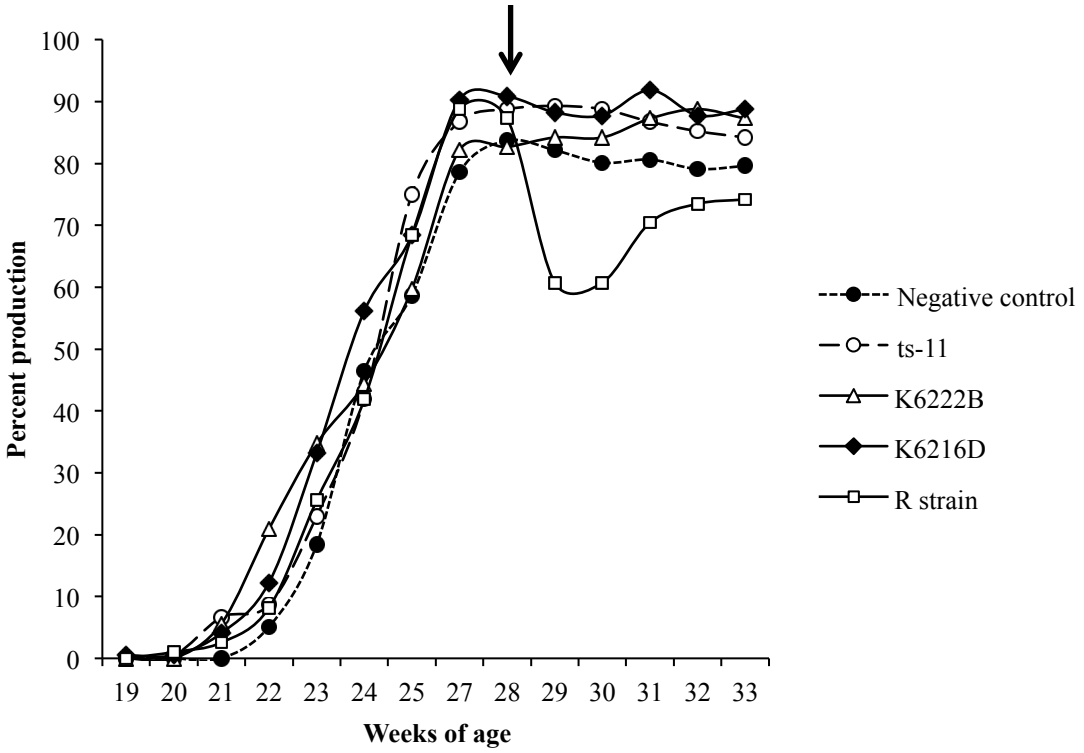


Figure 3.2. Egg production of groups inoculated with ts-11 vaccine, K6222B, K6216D and R strain. The arrow indicates the time of infection.

# CHAPTER 4

## A COMPARATIVE EVALUATION OF THE TRANSMISSIBILITY OF TWO *MYCOPLASMA SYNOVIAE* GENOTYPES[3]

[3] N.K. Armour, S.J. Gustin and N. Ferguson-Noel. To be submitted to *Avian Diseases*

Key words: *Mycoplasma synoviae*, MS, genotypes, transmissibility, quantitative real-time PCR

Abbreviations: CCU = color changing units; ELISA = enzyme-linked immunosorbent assay; HI = haemagglutination inhibition; MCN = mean DNA copy number; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; qPCR = quantitative real-time PCR; SPA = serum plate agglutination; SPF = specific pathogen free; WPI = weeks post infection of the seeders

## Summary

An outbreak of *Mycoplasma synoviae* (MS) in broilers and broiler breeders in the state of Arkansas was characterized by severe respiratory disease, mortality and airsacculitis condemnations in broilers, and egg production drops and mortality in breeders. Two MS isolates from this outbreak, K6191C and K6315D, belonging to the genotypes designated S-10 and S-17 respectively, were previously evaluated in broiler breeder pullets. The S-10 isolate was demonstrated to be significantly more virulent than the S-17 isolate ($P \leq 0.05$), and similar in virulence and transmissibility to the pathogenic MS reference strain K1968. The objectives of the current study were to evaluate and compare the transmissibility of S-10 and S-17 in sexually mature broiler breeders, and to assess the effect of prophylactic tylosin medication on MS transmission. MS transmission from infected seeder chickens was detected by real-time PCR at 6 weeks post infection in both the K6191C and K6315D groups. The transmission patterns of the two genotypes differed, however; S-10 transmitted to adjacent contact chickens, and S-17 to fomite contact chickens. S-17 fomite contact chickens medicated with tylosin remained MS negative. S-10 was significantly more immunogenic than S-17, based on antibody titers in infected chickens ($P \leq 0.05$). Quantitative real-time PCR data indicated that S-10 replicated more rapidly and to higher levels than S-17 in the upper respiratory tracts of directly or indirectly infected chickens. This is the first experimental evaluation of the horizontal transmission of MS in sexually mature chickens. Our results provide evidence for unique transmission patterns of different MS strains, and indicate that the transmission rate of an MS strain may be independent of its virulence, immunogenicity and *in vivo* replication rate.

## Introduction

*Mycoplasma synoviae* (MS), the causative agent of infectious synovitis of chickens and turkeys, is a pathogen of worldwide prevalence (10). Currently circulating strains of MS in the United States are more commonly implicated as a cause of respiratory disease than the synovitis for which this pathogen is named. The respiratory disease induced by MS ranges in severity from a subclinical upper respiratory infection to a more severe respiratory disease, involving airsacculitis (10). The wide variation in the presentation and severity of MS-induced disease is largely related to the strain of MS involved (10, 17, 29, 33).

An important characteristic of the avian mycoplasmas is their propensity to transmit both horizontally and vertically, via the egg. Horizontal transmission of MS is generally more rapid than that of *M. gallisepticum* (MG), and typically results in infection of 100% of birds in the flock (10). Differences in the transmission rates of different MS strains have been reported (9, 42). Horizontal transmission of MS occurs readily via the respiratory route; MS transmission from inoculated chickens to direct contact birds has been demonstrated (9, 25, 30, 35, 42). MS is capable of surviving for up to 3 days on feathers, 2 days on cotton and 8 hours on rubber (6), and has been shown to transmit indirectly to chicks placed in isolators previously contaminated with either MS broth culture, MS-infected chickens, or with material (dust, feathers and feed) collected from an MS positive flock (31). The horizontal transmission of MS has not previously been evaluated in sexually mature broiler breeder chickens.

MS isolates have demonstrated *in vitro* susceptibility to a number of antibiotics, including tetracycline, chlortetracycline, oxytetracycline, danofloxacin, enrofloxacin,

lincomycin, spectinomycin, spiromycin, tiamulin, tilmicosin, aivlosin and tylosin (4, 5, 11, 16, 19, 24, 28, 43). Tylosin and tetracyclines are currently the most commonly used antibiotics in the United States for MS control (22). While it cannot be relied upon to eliminate MS infection from a flock, antibiotic treatment of infected flocks has been used to prevent the development of airsacculitis and synovitis lesions and to reduce vertical transmission of MS (2, 14, 15, 34, 36). The use of antibiotic medication to prevent MS infection of indirect contact chickens has not been studied.

MS was identified as the cause of outbreaks of severe respiratory disease in broiler and broiler breeder chickens in the state of Arkansas between 2008 and 2009. The respiratory disease was associated with decreased feed efficiency, increased mortality and airsacculitis condemnations in affected broilers, and drops in egg production and increased mortality in broiler breeders. The severity of this outbreak was unusual for MS, which typically causes a subclinical upper respiratory disease (10). This apparent atypical virulence was accompanied by evidence of extensive vertical and horizontal transmission.

The pathogenicity two MS isolates from this outbreak, K6191C and K6315D, belonging to the genotypes designated S-10 and S-17 respectively, was previously investigated in broiler breeder pullets (1). The S-10 isolate was significantly more pathogenic than the S-17 isolate ($P \leq 0.05$), and similar in pathogenicity to the pathogenic reference strain K1968 (1). The rate of transmission of S-10 MS to direct, adjacent, downwind and fomite contact chickens was found to be similar to, but slightly slower than that of K1968 (1).

The objective of the present study was to compare the transmissibility of the S-10 and S-17 genotypes of MS in sexually mature broiler breeders. The transmission of MS to birds associated with infected seeder groups by proximity (adjacent contacts), airflow (downwind contacts) and fomite placement (fomite contacts) was evaluated. In addition, the effect of prophylactic tylosin medication on MS transmission to fomite contact birds (medicated fomite contacts) was investigated.

## Materials and Methods

**MS strains and isolates.** The MS isolates K6191C and K6315D were isolated from tracheal swabs collected from broiler breeders in 2008 and 2009 respectively during an MS outbreak in the state of Arkansas, USA. K6191C and K6315D belong to the genotypes designated S-10 and S-17 respectively (1), based on sequencing of the *vlhA* region (18). In a previous study, the S-10 isolate was found to be more pathogenic than the S-17 isolate, and similar in pathogenicity and transmissibility to the virulent MS reference strain K1968 (1). The inoculation doses (K6191C, $3.62 \times 10^5$ color-changing units [CCU]/ml; K6315D, $7.74 \times 10^4$ CCU/ml) were administered by a combination of intraocular (100µl), intranasal (200µl) and intratracheal (100µl) routes. Real-time PCR results revealed that the S-17 seeder birds were not infected 1 week after inoculation, necessitating re-inoculation of this group with a higher titered isolate ($7.74 \times 10^7$ CCU/ml). Both isolates used for inoculation were $9^{th}$ passage isolates, which had been propagated in Frey's modified broth medium.

**Serology.** All sera were analyzed for MS antibodies using the serum plate agglutination (SPA), haemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) tests. The SPA and HI tests were performed as previously described (23), using commercial antigen for the SPA test (Charles River Laboratories, Wilmington, MA) and antigen prepared from the WVU1853 strain and chicken erythrocytes for the HI test. ELISA tests were performed using a commercially available kit (IDEXX, Westbrook, ME). An SPA score ≥ 1 was considered positive. An HI titer of 1:20 was considered suspect, and ≥ 1:40 was considered positive. A geometric mean sample/positive (S/P) ratio > 0.5 on the ELISA test was considered positive.

**Isolation and identification of mycoplasma.** Cotton swabs used for tracheal swabbing were inoculated in Frey's modified broth and agar, and incubated at 37.5°C. *Mycoplasma* isolates were identified using direct immunofluorescence (23).

**DNA extraction.** DNA extraction was performed using the MagMAX$^{TM}$-96 Viral RNA Isolation kit (Applied Biosystems by Life Technologies, Carlsbad, CA) on the MagMAX$^{TM}$ Express-96 Magnetic Particle Processors (Applied Biosystems by Life Technologies), according to the manufacturer's instructions.

**Real-time PCR analysis.** MS quantitative real-time PCR analysis (qPCR) was performed following a previously described protocol (38) on choanal cleft swab and tracheal wash samples, collected from chickens sampled during the course of the study or at necropsy respectively, and prepared as described previously (37).

**Chickens and experimental design.** Two hundred, 51-week-old Cobb 700 broiler breeder hens were acquired from a commercial source. The birds were divided

into 10 groups, and placed in 10 floor pens (1.5 x $3m^2$) with pine shavings litter in a naturally ventilated, curtain-sided poultry house. For the evaluation of S-10 transmission, 100 birds were divided into one group of 12 "seeder" chickens and 4 groups of 22 chickens; one group for each transmission route to be evaluated (adjacent, fomite, medicated fomite and downwind contacts). One hundred birds were similarly divided for the evaluation of S-17 transmission. At placement, 10 randomly selected birds were screened by serology (SPA, HI and ELISA), real-time PCR and culture, to confirm that they were negative for avian *Mycoplasma* spp. One week after placement, all chickens in both seeder groups were inoculated with either the S-10 or S-17 MS isolates. The time of infection of the group was designated as the point at which at least 50% of the seeder birds were MS positive by real-time PCR, with a mean DNA copy number (MCN) $\log_{10}$ greater than 1.0 per group.

The seeder birds were in a pen adjacent to the adjacent contact birds, and upwind of the downwind contact birds. Airflow was generated by placing an industrial fan (1.0 m in diameter) to move air from the seeder pens towards the downwind contact pens (average air velocity, 2.4 m/s). Sixteen golf balls and 16 tennis balls used as fomites were placed in the S-10 and S-17 seeder pens at the time of inoculation. One week post infection (WPI) of the seeders, 8 golf balls and 8 tennis balls were moved from the seeder pens to each of the fomite and medicated fomite contact pens. Tylosin tartrate (Tylan® Soluble Powder, Elanco, Greenfield, IN) was administered in the drinking water to birds in the medicated fomite contact pens according to the manufacturer's recommendations, at a rate of 530 mg of tylosin base per liter of water. The medication was started 2 days before addition of the fomites to the medicated fomite contact pens, and was discontinued

30 days later. Fresh medicated drinking water was prepared twice weekly. All birds were bled for serology and sampled for real-time PCR at 2 and 6 WPI, and sampled for serology and real-time PCR at necropsy at 11 WPI.

All animal procedures in these experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia, Athens, GA. The bird welfare was adequate and in accordance with the IACUC, and all birds were provided with feed and water *ad libitum*. Chickens were euthanized by cervical dislocation or carbon dioxide inhalation.

**Statistical analysis.** SPA scores were analyzed using the Kruskal-Wallis rank sums test. HI titers $\log_{10}$, ELISA S/P ratios and MS DNA copy numbers $\log_{10}$ were analyzed using the Tukey-Kramer highly significant difference test (JMP® Pro 11.0.0, SAS Institute Inc., Cary, NC). A $P$-value $\leq 0.05$ was considered significant.

## Results

**Pre-Inoculation.** One bird (1/10) was positive for MG antibodies, and three birds (3/10) were positive for MS antibodies pre-inoculation by SPA testing. All birds (10/10) were, however, negative for MG and MS antibodies before inoculation by HI and ELISA serology. All of the birds (10/10) tested pre-inoculation were negative for MG and MS by qPCR and were confirmed to be free of avian *Mycoplasma* spp. by culture.

**Seeders.** The qPCR results for the seeder groups are presented in Table 4.1 and Figure 4.1; serology results are presented in Table 4.1. One week after inoculation of the

seeders, 82% (9/11) of the S-10 seeder group was qPCR positive for MS (MCN $\log_{10}$, 1.6), but only 8% (1/12) of the S-17 seeders was positive (MCN $\log_{10}$, 0.2). Two weeks after inoculation, MS DNA was detected in 7/12 (58%) of the S-17 seeders (MCN $\log_{10}$, 1.2); this level of infection was not significantly different from that of the S-10 seeder group 1 week after inoculation ($P > 0.05$). The time of infection (0 WPI; the point at which at least 50% of the seeder birds were MS positive by real-time PCR, with an MCN $\log_{10} > 1.0$ per group) was therefore designated as 1 and 2 weeks post inoculation of the S-10 and S-17 seeders respectively.

At 2 WPI, 91% (10/11) and 73% (8/11) of the S-10 and S-17 seeder chickens respectively were qPCR positive for MS; by 6 WPI, 100% of birds in both of these groups were qPCR positive (Table 4.1). MS DNA copy numbers $\log_{10}$ in the S-10 seeder group were significantly higher than in the S-17 seeder groups at 2 and 6 WPI ($P \leq 0.05$). Quantification of MS DNA in the upper respiratory tracts of the seeder chickens revealed a sharp increase in S-10 MCN $\log_{10}$ from 0 WPI, peaking at 4.1 at 2 WPI, compared with a more gradual increase in S-17 MCN $\log_{10}$, which peaked at 3.0 at 6 WPI (Figure 4.1). Average MS MCN $\log_{10}$ declined in both seeder groups from 6 WPI to 11 WPI, at which point they were not significantly different ($P > 0.05$).

At 2 WPI, 91% (10/11), 55% (6/11) and 82% (9/11) of the S-10 seeder group tested positive for MS antibodies by the SPA, HI and ELISA tests respectively (Table 4.1). All of the S-10 seeder chickens (10/10) were positive on the SPA, HI and ELISA tests at 11 WPI. MS antibodies were detected in 55% (6/11) and 18% (2/11) of S-17 seeder birds at 2 WPI by the SPA and ELISA tests respectively. Positive HI titers were first detected in this group at 11 WPI, when 30% (3/10) tested HI positive, and 60%

(6/10) and 50% (5/10) were SPA and ELISA positive respectively. MS HI and ELISA antibody titers were significantly higher in the S-10 seeder group compared with the S-17 seeder group for the duration of the study ($P \leq 0.05$).

**Adjacent Contacts.** The qPCR results for the adjacent contact groups are presented in Table 4.2 and Figure 4.2; serology results are presented in Table 4.2. Forty-two percent (8/19) of the S-10 adjacent contact chickens were qPCR positive at 6 WPI; by 11 WPI, 100% of this group (18/18) was MS positive (Table 4.2). S-17 adjacent contact chickens remained qPCR negative for the duration of the trial, apart from one chicken with a relatively low MS DNA copy number at 2 WPI. MS DNA copy numbers $\log_{10}$ were significantly higher in the S-10 adjacent contact group compared with the S-17 adjacent contacts at 6 and 11 WPI ($P \leq 0.05$). The graphical presentation of MS DNA levels in the upper respiratory tracts of the adjacent contact chickens indicates an exponential increase in MCN $\log_{10}$ from 0 to 3.4 between 2 and 11 WPI in the S-10 adjacent contact group (Figure 4.2).

The first clear serological evidence of transmission from the S-10 seeders occurred at 11 WPI, when MS antibodies were detected in 94% (17/18; SPA and HI) and 100% (18/18; ELISA) of the S-10 adjacent contact chickens (Table 4.2). One of the S-17 adjacent contact chickens was ELISA positive at 2 WPI; however this was not the same bird that was positive on real-time PCR, and the group otherwise tested consistently negative for MS DNA and antibodies.

**Fomite Contacts.** The qPCR results for the fomite contact groups are presented in Table 4.3 and Figure 4.3; serology results are presented in Table 4.3. While no MS

DNA was detected in the S-10 fomite contact group for the duration of the study, 90% (18/20) and 89% (16/18) of the S-17 fomite contact chickens were MS positive by qPCR at 6 and 11 WPI respectively (Table 4.3). MS DNA copy numbers $\log_{10}$ in the S-17 fomite contact group were significantly higher than in the S-10 fomite contact group at 6 and 11 WPI ($P \leq 0.05$). The MCN $\log_{10}$ of the S-17 fomite contact group peaked at only 1.1 at 6 WPI, with a slight decline to 0.9 by 11 WPI (Figure 4.3).

Low-level, weak SPA positivity was detected in the S-10 and S-17 fomite contact groups from 2 to 11 WPI, however HI and ELISA titers in both groups remained negative for the duration of the study, apart from 1/16 ELISA positive in the S-10 group at 11 WPI (Table 4.3).

**Medicated Fomite Contacts.** The qPCR and serology results for the medicated fomite contact groups are presented in Table 4.4. Both S-10 and S-17 medicated fomite contact groups remained qPCR negative for the duration of the study.

A low percentage of weak SPA and ELISA serological positives was detected in both medicated fomite contact groups at each sampling point, however all chickens tested negative for MS antibodies by HI for the duration of the study.

**Downwind Contacts.** The qPCR and serology results for the downwind contact groups are presented in Table 4.5. Both S-10 and S-17 downwind contact groups remained qPCR negative for the duration of the trial.

There were low percentages of SPA positives in the S-10 and S-17 downwind contact groups throughout the study, and 2/16 ELISA positives in the S-10 group at 11 WPI, but no MS antibodies were detected in either group by the HI test for the duration of the study.

**Clinical signs and mortality.** No clinical signs were observed in any of the groups for the duration of the study. Mortality over the course of the study was 2 to 6 birds per pen; mortality was attributed to miscellaneous causes not related to MS infection.

## Discussion

The ability of MS to spread rapidly in infected flocks is well-known, and is an important aspect of the epidemiology of this pathogen (10), however, few studies have evaluated the horizontal transmission of MS. These studies have demonstrated the horizontal transmission of MS by direct contact (9, 25, 30, 35, 42), and indirectly by contact with infected fomites (31). The current study was performed to elucidate the ability of two MS genotypes (S-10 and S-17) from a recent MS outbreak in Arkansas to transmit to naïve adult broiler breeders indirectly associated with infected chickens by proximity, airflow and fomite placement, and to evaluate the effect of prophylactic medication on fomite transmission.

Based on real-time PCR results, the transmission rates of the S-10 and S-17 MS isolates were similar, with transmission occurring from the seeder groups at 6 WPI for both genotypes. The transmission patterns, however, were different: transmission from the S-10 seeders occurred to adjacent contacts, while the S-17 genotype transmitted from seeders to fomite contact chickens. Transmission was restricted to pen-mates within these infected groups for the duration of the trial, except for one S-17 adjacent contact chicken, which was qPCR positive for MS at 2 WPI.

In a previous MS transmission trial comparing the transmission of the S-10 and K1968 MS genotypes in broiler breeder pullets, we detected S-10 transmission to adjacent and downwind contacts (1). In that study, S-10 fomite contact chickens remained MS negative throughout the study, with the exception of 2/19 fomite contacts with very low MS DNA copy numbers at 12 weeks after inoculation (1). Taken together, these results indicate that S-10 MS transmission occurs readily to adjacent contact chickens, while fomite transmission appears to be less efficient. It is tempting to speculate that the comparatively effective fomite transmission of S-17 MS seen in this study may be as a result of longer survival times of S-17 MS on the fomite surface (golf balls and tennis balls). The results of previous studies indicate that different MS strains exhibit differences in their ability to survive on various substrates (6, 32, 39).

MS transmission to downwind contact birds occurred in the previous study in pullets (S-10 and K1968 genotypes) (1) but not in the current study (S-10 and S-17 genotypes), indicating that pullets may be more readily infected by airborne MS than sexually mature birds. In both the current and previous studies, S-10 transmission from the infected seeder pens was detected 4 weeks after MS DNA copy numbers peaked in the upper respiratory tracts of the seeder chickens, demonstrating an association between MS infection level and transmission (1).

Marois *et al.* (2005) evaluated the indirect transmission of MS to SPF chicks in isolators, and reported that MS infection was first detected by PCR and culture 33 and 54 days respectively after exposure of the chicks to material (feed, feathers and dust) from an MS positive farm (31). These results showed that the interval between the exposure of chickens to infected material and the detection of MS infection can be surprisingly long

(31). It is interesting to note that in our study, S-17 fomite contact chickens were also first found to be MS positive by qPCR 35 days after exposure to fomites from the S-17 seeder pen (6 WPI of the seeders). Since the interval between this and the previous sampling was 4 weeks, it is of course also possible that the S-17 fomite contact chickens were infected some time before infection was detected in this group.

The fact that MS was detected in the S-17 fomite, but not in the S-17 medicated fomite contact group suggests that medication of chickens with tylosin via the drinking water prevented MS infection in this group. Another explanation could be that the fomites placed in the S-17 medicated fomite group were not infected with MS; however, identical fomites originating from the same seeder pen did transmit MS to the non-medicated S-17 fomite contacts. It could also be argued that the S-17 fomite contact chickens were infected by airborne MS transmission from the seeder pen. This is also unlikely, given that the S-17 downwind contacts, situated between the seeder and fomite contact pens, remained MS negative throughout the study. The efficacy of tylosin against S-10 MS could not be evaluated, as neither of the S-10 fomite contact groups was infected with MS.

Several authors have reported negative isolation results after MG (20, 21, 40) or MS (36) infection in chickens prophylactically treated with antibiotics. The absence of PCR quantification in these studies, however, raises the question of whether treatment prevented infection or reduced organism levels below the detection limit of routine isolation techniques. Cummings *et al.* (1986) studied the effect of in-feed tylosin and chlortetracycline medication on the tracheal MG population of infected birds, and concluded that medication reduced tracheal counts but did not prevent infection (8). In

the current study we use quantitative PCR to demonstrate the absence of detectable MS DNA in the tylosin-medicated S-17 fomite contact group, compared with 89-90% MS PCR positivity in the non-medicated S-17 fomite group. These data indicate that tylosin tartrate administered in the drinking water prevented the establishment of fomite-transmitted S-17 MS infection under the conditions of this study. It should be noted that the level of MS infection in the S-17 fomite contact group was low (MCN $\log_{10}$, 0.9 to 1.1); further investigation would therefore be required to determine the efficacy of tylosin prophylaxis at higher MS infection levels.

Seroreactivity to S-17 infection was significantly less than that to S-10 infection, with SPA, HI and ELISA titers of the S-17 seeders being consistently significantly lower than those of the S-10 seeders ($P \leq 0.05$). Antibodies were only detected in S-17 seeder group at 11 WPI by HI (9 weeks after positive HI serology in the S-10 seeder group), despite the fact that 58% of the S-17 seeders were MS positive by PCR at the time of infection. In addition, no MS antibodies were detected by either ELISA or HI in the S-17 fomite contact group, despite real-time PCR evidence that this group was infected at 6 WPI of the seeders (i.e. 5 weeks after placement of the fomites from the seeder pen). Using PCR and culture, Ewing *et al.* (1998) found no difference in bird-to-bird transmission of a virulent MS strain and an isolate (K1858) of low virulence, which had been previously described as being of low transmissibility based on serological evidence (9, 42). In that study, 5-week-old SPF chickens inoculated with K1858 were PCR positive within 3 days but only became HI positive 6 weeks after inoculation, while commingled birds which were PCR positive 7 days after exposure showed no HI positivity up to 6 weeks after inoculation (9). Our findings support this evidence for the

existence of MS strains of low immunogenicity and but comparable transmission rates to virulent MS strains.

Interpretation of serology results in this study was confounded by SPA and ELISA positives in the S-10 and S-17 downwind and medicated fomite and S-10 fomite contact groups, which were consistently MS HI and qPCR negative. There were also weak SPA positives in sera tested pre-inoculation from birds that were confirmed to be MS and MG negative by real-time PCR and culture. These results are consistent with false positive serological reactions, which are relatively common with these tests (3, 13). Non-specific serological reactions may be attributed to prior vaccination of the birds used in this study with oil-emulsion vaccines, which are known to increase the incidence of false positive serological reactions (7, 12). Our results highlight the usefulness of real-time PCR as a sensitive and specific detection method in cases where the incidence of non-specific serological reactors is high. In this study, real-time PCR gave a more accurate indication than serology of MS infection and of the rate of MS transmission within and between groups, in agreement with the findings of Ewing *et al.* (1998) (9).

Using qPCR, we were able to investigate the kinetics of MS replication in the upper respiratory tract of infected chickens. S-10 MS replicated rapidly in the seeder birds to peak at an MCN $\log_{10}$ of 4.1 at 2 WPI, ten times higher and 4 weeks earlier than the peak mean DNA copy number of the S-17 seeder group. Interestingly, the peak MS DNA copy numbers in this study for S-10 and S-17 were similar to those we have obtained in previous studies, with different inoculation doses and routes and bird ages. S-10 and S-17 MCNs $\log_{10}$ peaked at 4.0 and 3.2 respectively in a pathogenicity trial performed in 3-week-old broilers, and the S-10 MCN $\log_{10}$ peaked at 3.9 in a

transmission study with broilers of the same age (1). DNA copy numbers in the birds secondarily infected by S-10 in this study were also substantially higher than in those secondarily infected by S-17; An MCN $\log_{10}$ of 3.4 was detected in the S-10 adjacent contact birds at 11 WPI, compared with an MCN $\log_{10}$ of only 0.9 in the S-17 fomite contact group at the same time point. These data indicate that S-10 consistently replicates faster and to higher levels than S-17 MS in the respiratory tract, with S-10 and S-17 DNA levels peaking at MCNs $\log_{10}$ of approximately 4 and 3 respectively. This differential infection level is seen in conjunction with significantly higher virulence and immunogenicity (based on serology results) of S-10 compared with S-17, as evidenced by this study and a previous pathogenicity study ($P \leq 0.05$) (1).

Infection of the S-17 seeder chickens to achieve a statistically similar level of infection to that of the S-10 seeders necessitated infecting the S-17 seeders with approximately 100x more organism, and waiting an additional week after inoculation. To facilitate comparison of S-10 and S-17 transmission after established infection, the time of infection was determined to be the point at which real-time PCR positivity in the seeder groups exceeded 50%, with an MCN $\log_{10}$ greater than 1.0 per group. At this point (1 and 2 weeks post inoculation for the S-10 and S-17 seeders respectively), the DNA copy numbers of the S-10 and S-17 seeders were statistically similar ($P > 0.05$), permitting reliable comparison of the transmissibility of these genotypes.

The difficulty in achieving statistically comparable levels of infection of the S-10 and S-17 seeders suggests that the infectious dose of the S-17 genotype is considerably higher than that of S-10 MS in sexually mature broiler breeders. We subsequently performed a trial to establish the infectious dose ($ID_{50}$) of S-10 and S-17 MS in 28-week-

old commercial layers. The $ID_{50}$ of S-10 was calculated as $2.0 \times 10^4$ CCU/100 µl dose ($2.0 \times 10^5$ CCU/ml). Sufficient birds could not, however, be infected with S-17 MS to allow the determination of the $ID_{50}$, indicating that the $ID_{50}$ of S-17 is greater than $2.4 \times 10^5$ CCU/100 µl dose ($2.4 \times 10^6$ CCU/ml) (*unpublished data*). These data suggest that the infectivity of the S-17 genotype of MS is substantially less than that of the S-10 genotype, explaining the difficulty in infecting birds with S-17 MS in the current study. Further research is necessary to establish the genetic and phenotypic basis for the differences in infectivity of the S-10 and S-17 genotypes.

The mature age of the chickens used in this study probably played a role in the reduced ability of the S-17 isolate to infect chickens and to elicit a detectable immune response. Older chickens are known to be less susceptible to the development of clinical MS disease (10), presumably because the pathogen is more effectively controlled by their mature immune systems. It is plausible that reduced clearance and increased systemic invasion of MS in younger chickens would respectively facilitate MS colonization and stimulate a greater humoral immune response. In addition, factors such as immunosuppression, concurrent environmental stressors and infection with other respiratory pathogens, which were not present in this study but are common under field conditions, may have exacerbated MS infection and transmission. Newcastle Disease and Infectious Bronchitis vaccination exacerbates MS clinical disease (27), and has been used in MS studies to potentiate lesions, seroconversion and transmission of MS (9, 15, 26, 31, 41). In order to be consistent with industry practices for breeders of this age (at which breeders are not routinely vaccinated for respiratory viruses), and to avoid introducing an

additional variable that could complicate data interpretation, we elected not to vaccinate the breeders in this study.

Once MS infection was established in the sexually mature broiler breeders, the transmission rate of the virulent S-10 isolate was similar to that of the less virulent S-17 isolate, although the transmission pattern of these genotypes differed. The S-17 isolate replicated more slowly and to lower levels in the trachea, and was significantly less immunogenic than the S-10 isolate ($P \leq 0.05$), necessitating the use of real-time PCR, rather than serology for the effective evaluation of transmission. Our results suggest that, while the virulence of an MS strain is related to its immunogenicity and *in vivo* replication rate, virulence and transmissibility appear to be independent characteristics. The value of prophylactic medication with tylosin in limiting indirect transmission of MS is supported by our data, but more research is required to evaluate the efficacy of tylosin prophylaxis at higher infection levels. In conclusion, these results improve our understanding of MS transmission dynamics, and have implications for the design of rational and effective MS control programs.

## Acknowledgements

The authors would like to thank Mrs. Victoria Laibinis, Mrs. Ruth Wooten and Ms. Michelle Farrar for their assistance with this study.

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Table 4.1. Detection of MS DNA and antibodies in S-10 and S-17 seeder groups at 2, 6 and 11 weeks post infection.[A]

| WPI | MS strain | Real-time PCR[BC] | SPA[BD] | HI[BE] | ELISA[BF] |
|---|---|---|---|---|---|
| 2 | S-17 | 8/11 (2.6 ± 1.8)[a] | 6/11 (0.6)[a] | 0/11 (0.1)[a] | 2/11 (0.2)[a] |
| | S-10 | 10/11 (4.1 ± 1.4)[b] | 10/11 (2.1)[b] | 6/11 (1.0)[b] | 9/11 (1.8)[b] |
| 6 | S-17 | 10/10 (3.0 ± 1.2)[a] | 3/10 (0.3)[a] | 0/10 (0.8)[a] | 0/10 (0.2)[a] |
| | S-10 | 11/11 (4.0 ± 0.8)[b] | 10/11 (1.7)[b] | 10/11 (2.0)[b] | 11/11 (2.1)[b] |
| 11 | S-17 | 10/10 (2.5 ± 1.5) | 6/10 (0.8) | 3/10 (1.0)[a] | 5/10 (0.6)[a] |
| | S-10 | 10/10 (3.2 ± 0.7) | 10/10 (1.4) | 10/10 (2.2)[b] | 10/10 (5.6)[b] |

[A] Values within a column at a specific time period with a different lowercase, superscripted letter are significantly different ($P \leq 0.05$).
[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥1:40, and ELISA: >0.5).
[C] Mean DNA copy no. $\log_{10}$ ± SD.
[D] Mean agglutination grade (from 0 to 4).
[E] Mean titer $\log_{10}$.
[F] Mean sample to positive ratio.

Table 4.2. Detection of MS DNA and antibodies in S-10 and S-17 adjacent contact groups at 2, 6 and 11 weeks post infection of the seeders.[A]

| WPI | MS strain | Real-time PCR[BC] | SPA[BD] | HI[BE] | ELISA[BF] |
|---|---|---|---|---|---|
| 2 | S-17 | 1/19 (0.1 ± 0.4) | 0/19 (0.0) | 0/19 (0.0) | 1/19 (0.1) |
| | S-10 | 0/20 (0.0) | 0/20 (0.0) | 0/20 (0.0) | 1/20 (0.1) |
| 6 | S-17 | 0/19 (0.0)[a] | 0/19 (0.0) | 0/19 (0.0) | 0/19 (0.1) |
| | S-10 | 8/19 (1.8 ± 2.2)[b] | 0/19 (0.0) | 0/19 (0.1) | 1/19 (0.2) |
| 11 | S-17 | 0/16 (0.0)[a] | 0/16 (0.0)[a] | 0/16 (0.0)[a] | 0/16 (0.0)[a] |
| | S-10 | 18/18 (3.4 ± 0.6)[b] | 17/18 (1.6)[b] | 17/18 (1.9)[b] | 18/18 (4.1)[b] |

[A] Values within a column at a specific time period with a different lowercase, superscripted letter are significantly different ($P \leq 0.05$).
[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥1:40, and ELISA: >0.5).
[C] Mean DNA copy no. $\log_{10}$ ± SD.
[D] Mean agglutination grade (from 0 to 4).
[E] Mean titer $\log_{10}$.
[F] Mean sample to positive ratio.

Table 4.3. Detection of MS DNA and antibodies in S-10 and S-17 fomite contact groups at 2, 6 and 11 weeks post infection of the seeders.[A]

| WPI[G] | MS strain | Real-time PCR[BC] | SPA[BD] | HI[BE] | ELISA[BF] |
|---|---|---|---|---|---|
| 2 | S-17 | 0/21 (0.0) | 4/21 (0.2) | 0/21 (0.0) | 0/21 (0.1) |
| | S-10 | 0/20 (0.0) | 1/20 (0.1) | 0/20 (0.0) | 0/20 (0.1) |
| 6 | S-17 | 18/20 (1.1 ± 0.4)[b] | 2/20 (0.1) | 0/20 (0.0) | 0/20 (0.1) |
| | S-10 | 0/18 (0.0)[a] | 5/18 (0.4) | 0/18 (0.0) | 0/18 (0.1) |
| 11 | S-17 | 16/18 (0.9 ± 0.4)[b] | 2/18 (0.2) | 0/18 (0.0) | 0/18 (0.0)[a] |
| | S-10 | 0/16 (0.0)[a] | 0/16 (0.0) | 0/16 (0.0) | 1/16 (0.2)[b] |

[A] Values within a column at a specific time period with a different lowercase, superscripted letter are significantly different ($P \leq 0.05$).
[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥1:40, and ELISA: >0.5).
[C] Mean DNA copy no. $\log_{10}$ ± SD.
[D] Mean agglutination grade (from 0 to 4).
[E] Mean titer $\log_{10}$.
[F] Mean sample to positive ratio.
[G] Fomites were moved from the seeder to the fomite contact pens 1 WPI.

Table 4.4. Detection of MS DNA and antibodies in S-10 and S-17 medicated fomite contact groups at 2, 6 and 11 weeks post infection of the seeders.[A]

| WPI[G] | MS strain | Real-time PCR[BC] | SPA[BD] | HI[BE] | ELISA[BF] |
|---|---|---|---|---|---|
| 2 | S-17 | 0/20 (0.0) | 1/20 (0.1) | 0/20 (0.0) | 1/20 (0.1) |
| | S-10 | 0/19 (0.0) | 1/19 (0.1) | 0/19 (0.0) | 1/19 (0.2) |
| 6 | S-17 | 0/19 (0.0) | 1/19 (0.1) | 0/19 (0.0) | 1/19 (0.2) |
| | S-10 | 0/18 (0.0) | 0/18 (0.0) | 0/18 (0.0) | 2/18 (0.2) |
| 11 | S-17 | 0/19 (0.0) | 0/19 (0.0) | 0/19 (0.0) | 3/19 (0.2) |
| | S-10 | 0/17 (0.0) | 0/17 (0.0) | 0/17 (0.0) | 2/17 (0.3) |

[A] Values within a column at a specific time period with a different lowercase, superscripted letter are significantly different ($P \leq 0.05$).
[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥1:40, and ELISA: >0.5).
[C] Mean DNA copy no. $\log_{10}$ ± SD.
[D] Mean agglutination grade (from 0 to 4).
[E] Mean titer $\log_{10}$.
[F] Mean sample to positive ratio.
[G] Fomites were moved from the seeder to the medicated fomite contact pens 1 WPI.

Table 4.5. Detection of MS DNA and antibodies in S-10 and S-17 downwind contact groups at 2, 6 and 11 weeks post infection of the seeders.[A]

| WPI | MS strain | Real-time PCR[BC] | SPA[BD] | HI[BE] | ELISA[BF] |
|---|---|---|---|---|---|
| 2 | S-17 | 0/21 (0.0) | 3/21 (0.3) | 0/21 (0.0) | 0/21 (0.1) |
| | S-10 | 0/18 (0.0) | 0/18 (0.0) | 0/18 (0.0) | 0/18 (0.1) |
| 6 | S-17 | -[G] | 0/21 (0.0)[a] | 0/21 (0.0) | 0/21 (0.1) |
| | S-10 | 0/17 (0.0) | 4/17 (0.5)[b] | 0/17 (0.0) | 0/17 (0.1) |
| 11 | S-17 | 0/20 (0.0) | 1/20 (0.1) | 0/20 (0.0) | 0/20 (0.0)[a] |
| | S-10 | 0/16 (0.0) | 3/16 (0.3) | 0/16 (0.0) | 2/16 (0.2)[b] |

[A] Values within a column at a specific time period with a different lowercase, superscripted letter are significantly different ($P \leq 0.05$).
[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥1:40, and ELISA: >0.5).
[C] Mean DNA copy no. $\log_{10} \pm$ SD.
[D] Mean agglutination grade (from 0 to 4).
[E] Mean titer $\log_{10}$.
[F] Mean sample to positive ratio.
[G] Results not included due to sample contamination.

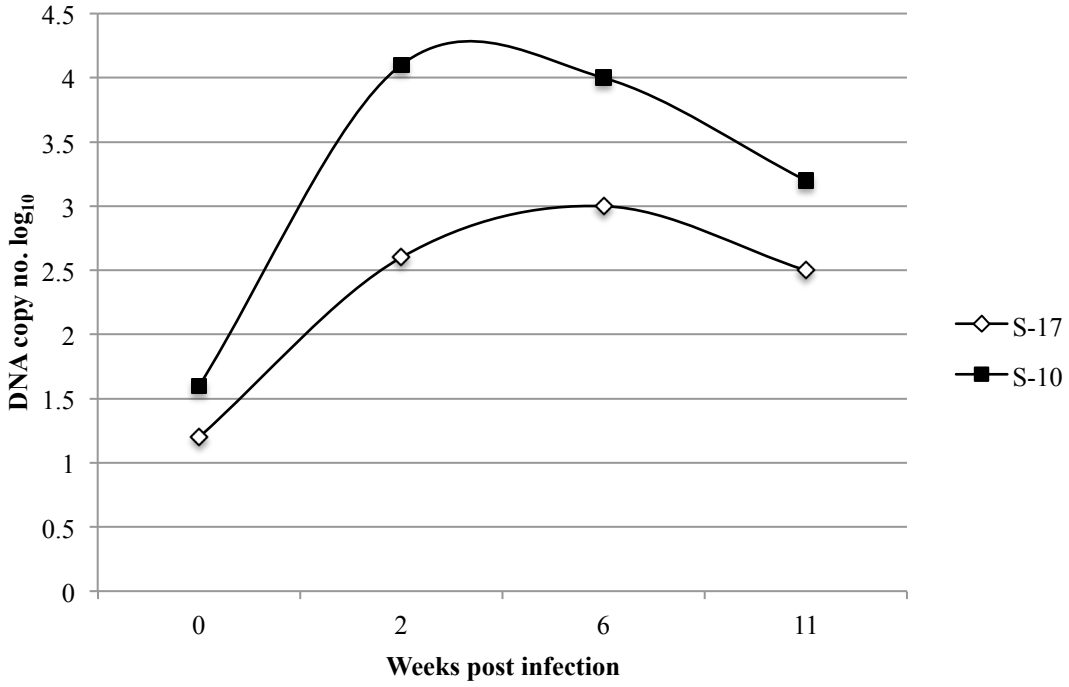


Figure 4.1. Mean DNA copy numbers $\log_{10}$ in the S-10 and S-17 seeder groups at 2, 6 and 11 weeks post infection.

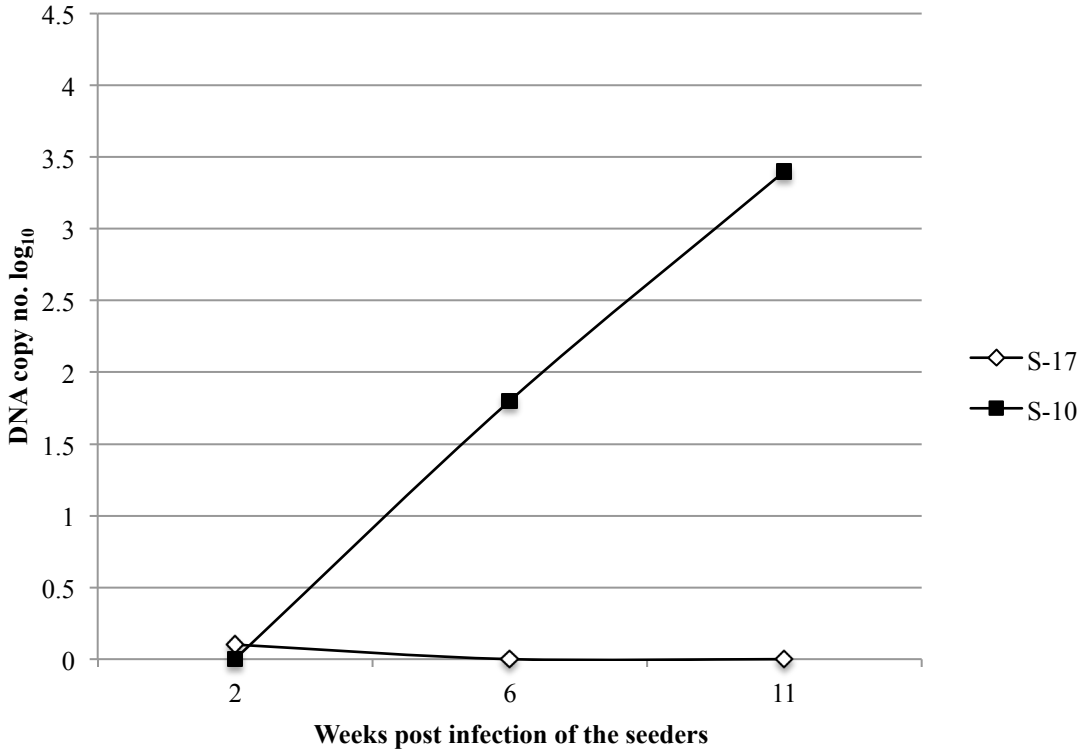


Figure 4.2. Mean DNA copy numbers $\log_{10}$ in the S-10 and S-17 adjacent contact groups at 2, 6 and 11 weeks post infection of the seeders.

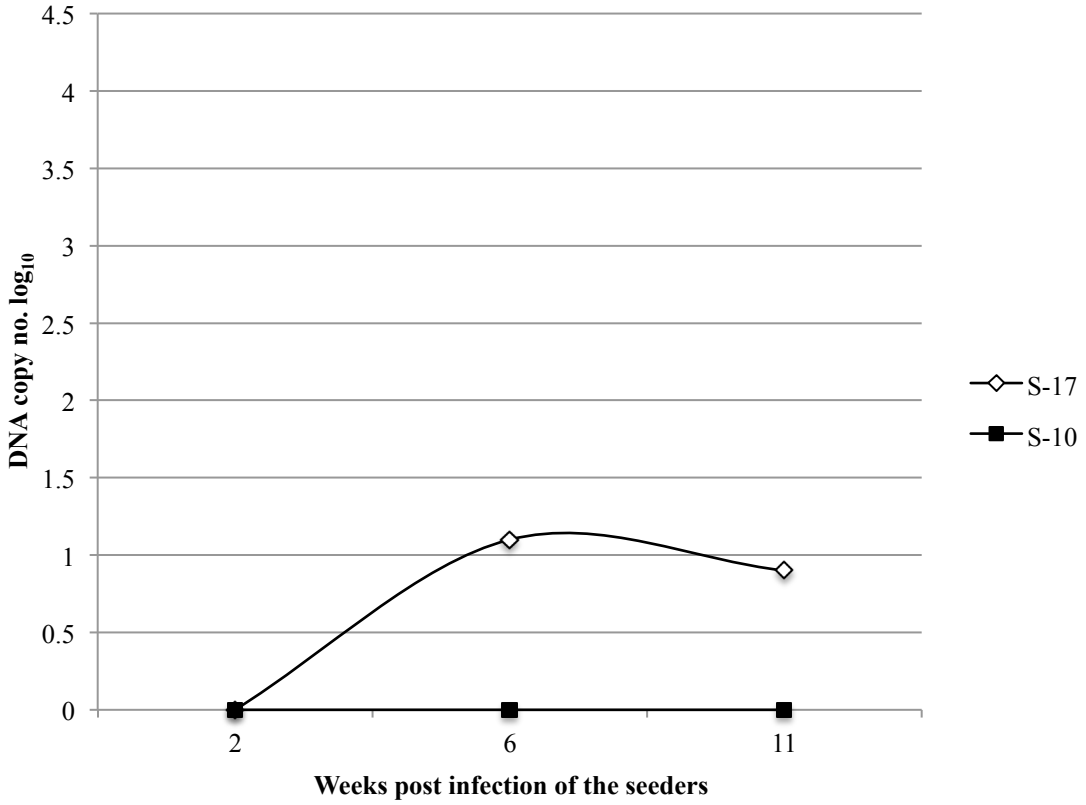


Figure 4.3. Mean DNA copy numbers $\log_{10}$ in the S-10 and S-17 fomite contact groups at 2, 6 and 11 weeks post infection of the seeders.

# CHAPTER 5

# AN INFECTION MODEL TO EVALUATE THE FOMITE TRANSMISSION OF *MYCOPLASMA SYNOVIAE*[4]

[4] N.K. Armour and N. Ferguson-Noel. To be submitted to *Avian Diseases.*

Key words: *Mycoplasma synoviae*, MS, fomite, transmission, model, mucin

Abbreviations: CCU = color changing units; FMS = modified Frey's medium with swine serum; HI = haemagglutination inhibition; MCN = mean DNA copy number; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; PI = post inoculation; qPCR = real-time quantitative PCR; SPA = serum plate agglutination; SPF = specific pathogen free; WPP = weeks post placement of inoculated fomites

## Summary

*Mycoplasma synoviae* (MS) is a prevalent and economically significant poultry pathogen, causing respiratory tract infections and infectious synovitis. MS was identified as the causative agent of an outbreak of severe respiratory disease in broilers and broiler breeders in the state of Arkansas during 2008 and 2009. Two MS isolates from this outbreak, K6191C and K6315D, belonging to the genotypes designated S-10 and S-17 respectively, were previously evaluated. K6191C was demonstrated to be significantly more virulent than K6315D, and similar in virulence and transmissibility to the pathogenic MS reference strain K1968. The objective of the current study was to evaluate and compare the transmission of K6191C and K6315D to sexually mature broiler breeder chickens from various directly inoculated materials commonly encountered in the poultry house environment, which could serve as fomites facilitating the indirect transmission of MS. Evaluation of K6191C and K6315D growth curves revealed substantially different replication kinetics, with K6315D titers peaking 40 hours after K6191C titers. Mucin was added to the infection cultures, to simulate the consistency and composition of respiratory secretions; evaluation showed that mucin did not negatively impact media pH and sterility or MS survivability on a plastic substrate at an inclusion level of at 5 mg/ml. Groups of broiler breeder chickens were exposed to feathers, cotton fabric or plastic fomites inoculated with K6191C or K6315D cultures infused with 5 mg/ml of mucin. Low-level MS transmission (6.7% - 20.0%) from K6315D-inoculated cotton fabric and from K6191C-inoculated feather, cotton fabric and plastic fomites was detected at 3 weeks post fomite placement by real-time PCR, but not by culture or serology. This study demonstrates the application of a novel *Mycoplasma* infection model, and is the first

report of the transmission of MS to sexually mature chickens from directly inoculated fomite materials.

## Introduction

*Mycoplasma synoviae* (MS) infects the respiratory tract of chickens and turkeys, causing subclinical or clinical respiratory disease with airsacculitis, and may infect the synovial tissues, resulting in infectious synovitis (9). Different MS strains display differences in pathogenicity (9), transmissibility (8, 28) and ability to survive on substrates (7, 18, 26). MS and *M. gallisepticum* (MG) are both vertically and horizontally transmitted; horizontal transmission of MS is, however, generally more rapid than that of MG (9). Understanding MS transmission dynamics is critical to effectively controlling this pathogen.

Several authors have reported direct lateral transmission of MS to birds in the same pen as infected birds (8, 14, 15, 21, 28), but there are few reports providing experimental evidence for the indirect transmission of MS. MS has been demonstrated to survive for up to 3 days on feathers, 2 days on cotton, 4 hrs on wood shavings, 8 hrs on rubber and 12 hrs in the human nasal passage (7), and has been detected in the environments of poultry naturally or experimentally infected with MS (16-18). Some mycoplasmas have the ability to form biofilms, which may facilitate their survival in the environment (6, 19). MS transmission to specific pathogen-free (SPF) chicks placed in contaminated isolators or exposed to feed, feathers and dust collected from an MS positive flock has been demonstrated (17). Susceptible flocks may thus be infected not only directly, by the introduction of infected birds, but also indirectly, by contact with

contaminated materials serving as fomites, making fomite transmission of MS an important risk to poultry operations.

Between 2008 and 2009, an epidemic of MS caused severe respiratory disease in broiler and broiler breeder chickens in the state of Arkansas. The rapid spread of the MS infection indicated that the strains involved may have been unusually transmissible. We previously investigated the pathogenicity and transmissibility of two isolates from this outbreak, K6191C and K6315D, belonging to the genotypes designated S-10 and S-17 respectively (4, 5). MS transmission from seeder chickens to naïve chickens in the same pen, in pens adjacent to or downwind of the seeder pen, or in pens to which fomites from the seeder pen were added was evaluated (4, 5). K6191C was found to be significantly more pathogenic than K6315D (4), and to display a similar rate but different pattern of transmission to K6315D (5). Transmission of K6315D, but not of K6191C, from naturally-infected fomites to sexually mature chickens was demonstrated, and prophylactic tylosin medication of fomite-exposed chickens appeared to prevent K6315D infection (5).

The current study was performed to further investigate the potential for K6191C and K6315D to transmit to chickens on infected fomites, given the suspected role of fomite transmission in contributing to the rapid spread of MS during the 2008/2009 epidemic in Arkansas. Lateral transmission of MS occurs via the respiratory route (9), in respiratory aerosols/secretions. The glycoprotein mucin is an important component of mucus, and is responsible for its characteristic consistency (30). Mucin of porcine or bovine origin has been used as a component of suspension media to simulate saliva or sputum in transmission, survival and gene expression studies with several human

respiratory pathogens, including *Pseudomonas aeruginosa*, human parainfluenza virus and rhinovirus (2, 11, 25, 27). MG was reported to survive for up to 7 to 14 days at room temperature on paper discs inoculated with media containing mucin (20). Based on the hypothesis that the composition and consistency of respiratory mucus facilitates the survival of MS on substrates, and in order to simulate these characteristics in an infection model, the feasibility of adding mucin to MS infection cultures was investigated.

The next objective was to evaluate the transmission of K6191C and K6315D from infected fomite materials commonly encountered in the poultry house environment to naïve sexually mature broiler breeder chickens. Feathers, cotton fabric and plastic bootcovers were evaluated for their potential to act as fomites facilitating the indirect transmission of MS. Our results demonstrate the application of mucin in a *Mycoplasma* infection model, and provide the first experimental evidence for MS transmission from inoculated fomite materials to sexually mature chickens.

## Materials and Methods

**MS isolates, replication kinetics and inoculation cultures.** The MS isolates K6191C and K6315D have been previously described; these isolates belong to the genotypes designated S-10 and S-17 respectively (4, 5). In previous studies, K6191C was found to be significantly more virulent than K6315D, similar in virulence and transmissibility to the pathogenic MS reference strain K1968 (4), and to have a similar rate but different pattern of transmission to K6315D (5).

Growth curves were constructed to assess the replication kinetics of the MS isolates in this study. Frozen K6191C and K6315D broth cultures, which were 7th and 8th passage isolates respectively, were thawed and one part culture was inoculated into nine parts modified Frey's medium with swine serum (FMS) broth. The cultures were incubated at 37.5°C, and each culture was sampled for titration immediately, and thereafter 17 times at 4 to 10 hourly intervals over a 102 hour period. Titrations were performed in duplicate, and the titer of each inoculum (expressed in color changing units (CCU)/ml) was determined as previously described (24). Titration results at each sampling point were averaged to construct growth curves for each isolate.

For the infection cultures, the MS isolates were grown to their predicted peak titers based on growth curve results (30 and 70 hrs post inoculation (PI) for the K6191C and K6315D cultures respectively), and the titer of each inoculum was determined (24). At this point, a 55 mg/ml solution of mucin, prepared by dissolution of porcine stomach mucin (Sigma-Aldrich®, Saint Louis, MO) in sterile, deionized water, was added to infection cultures to a final concentration of 5 mg mucin/ml of culture. This mucin concentration was selected based on its use in other studies (2, 11, 25, 27), and on the mucin evaluation results of this study.

**Mucin evaluation: Effect on pH and sterility of FMS broth media.** Triplicate samples of FMS broth containing 0, 2.5, 5 or 10 mg/ml of mucin were tested to evaluate the effect of mucin addition on media pH and sterility. The pH of the media was measured using a Thermo Scientific™ Orion Star™ pH meter (Fisher Scientific, Pittsburgh, PA). For the evaluation of media sterility, FMS broth samples were incubated at 37.5°C and evaluated daily in comparison with control broth for a change in color from

red to orange or yellow (indicating a shift in pH). Broth media was plated on FMS agar when a color change was observed or after 2 weeks if no color change occurred; agar plates were then incubated and examined for bacterial colonies after 5 days.

**Mucin evaluation: Effect on MS survivability on a plastic substrate.** A 55 mg/ml solution of mucin, prepared as described above, was added to aliquots of log-phase cultures of K6191C and K6315D, to a final concentration of 5 mg/ml. 400 µl aliquots of K6191C or K6315D cultures with or without mucin were evenly applied to the surface of each of 5 plastic petri dishes (BD Falcon™ Petri Dish, BD Biosciences, Durham, NC), divided into 8 equal sections. Petri dishes were sampled for titration 0, 12 and 24 hrs after inoculation, and thereafter every 24 hrs for a total of 4 days. Petri dishes were sampled using polyester swabs (Polyester Dacron Swab Pack, Medical Packaging Corp., Camarillo, CA) pre-wet in sterile FMS broth to swab 4 sections (representing 200 µl of inoculate) at each sampling; sections to be sampled were randomly pre-determined using Research Randomizer© (www.randomizer.org). Swabs were inoculated into sterile FMS broth, and titers were determined as previously described (24). Petri dishes were maintained at room temperature (21°C) between samplings.

**Serology.** All sera were analyzed for MS antibodies using the serum plate agglutination (SPA) and haemagglutination inhibition (HI) tests. The SPA and HI tests were performed as previously described (13), using commercial antigen for the SPA test (Charles River Laboratories, Wilmington, MA) and antigen prepared from the WVU1853 strain and chicken erythrocytes for the HI test. An SPA score $\geq 1$ was considered positive. An HI titer of 1:20 was considered suspect, and $\geq$ 1:40 was considered positive.

**Isolation and identification of mycoplasma.** Cotton swabs used for tracheal swabbing were inoculated in FMS broth and agar, and incubated at 37.5°C. *Mycoplasma* isolates were identified using direct immunofluorescence (13).

**DNA extraction.** DNA extraction was performed according to the manufacturers' instructions using the Mag-Bind® Viral DNA/RNA 96 Kit (Omega Bio-Tek, Norcross, GA) on the MagMAX™ Express-96 Magnetic Particle Processors (Applied Biosystems by Life Technologies) or the QIAGEN DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, CA).

**Real-time PCR analysis.** MS quantitative real-time PCR (qPCR) analysis was performed following a previously described protocol (23) on choanal cleft swab and tracheal wash samples, collected at 3 and 5 weeks post fomite placement (WPP) respectively, and prepared as described previously (22).

**PCR amplification and sequencing of the 16S rRNA gene of *Acholeplasma laidlawii*.** The primer sequences used for amplification of the 16S rRNA gene of *A. laidlawii* were as follows: 16S UF: 5'-ACT CCT ACG GGA GGC AGC AG-3'; 16S UR: 5'-CTC ACG ACA CGA GCT GAC GA-3' (Raviv, unpublished). The reaction mix (EPICENTRE Biotechnologies, Madison, WI) contained 25 µl of FailSafe PCR 2x PreMix B, 5 µM primers, 0.5 µl FailSafe PCR Enzyme Mix, 5 µl DNA template and water to a total volume of 50 µl. DNA was amplified in a Bio-Rad C1000™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) at 95°C for 3 min; 35 cycles of 94°C for 20 sec, 55°C for 40 sec and 72°C for 60 sec; and ending with 72°C for 5 min (Raviv, unpublished). PCR products were visualized under UV transillumination in a 2% agarose

gel containing 1 μg/ml ethidium bromide. Amplified DNA was purified and sequenced, and sequences were analyzed as previously described (3).

**Chickens and experimental design.** One hundred and thirty-three, 65-week-old Ross 708 broiler breeder hens acquired from a commercial source were divided into 9 groups, and placed in 9 floor pens (1.5 x $3m^2$) with pine shavings litter in a naturally ventilated, curtain-sided poultry house. For the evaluation of K6191C transmission, 60 birds were divided into 4 groups of 15; one group for each fomite to be evaluated (plastic, cotton fabric, feathers) and one sentinel group. Sixty birds were similarly divided for the evaluation of K6315D transmission, and one group of ten birds served as negative controls. Three cull hens were euthanized, and their feathers were plucked for use in the study. Pens were set up and positioned to avoid cross-infection, and the sentinel pens were positioned to facilitate detection of inadvertent MS cross-infection. At placement, 20 birds were screened by serology (SPA and HI), real-time PCR and culture to confirm that they were negative for MS and MG. One week after placement, pens were infected by the addition of plastic, cotton fabric or feather fomites inoculated with K6191C (4.5 x $10^7$ CCU/ml) or K6315D (7.7 x $10^7$ CCU/ml) cultures infused with 5 mg/ml mucin. Fomites were not placed in the negative control pen.

For both the plastic and cotton fabric infection, clean rubber boots were covered with boot covers made of red cotton fabric. For the plastic fomite group, the boots were then covered with clear plastic boot covers (Elastic-top boots, Agri-Pro Enterprises of Iowa, Inc., Iowa Falls, IA). The infection dose (2 ml per boot) was applied evenly onto each cotton fabric boot cover (cotton fabric fomite) or plastic boot cover (plastic fomite). Five inoculated fomites were evenly placed in the same location in each fomite contact

pen (total infection dose; 10 ml per pen). For the feather fomite inoculation, a pipette was used to apply the infection dose (10 ml) to 3g of feathers. The infected feathers were spread evenly over the surface of the wood shavings in the feather fomite pen. All fomites were allowed to dry for approximately 30 minutes after inoculation, before placement in the fomite contact pens.

Birds were bled for serology and sampled for real-time PCR at 3 WPP, and sampled for serology, real-time PCR and culture at necropsy, 5 WPP. All animal procedures in these experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia, Athens, GA. The bird welfare was adequate and in accordance with the IACUC, and all birds were provided with feed and water *ad libitum*. Chickens were euthanized by cervical dislocation or carbon dioxide inhalation.

**Statistical analysis.** DNA copy numbers $\log_{10}$ were analyzed using the Tukey-Kramer highly significant difference test (JMP® Pro 11.0.0, SAS Institute Inc., Cary, NC). A $P$-value $\leq 0.05$ was considered significant.

## Results

**MS replication kinetics.** The K6191C and K6315D growth curves are presented in Figure 5.1. Exponential growth of the K6191C culture commenced 16 hrs post inoculation (PI) of the thawed isolate into FMS broth, and the highest titer ($10^{8.0}$ CCU/ml) was attained 30 hrs PI. The rapid decline in the K6191C titer after 36 hrs paralleled a change in color of the phenol red pH indicator in the FMS broth from orange

to yellow (indicating a pH less than 6.8). No viable K6191C MS was detected by 58 hrs PI. The K6315D titer at the time of inoculation was approximately $10^2$ CCU/ml lower than the K6191C titer, and exponential growth began 36 hrs PI. The K6315D titer peaked at 70 hrs PI ($10^{7.9}$ CCU/ml), at a similar level to the highest K6191C titer, after which it declined to $10^{4.6}$ CCU/ml by 102 hrs PI.

**Effect of mucin on FMS broth media pH and sterility.** The addition of mucin to FMS broth resulted in a slight decrease in the pH of the media; which was proportional to the mucin concentration of the final solution (Mean pH: FMS only, 7.68; FMS + 2.5 mg/ml mucin, 7.65; FMS + 5 mg/ml mucin, 7.62; FMS + 10 mg/ml mucin, 7.58). After 2 weeks of incubation at 37.5°C, only the FMS solution containing 10 mg/ml of mucin showed a color change (red to orange-yellow); no color change was observed after incubation of FMS broth containing 2.5 mg/ml or 5.0 mg/ml of mucin. There was no bacterial growth on FMS agar at any of the mucin concentrations tested.

**Effect of mucin on MS survivability on a plastic substrate.** The results of the MS survivability experiment are presented in Figure 5.2. The titers of all inocula were initially similar (0 hr, $10^{5.4}$-$10^{5.7}$ CCU/ml); thereafter the titers of the K6191C, K6191C+mucin and K6315D inocula increased until 12 hours post inoculation (PI) of the petridish surfaces. The increase in K6191C titers was greater than that of K6315D titers (increase of $10^{0.6}$-$10^{0.8}$ CCU/ml compared with $10^{0.4}$ CCU/ml respectively), and there was no increase in the titer of the K6315D+mucin inoculum. All titers decreased after 24 hours, and no live MS organisms were detected in the K6191C, K6191C+mucin and K6315D inocula by 48 hr PI. MS in the K6315D+mucin inoculum survived the longest ($10^{3.0}$ CCU/ml at 48 hours); no live MS was detected in this inoculum by 72 hr PI.

**Serology.** All birds tested (20/20) were negative for MG and MS antibodies before fomite placement by SPA and HI serology. The serology results for the trial are presented in Table 5.1. All birds in all groups (130/130) tested negative for MS antibodies by the SPA and HI tests at 3 and 5 WPP.

**Isolation and identification of mycoplasmas.** *Mycoplasma* spp. was not isolated from the tracheal swabs of any chickens tested before fomite placement (20/20). MS tracheal culture results for the trial are presented in Table 5.1. No MS was isolated at 5 WPP from any of the chickens (124/124) in the study. However, an organism with similar colony morphology to *Mycoplasma* spp. was isolated from birds in the negative control (2 birds), K6191C sentinel, plastic and cotton fabric groups (1, 6 and 7 birds respectively), and in the K6315D plastic group (2 birds); this organism was determined not to be MS or MG by direct immunofluorescence, and was identified as *Acholeplasma laidlawii* by 16S rRNA PCR and sequencing. In addition, several cultures were overgrown by contaminant bacteria, as follows: K6191C plastic (2), cotton fabric (1) and feather (5) cultures and K6315D sentinel (1) and feather (2) cultures.

**Real-time PCR analysis.** All birds tested (20/20) were negative for MG and MS DNA before fomite placement by qPCR. The real-time PCR results for the trial are presented in Table 5.1. MS DNA was detected in a small percentage of the K6191C plastic (1/15; 6.7%), cotton fabric (3/15; 20.0%) and feather (1/14; 7.1%) contact chickens at 3 WPP, and in only one K6315D cotton fabric contact chicken (1/15; 6.7%) at 3 WPP. MS DNA levels were relatively low in each case, with mean DNA copy numbers (MCNs) $\log_{10}$ of 0.1, 0.3 and 0.1 for the K6191C plastic, cotton fabric and feather groups respectively, and 0.1 for the K6315D cotton fabric group. There were no

statistically significant differences in DNA copy numbers $\log_{10}$ when comparing different isolates (same fomite material) or different fomite materials (same isolate) ($P > 0.05$). All birds tested negative for MS by real-time PCR at necropsy (5 WPP); manual DNA extraction (using the QIAGEN DNeasy® Blood and Tissue Kit) and re-testing was performed, with the same results. All negative control and sentinel chickens were PCR negative for MS at 3 and 5 WPP.

**Clinical signs and mortality.** No clinical signs were observed in any of the groups for the duration of the study. Mortality over the course of the study was 0 to 3 birds per pen; mortality was attributed to miscellaneous causes not related to MS infection.

## Discussion

The ability of MS to survive outside of the host for up to several days on a number of different organic and inorganic substrates (1, 7, 16, 18, 26) provides a mechanism for its indirect transmission to naïve poultry via contaminated fomites. MS strains exhibit differences in their ability to survive on various substrates (7, 18, 26); this could potentially influence the fomite transmissibility of different strains. Indirect transmission of MS to SPF chicks in contaminated isolators has previously been demonstrated (17). The current study is the first report of indirect MS transmission from inoculated fomite materials to sexually mature chickens, and demonstrates the application of a unique model for the evaluation of MS fomite transmission.

Growth curves constructed to elucidate the replication kinetics of the MS isolates K6191C and K6315D revealed markedly different replication rates and patterns.

Exponential replication of the K6315D culture commenced at 36 hr PI, 20 hrs later than the K6191C culture; and peak K6315D titers were attained at 70 hr PI, 40 hrs after peak K6191C titers. After peaking, the rate of titer decrease of K6315D was slower than than that of K6191C, which had a rapid decrease in titer after 36 hr PI. MS is highly susceptible to low pH (9), and it is likely that the rapid decrease in titer with K6191C was due to higher levels of acid accumulation (evidenced by the color change of the phenol red pH indicator) associated with more rapid growth of this strain. Interestingly, the K6191C and K6315D cultures peaked at similar titers ($10^{8.0}$ and $10^{7.9}$ CCU/ml respectively). These results highlight the importance of constructing growth curves to obtain equivalent, high-titered inoculation cultures for strains with substantially different replication kinetics.

Mucin was added to infection cultures to simulate the consistency and composition of avian respiratory secretions, based on its reported use in suspension media for several human respiratory pathogens (2, 11, 25, 27). A report on the survival of MG for up to 7 to 14 days at room temperature on paper discs inoculated with a mucin-containing broth medium indicated that mucin may extend mycoplasma survival; however, the substrate survival of MG suspended in medium without mucin was not evaluated in that study (20). In the current study, *in vitro* testing confirmed that the inclusion of mucin at 5 mg/ml would not adversely affect the pH and sterility of FMS broth media, or the survivability of MS on a plastic substrate. The survival of K6315D in mucin-containing media was, in fact, extended by 24 hrs compared with media without mucin. It is possible that the more viscous consistency of the culture medium containing mucin protected the K6315D organisms from desiccation.

The materials used for inoculation in this study were selected on the basis of their frequent presence in the poultry house environment, and their potential as fomites. Feathers remaining in the house after depopulation of the previous flock, or becoming wind-borne during transit of flocks to the processing facility, as well as cotton fabric clothing/coveralls and plastic bootcovers worn by people moving between poultry houses could serve as fomites, facilitating the indirect transmission of MS. Christensen *et al.* (1994) reported MS survival times of up to 3 days on feathers and 2 days on cotton, while MG survived up to 1 day on a plastic substrate (7).

In the current study, MS transmission from directly inoculated fomite materials (plastic boot cover, cotton fabric and feathers) to adult broiler breeder chickens was demonstrated at 3 WPP. MS transmission occurred at a low level; only 6.7 - 20% of K6191C exposed chickens and 0 – 6.7% of K6315D exposed chickens tested positive by real-time PCR at 3 WPP, and MS DNA levels in infected groups were low (MCN $\log_{10}$, 0.1 – 0.3 for K6191C and 0.1 for K6315D). MS DNA was not detected in any tracheal samples collected at necropsy (5 WPP); these results were confirmed by repeated DNA extraction and testing. Low-level PCR positivity was not unexpected, given the low DNA copy numbers, and delayed detection of MS in chickens exposed to fomites naturally contaminated with K6191C and K6315D in previous studies (4, 5). Fomite transmission of K6191C was previously only detected by real-time PCR at 7 WPP (4); while in another study, fomite-transmitted K6315D was first detected at 5 WPP (5). The reasons for the negative PCR results at 5 WPP in the current study in groups that previously tested positive for MS DNA are not clear, but we suspect that large amounts of mucus and some feed present in the tracheal samples at necropsy caused PCR inhibition in these

samples, resulting in negative PCR results in samples which may have contained low concentrations of MS DNA. Components of respiratory secretions and various foods have been reported to inhibit PCRs targeting a number of different organisms (29).

The chickens remained serologically negative for MS for the duration of the study, and MS was not isolated from any tracheal samples at necropsy. Delayed serologic responses to MS infection have been reported for chickens inoculated with MS or contact-exposed to MS infected chickens; Ewing *et al*. (1998) reported positive SPA results 3-4 weeks after positive PCR and/or culture results, and no HI positivity for up to 6 wks in chickens commingled with MS-infected chickens (8). A lack of seroconversion 5-6 weeks after detection of MS DNA was previously found in chickens exposed to fomites from MS-infected seeder pens (4, 5). *A. laidlawii* infection of fourteen K6191C and two K6315D fomite-exposed chickens and contaminant overgrowth in eight K6191C and three K6315D cultures may have interfered with MS isolation in the current study. The lack of MS isolation from PCR positive chickens may also be a consequence of very low-level tracheal colonization. Marois *et al.* (2005) reported that MS was first detected by isolation 33 days after placement of chicks in contaminated isolators, and 54 days after exposure of chicks to material from an MS positive laying farm (17). It is possible that MS organism and antibodies would have been detected had the study been extended; unfortunately, this was not possible for logistical reasons.

Fomite transmission of K6191C was more effective than that of K6315D, with MS detected in 11.4% of K6191C-exposed chickens, compared with only 2.3% of K6315D-exposed chickens at 3 WPP. This is in contrast with the results of a previous study, in which K6191C fomite-exposed chickens remained negative; whereas 90% of

K6315D fomite-exposed chickens tested positive for low levels of MS DNA at 5 WPP (5). In that study, broiler breeder chickens were exposed to fomites transferred from pens in which seeder chickens had been infected with MS; the fomites were not directly inoculated with MS cultures as in the current study. It is therefore possible that fomites transferred from the K6191C seeder pens in that study were not infected with sufficient MS organisms to facilitate the fomite transmission of K6191C (5).

Cotton fabric was the most effective fomite for MS transmission; 3/5 K6191C- and 1/1 K6315D-positive chickens had been exposed to inoculated cotton fabric. Plastic and feathers were less effective as fomites for MS transmission (1/5 K6191C-positive chickens had been exposed to either plastic or feather fomites); however, these differences were not statistically significant ($P > 0.05$). It was expected that the efficiency of the materials as fomites for MS would correlate with previously reported *Mycoplasma* survival times (feathers, 3 days; cotton fabric, 2 days; plastic, 1 day) (7); however, this was not the case. These differences could be attributed to differences in study design (e.g. different MS strains, different types and preparation of fomite materials, different suspension media), but probably indicate that indirect transmission of MS is a function not only of the ability of the organism to survive on the fomite substrate, but also of its ability to transmit from the fomite to the susceptible chicken.

MS transmission from the infected fomite to a susceptible chicken requires contact between the chicken and the fomite. In order to facilitate optimum bird contact with the fomites, and to reduce variability in the attractiveness of fomites to birds, the fomites used in the plastic and cotton fabric exposure pens were identical in shape, color and distribution within the pens. Clear plastic boot covers placed over the cotton fabric

boot covers served as the plastic fomites. Red cotton fabric was selected to cover rubber boots for both the cotton fabric and plastic fomites because previous studies have reported that chicks exhibit a preference for red objects (10, 12).

In conclusion, a novel infection model was used to compare the potential of two genotypically distinct MS isolates, K6191C and K6315D, to transmit from infected cotton fabric, plastic and feathers to sexually mature broiler breeder chickens. Low-level MS transmission was detected; K6191C transmitted more effectively from infected fomites than K6315D, and the highest infection levels occurred in groups exposed to cotton fabric fomites. Fomite transmission of MS is likely to be more rapid in younger chickens and under commercial conditions, with environmental stressors and concurrent infection with other respiratory pathogens. Future studies using this infection model should include some of these variables, as well as a larger infection dose and longer study duration. Our results highlight the potential of diverse materials to act as fomites facilitating the indirect transmission of MS, and emphasize the importance of adequate biosecurity to prevent MS fomite transmission.

## Acknowledgements

The authors would like to thank Dr. Margie Lee, for suggesting the use of mucin in this infection model, and Mrs. Ruth Wooten, Mr. Patrick Black and Dr. Vijay Durairaj for their assistance with this study.

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Table 5.1. Detection of MS antibodies, live organism and genomic DNA in negative control and K6191C and K6315D sentinel, plastic boot cover, cotton fabric and feather contact groups at 3 and 5 WPP.[A]

| WPP | Group | MS isolate | SPA[BC] | HI[BD] | MS isolation[BEF] (trachea) | Real-time qPCR[BGH] |
|---|---|---|---|---|---|---|
| 3 | Negative control | - | 0/10 (0.0) | 0/10 (0.0) | ND | 0/10 (0.0) |
| | K6191C Sentinel | - | 0/15 (0.0) | 0/15 (0.0) | ND | 0/15 (0.0) |
| | Plastic | K6191C | 0/15 (0.0) | 0/15 (0.0) | ND | 1/15 (0.1 ± 0.2) |
| | Cotton fabric | K6191C | 0/15 (0.0) | 0/15 (0.0) | ND | 3/15 (0.3 ± 0.7) |
| | Feather | K6191C | 0/14 (0.0) | 0/14 (0.0) | ND | 1/14 (0.1 ± 0.4) |
| | K6315D Sentinel | - | 0/15 (0.0) | 0/15 (0.0) | ND | 0/15 (0.0) |
| | Plastic | K6315D | 0/15 (0.0) | 0/15 (0.0) | ND | 0/15 (0.0) |
| | Cotton fabric | K6315D | 0/15 (0.0) | 0/15 (0.0) | ND | 1/15 (0.1 ± 0.3) |
| | Feather | K6315D | 0/14 (0.0) | 0/14 (0.0) | ND | 0/14 (0.0) |
| 5 | Negative control | - | 0/10 (0.0) | 0/10 (0.0) | 0/10 | 0/10 (0.0) |
| | K6191C Sentinel | - | 0/14 (0.0) | 0/14 (0.0) | 0/14 | 0/14 (0.0) |
| | Plastic | K6191C | 0/15 (0.0) | 0/15 (0.0) | 0/13 | 0/15 (0.0) |
| | Cotton fabric | K6191C | 0/15 (0.0) | 0/15 (0.0) | 0/14 | 0/15 (0.0) |
| | Feather | K6191C | 0/13 (0.0) | 0/13 (0.0) | 0/8 | 0/13 (0.0) |
| | K6315D Sentinel | - | 0/15 (0.0) | 0/15 (0.0) | 0/14 | 0/15 (0.0) |
| | Plastic | K6315D | 0/15 (0.0) | 0/15 (0.0) | 0/15 | 0/15 (0.0) |
| | Cotton fabric | K6315D | 0/15 (0.0) | 0/15 (0.0) | 0/15 | 0/15 (0.0) |
| | Feather | K6315D | 0/12 (0.0) | 0/12 (0.0) | 0/10 | 0/12 (0.0) |

[A] Values within a column at a specific time period with a different lowercase, superscripted letter are significantly different ($P \leq 0.05$).
[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥1:40).
[C] Mean agglutination grade (from 0 to 4).
[D] Mean titer $\log_{10}$.
[E] ND = not done.
[F] Culture results for contaminated cultures are excluded.
[G] Mean DNA copy no. $\log_{10}$ ± SD.
[H] Samples tested were choanal cleft swabs at 3 WPP and tracheal washes at 5 WPP.

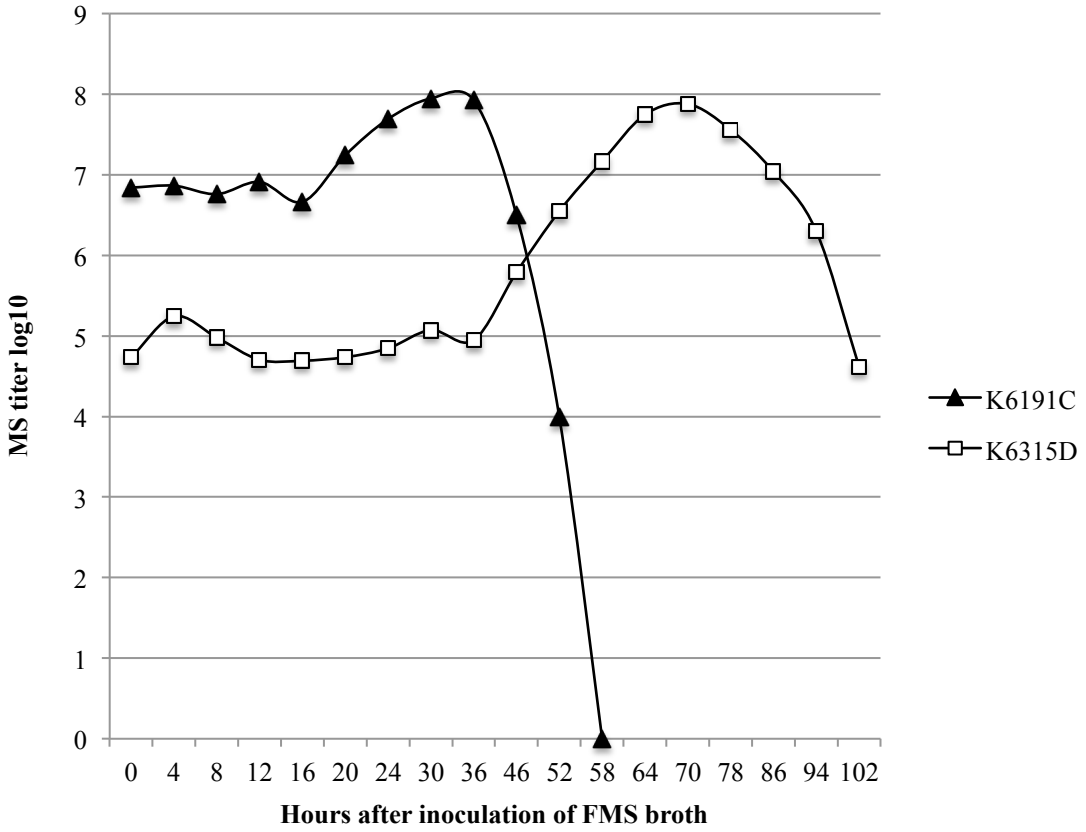


Figure 5.1. MS K6191C and K6315D growth curves in FMS broth

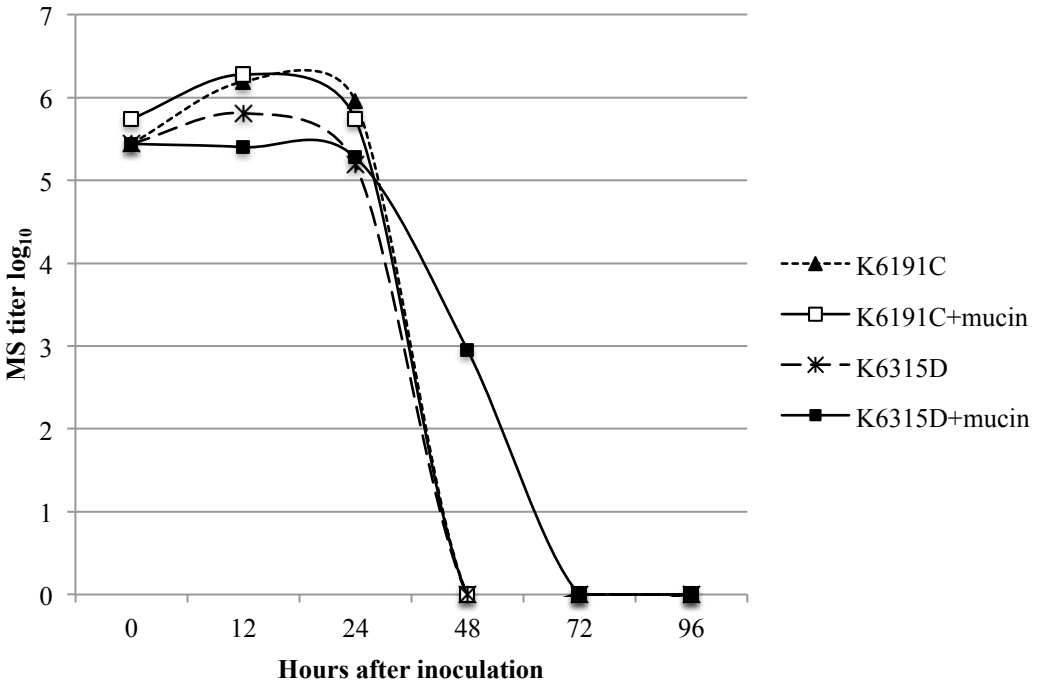


Figure 5.2. Survivability of K6191C and K6315D MS cultures with and without mucin on plastic petri dish surfaces.

# CHAPTER 6

# DISCUSSION AND CONCLUSIONS

*Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) are significant pathogens of commercial poultry, which are capable of both horizontal and vertical transmission (16, 40). PCR and sequencing of specific genomic targets has been successfully used to elucidate the molecular epidemiology of MG and MS, facilitating strain differentiation, outbreak investigations and the development of sequence databases (15, 19, 22-25, 27, 38, 39, 46). Targeted genetic sequencing was instrumental in investigating recent cases of suspected reversion to virulence and vertical transmission of the live MG vaccine ts-11 in Georgia (12), and an unusually severe outbreak of MS in Arkansas (5). A number of studies have contributed to current knowledge of the vertical transmission of MG; these studies have shown that that the transmission rate is highest when levels of MG in the respiratory tract peak, decreases with time (14, 20, 28, 29) and is highly variable, depending on factors such as inoculation strain, timing and route of inoculation and previous MG exposure (20, 21, 29, 37, 41). The horizontal transmission of MS is generally more rapid than that of MG (16), and has been demonstrated to occur by direct contact with infected chickens (13, 26, 30, 36, 48), and by indirect contact with contaminated material (32), and is facilitated by the ability of MS to survive for up to several days on contaminated materials (2, 10, 31, 43).

In Chapter 2, an MG sequence database, developed by sequencing of previously identified genomic targets, was used to investigate the epidemiology of MG in South Africa, and to identify genomic targets facilitating optimum strain differentiation. MG and MS genotypes characterized by targeted sequence analysis were next evaluated to elucidate their vertical and horizontal transmission characteristics respectively (Chapters 3-5). In Chapter 3, the egg transmission potential and pathogenicity of ts-11 vaccine and ts-11-like isolates from vaccinated broiler breeders and their progeny in northeastern Georgia were investigated. The horizontal transmission of two MS genotypes isolated from the outbreaks in Arkansas was evaluated in sexually-mature broiler breeder chickens in Chapter 4, and an infection model was designed to evaluate the ability of these genotypes to transmit on contaminated fomite materials in Chapter 5.

A model for the development and application of an MG sequence database was presented in Chapter 2 (4). A database of South African MG sequences was developed by targeted sequencing of four previously identified genomic targets; the 16S-23S rRNA intergenic spacer region (IGSR) (39) and the MG cytadhesin 2 (*mgc2*), MGA_0319 and *gapA* genes (15, 18). Twelve unique MG genotypes were identified, originating from commercial poultry samples from seven South African provinces collected over a four year period between 2009 and 2012. The most widespread genotype (SA-WT#7), which was present in 43% of the samples analyzed, was detected in samples from broilers, broiler breeders and layers from five provinces.

The South African genotypes were distinct from all other MG genotypes in a database comprised of sequences from multiple countries. Phylogenetic analysis of the genotypes from South Africa and from other countries revealed distinct geographical

clustering, which is consistent with the correlation of epidemiologically-linked isolates previously reported for sequencing of multiple genomic targets (15).

The similarity of the South African wild-type genotypes to each other and to the available live vaccine sequences necessitated IGSR/*mgc2*/MGA_0319 sequencing and IGSR/*mgc2* sequencing to differentiate these genotypes from each other and from the live vaccine strains respectively. The sequence similarity of the South African MG genotypes to commonly used vaccine strains, particularly ts-11, suggested that horizontal gene transfer or homologous recombination may have occurred between field and vaccinal MG strains. The South African genotypes were readily differentiated from the live F strain vaccine, which is not registered for use in South Africa; facilitating differentiation from field strains should F strain vaccine be introduced in the future.

Sequencing of the *mgc2* gene provided the best discriminatory power for strain differentiation of the South African genotypes, followed by IGSR and MGA_0319; while *gapA* was dispensible for strain differentiation. A previous study reported that higher discriminatory power was achieved by sequencing of IGSR than by *mgc2* sequencing (39). The superior efficacy of *mgc2* genotyping for differentiation of South African genotypes may be related to the specific deletions of this gene in some South African genotypes, resulting in increased variability of this target. This highlights the importance of developing national sequence databases to identify genomic targets which will facilitate optimum strain differentiation for particular geographic regions.

Chapter 2 is the first report on the diversity and distribution of MG wild-types in South Africa (4). This research can serve as a model to guide the development of other

MG sequence databases, and illustrates the application of these databases to characterize MG genotypes, select diagnostic tests and better understand the epidemiology of MG.

The application of targeted genetic sequencing was also instrumental in investigating the epidemiology of the first field cases of apparent reversion to virulence and vertical transmission of the ts-11 vaccine, which occurred recently in northeastern Georgia (12). “ts-11-like” MG isolates, which could not be distinguished from ts-11 vaccine by the genotyping methods used, were isolated from broiler breeders previously vaccinated with ts-11, and their clinically-ill broiler progeny. A ts-11-like isolate from broilers (K6216D) was characterized as significantly more pathogenic than ts-11 vaccine ($P \leq 0.05$) (12). A study performed to evaluate the egg transmission and pathogenicity of K6216D, K6222B (a ts-11-like isolate from the broiler breeder parent flock of these broilers) and ts-11 vaccine is presented in Chapter 3.

The ts-11 vaccine, one of the most widely used live MG vaccines worldwide, is recognized as a strain of low or no virulence (52), and has been demonstrated to provide protection against respiratory disease, egg production drops and vertical transmission associated with virulent MG infection (1, 7, 49, 51). ts-11 was produced by chemical mutagenesis of a virulent Australian MG field isolate, and selected for its temperature sensitive ($ts^+$) phenotype (growth at 33°C) (45, 52). The ts-11 vaccine’s lack of virulence is not contingent on the $ts^+$ phenotype (49); the genetic basis for its attenuation has not been fully elucidated.

K6216D transmitted via the egg at a rate of 4% in the third and fourth weeks after inoculation of the hens, but no egg transmission of K6222B or of ts-11 vaccine was detected. K6216D and K6222B induced respiratory signs and significantly more tracheal

and airsac/ovary/oviduct colonization and more severe tracheal and airsac lesions than ts-11 vaccine ($P \leq 0.05$), which was apathogenic to birds in this study. There were no substantial differences in the egg production of ts-11, K6216D and K6222B infected groups. Transovarian transmission of K6222B was not detected, despite the fact that the virulence and invasiveness of this isolate was statistically similar to that of K6216D ($P > 0.05$), which did transmit via the egg. These results suggested that, while virulence and invasiveness appear to be required for egg transmission, additional factors may be necessary to facilitate infection of the embryo.

Egg transmission of ts-11 vaccine was not detected in this study, despite the application of a high-dose, aerosol inoculum, and the testing of all settable egg laid for 6 weeks after infection. Detection of low-level egg transmission is limited by the number of eggs that can practically be tested. In addition, the evaluation of virulence characteristics which may have uniquely affected particular vaccine lots (e.g. due to virulent sub-populations) was not possible because the vaccine lots administered in northeastern Georgia between 2008 and 2011 were no longer available. Another factor which may have affected our ability to detect egg transmission of ts-11 was the length of the study. In the field cases described, vertical transmission was suspected of having occurred approximately 6 months after vaccination (12); this period may have been required for processes such as bird-to-bird transmission, back passage, selection of sub-populations and genetic mutations to occur. Other variables that may have precipitated egg transmission which were not evaluated in this study include concurrent respiratory infections and uneven vaccine application, which could potentially facilitate vaccine back-passage and increased virulence.

Whithear (1996) did not detect egg transmission of ts-11 following intra-abdominal or intraocular inoculation of hens in production (49). In another study, MG was detected in the eggs of ts-11 vaccinated hens, although vertical transmission could not be confirmed due to the possibility of contamination (49). The isolation of an MG strain from ts-11 vaccinated layers in Australia, which had an identical restriction endonuclease analysis banding pattern to ts-11 and its parent strain 80083, and which was intermediate in virulence between ts-11 and 80083 has been reported (50). Considering the extensive use of ts-11 and the paucity of reports indicating the possibility of reversion to virulence and/or egg transmission, egg transmission of ts-11 is probably a rare event.

The results presented in Chapter 3 provide the first conclusive evidence of transovarian transmission of an MG isolate of the ts-11 genotype, and indicate the existence of a spectrum of virulence and egg transmission potential for ts-11-like isolates. Analysis of the full genome sequences of ts-11 vaccine and several ts-11-like isolates is ongoing, to investigate whether the genetic relatedness of ts-11 vaccine and the ts-11-like isolates supports their epidemiological links; and to identify the genetic changes which may have precipitated reversion to virulence and vertical transmission of ts-11 vaccine.

The study detailed in Chapter 4 was performed to evaluate the horizontal transmission of two MS isolates, K6191C and K6315D (belonging to the genotypes designated S-10 and S-17 respectively), from an MS outbreak in the state of Arkansas. The outbreak was characterized by severe respiratory disease, mortality and airsacculitis condemnations in broilers, and egg production drops and mortality in breeders, and by evidence of extensive vertical and horizontal transmission. In a previous study in broiler breeder pullets, the S-10 isolate was demonstrated to be significantly more pathogenic

than the S-17 isolate ($P \leq 0.05$), and similar in transmissibility to the pathogenic reference strain K1968 (5). The objectives of the study reported in Chapter 4 were to evaluate and compare the transmissibility of S-10 and S-17 in sexually mature broiler breeders, and to assess the effect of prophylactic tylosin medication on MS transmission.

The rate of S-10 and S-17 transmission appeared to be similar, as both genotypes were detected in secondarily infected chickens 6 weeks after inoculation of the seeders. The pattern of S-10 and S-17 transmission differed, however; S-10 transmitted to adjacent contact chickens and S-17 to fomite contact chickens. It was hypothesized that the comparatively effective fomite transmission of S-17 could be as a result of longer survival time of S-17 on the fomite surface. Strains differences in MS survival times on various substrates have been reported (10, 33, 43).

MS was not detected in S-17 fomite contact chickens which were medicated with tylosin, suggesting the value of prophylactic tylosin medication in preventing low level MS transmission. Medication with tylosin has been reported to reduce tracheal counts of MG (11), but not to prevent infection. S-10 transmission to downwind contacts occurred in a previous study in pullets (5) but was not detected in the sexually mature broiler breeders in this study, suggesting possible age-related differences in airborne MS transmission.

Quantitative PCR (qPCR) analyis indicated that S-10 replicated more rapidly and to substantially higher levels in the upper respiratory tracts of directly and indirectly infected chickens than S-17. S-17 was also significantly less immunogenic than S-10, based on serum antibody titers in directly and indirectly infected chickens ($P \leq 0.05$). The existence of MS strains of low immunogenicity but comparable transmission rates to

virulent strains is supported in other research (13). The detection of S-10 transmission from the infected seeder pens 4 weeks after peak MS DNA copy numbers in the upper respiratory tracts of the seeder chickens in this study and in a previous study (5), demonstrates an apparent association between MS infection level and transmission.

In summary, Chapter 4 details the first experimental evaluation of the horizontal transmission of MS in sexually mature broiler breeder chickens. The results provide evidence for unique transmission patterns of different MS strains, and indicate that the transmission rate of an MS strain may be independent of its virulence, immunogenicity and *in vivo* replication rate.

The study described in Chapter 5 was performed to develop an infection model to further investigate the fomite transmission of the S-10 and S-17 genotypes of MS in sexually mature broiler breeder chickens. Feathers, cotton fabric and plastic bootcovers were evaluated for their potential to serve as fomites, based on their frequent presence in the poultry house environment.

An evaluation of S-10 and S-17 growth in Frey's modified broth medium revealed the markedly different replication kinetics of these genotypes. While peak titers were similar, S-10 titers peaked 40 hours before S-17 titers. Interestingly, the differential growth kinetics of S-10 and S-17 *in vitro* mirror their *in vivo* colonization of the upper respiratory tract reported in Chapter 4 (6). These results further highlight the importance of constructing growth curves to obtain high-titered, equivalent inoculation titers for strains with substantially different replication kinetics.

Based on the hypothesis that the consistency and composition of avian respiratory secretions could facilitate the survival of MS in the environment, the feasibility of adding

mucin to the inoculation cultures was evaluated. The use of mucin to simulate saliva in transmission, survival and gene expression studies with several human respiratory pathogens was previously reported (3, 17, 42, 47). In one study, the survival of MG appeared to be extended on paper discs inoculated with mucin-containing media (35). Mucin did not negatively impact media pH and sterility or MS survivability on a plastic substrate at an inclusion level of at 5 mg/ml. The substrate survival of K6315D was, in fact, extended by 24 hours by the addition of mucin to the culture medium, possibly because the K6315D organisms were protected from desiccation.

Low-level MS transmission (6.7% - 20.0%) from K6315D-inoculated cotton fabric and from K6191C-inoculated feather, cotton fabric and plastic fomites was detected at 3 weeks, but not at 5 weeks post fomite placement by real-time PCR, but not by culture or serology. The inability to detect MS infection by culture or serology in this study was likely a consequence of very low-level infection, while the presence of inhibitors may have affected MS DNA detection at necropsy. Extended study durations may be required to facilitate the replication of MS to detectable levels in the respiratory tract (32). Fomite transmission of K6191C appeared to be more effective than that of K6315D, and MS was detected in more chickens exposed to cotton fabric fomites than to either feather or plastic fomites, although these results were not statistically significant ($P > 0.05$).

The less efficient fomite transmission of K6315D is in contrast to its extended survival on a plastic substrate in a mucin-containing medium, and to its fomite transmission ability demonstrated in Chapter 4. The fomite transmission of MS is probably a function of multiple variables; including fomite material (10, 33, 43), MS

strain (13, 48), suspension medium (10), organism density on the fomite, biofilm formation (9, 34), environmental conditions (temperature, humidity and pH) (8, 16, 35, 43, 44), bird contact with the fomite (e.g. influenced by stocking density, fomite surface area and attractiveness), bird susceptibility to infection (e.g. influenced by age, stressors and concurrent respiratory and immunosuppressive disease), as well as other unidentified factors influencing the ability of the organism to physically transmit from the fomite to a susceptible chicken.

The results presented in Chapter 5 demonstrate the application of mucin in a *Mycoplasma* infection model, and provide the first experimental evidence for MS transmission from inoculated fomite materials to sexually mature chickens. Fomite transmission of MS in future studies with this infection model may be enhanced by the use of younger chickens, concurrent viral respiratory infection, larger MS infection doses and longer study durations.

In conclusion, an MG sequence database was developed as a model to facilitate epidemiological investigations (Chapter 2). The vertical transmission and virulence of MG isolates of the ts-11 genotype (Chapter 3) and the horizontal transmission of MS isolates of the S-10 and S-17 genotypes (Chapter 4) were evaluated, and an infection model was developed to faciliate the evaluation of MS fomite transmission (Chapter 5). These studies have improved our understanding of the molecular epidemiology and transmission of avian mycoplasma genotypes in commercial poultry, and provide a foundation for future research.

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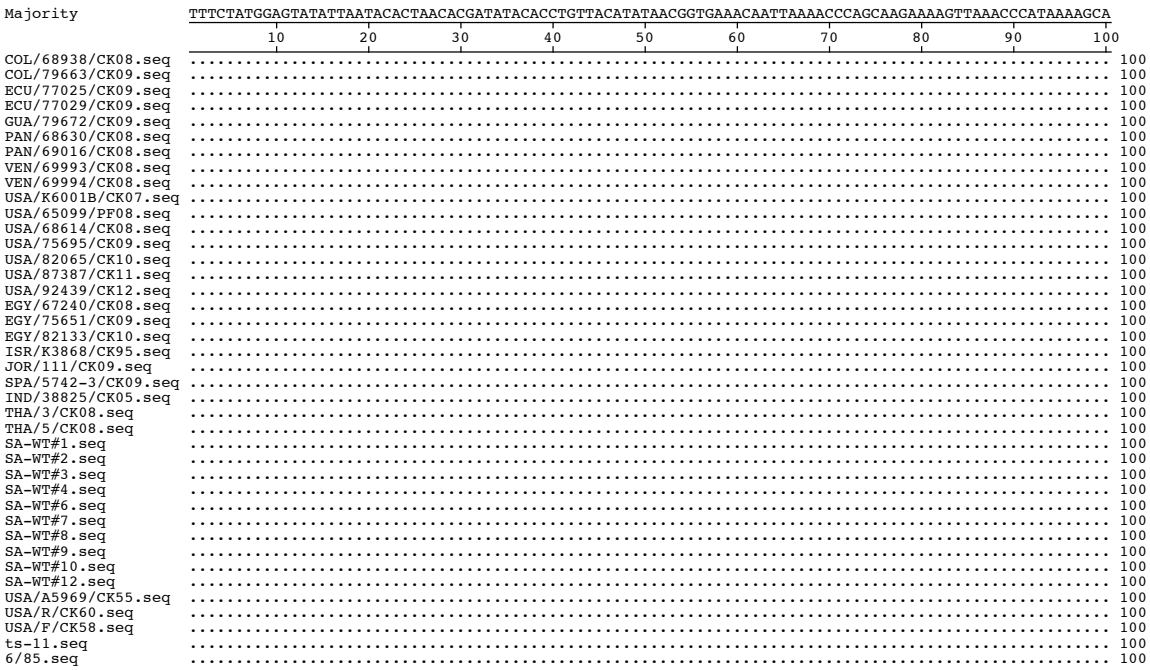
# APPENDIX A

## SUPPLEMENTARY FIGURES 1 TO 5

(Supplement to Chapter 2)

Supplementary Figure 1. Clustal-W alignment of the IGSR nucleotide sequences of South African *M. gallisepticum* wild-types, international isolates and vaccine and reference strains. Nucleotides identical to the consensus sequence or absent are represented by '.' or '-' respectively.



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Majority            AATTGATAACTTAAAAATTGTTGGTCGGATTCTATTCAGTTCTCAAGGGGTATTTTAAAAGCAGTTATTAAGTTTTTTT--CTTTAAAGCTTTTGAAACT
                              110       120       130       140       150       160       170       180       190       200
COL/68938/CK08.seq  ..........................................................T.................--..................... 198
COL/79663/CK09.seq  ..........A.............................A.....................................--................C... 198
ECU/77025/CK09.seq  ..........A.............................A.....................................--................C... 198
ECU/77029/CK09.seq  ..........A.....................................................................--................C... 198
GUA/79672/CK09.seq  ..........................................................................TT..............-... 199
PAN/68630/CK08.seq  ..........A.....................................................................--................-... 197
PAN/69016/CK08.seq  ..........A.....................................................................--................C... 198
VEN/69993/CK08.seq  ..........A.....................................................................--................C... 198
VEN/69994/CK08.seq  ..........A.....................................................................--................C... 198
USA/K6001B/CK07.seq ..........................................................................--..................-.. 197
USA/65099/PF08.seq  ..........................................................................--..................-... 197
USA/68614/CK08.seq  ..........................................................................--..................-... 197
USA/75695/CK09.seq  ..........................................................................--..................-... 197
USA/82065/CK10.seq  ..........................................................................--..................-... 197
USA/87387/CK11.seq  ..........................................................................--..................-... 197
USA/92439/CK12.seq  ..........................................................................---.................T..-.. 196
EGY/67240/CK08.seq  ..........................................................T.................--..................... 198
EGY/75651/CK09.seq  ..........................................................T.................--..................... 198
EGY/82133/CK10.seq  ..........................................................T.................--..................... 198
ISR/K3868/CK95.seq  ..................................................A.....T.................--..................... 198
JOR/111/CK09.seq    ..................................................A.....T.................--..................... 198
SPA/5742-3/CK09.seq ..........................................................T.................--..................-... 197
IND/38825/CK05.seq  ..........................................................T.................--..................-... 197
THA/3/CK08.seq      ..........................................................T.................--..................... 198
THA/5/CK08.seq      ..........................................................T.................--..................... 198
SA-WT#1.seq         ..........................................................................---.................T..-.. 196
SA-WT#2.seq         ..........................................................................---.................T..-.. 196
SA-WT#3.seq         ..........................................................T.................--..................... 198
SA-WT#4.seq         ..........................................................T.................--..................... 198
SA-WT#6.seq         ..........................................................................---.................T..-.. 196
SA-WT#7.seq         ..........................................................................---.................T..-.. 196
SA-WT#8.seq         ..........................................................................---.................T..-.. 196
SA-WT#9.seq         ..........................................................T.................--..................... 198
SA-WT#10.seq        ..........................................................T.................--..................... 198
SA-WT#12.seq        ..........................................................................---.................T..-.. 196
USA/A5969/CK55.seq  ..........................................................T.................--..................-... 197
USA/R/CK60.seq      ..........................................................................---.................T..-.. 196
USA/F/CK58.seq      ..........A.....................................................................--................C... 198
ts-11.seq           ..........................................................T.................--..................... 198
6/85.seq            ..........................................................T.................--..................... 198
```

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Majority            TAATAACACTTTAAGTTAACCTT-GTXAAXATCATTAAGTTTACCTTTCAAAGATCAAAGAACAATTATTAAAACCTTTTTGATCAGATAACTAATAAAC
                              210       220       230       240       250       260       270       280       290       300
COL/68938/CK08.seq  ......................-..T..G........T.....................G...........T......CG..T.............. 297
COL/79663/CK09.seq  ......................A..A..C.....................G.....................T.....................  298
ECU/77025/CK09.seq  ......................A..A..C.....................G.....................T.....................  298
ECU/77029/CK09.seq  ......................A..A..C.....................................T.....................  298
GUA/79672/CK09.seq  ......................-..A..C.....................................................  298
PAN/68630/CK08.seq  ......................A..A..C.......T.....................................T.....................  297
PAN/69016/CK08.seq  ......................A..A..C.....................................T.....................  298
VEN/69993/CK08.seq  ......................A..A..C.....................................T.....................  298
VEN/69994/CK08.seq  ......................A..A..C.....................................T.....................  298
USA/K6001B/CK07.seq ......................-..A..C..T..................................  296
USA/65099/PF08.seq  ......................-..A..C..T..................................  296
USA/68614/CK08.seq  ......................-..A..C..T..................................  296
USA/75695/CK09.seq  ......................-..A..C..T..................................  296
USA/82065/CK10.seq  ......................-..A..C..T..................................  296
USA/87387/CK11.seq  ......................-..A..C..T..................................  296
USA/92439/CK12.seq  ......................-..A..C.....................C...............  295
EGY/67240/CK08.seq  ......................-..T..G........T.....................G...........T......C...T.....  297
EGY/75651/CK09.seq  ......................-..T..G........T.....................G...........T......C...T.....  297
EGY/82133/CK10.seq  ......................-..T..G........T.....................G...........T......C...T.....  297
ISR/K3868/CK95.seq  ......................-A.T..G........T.....................G...........T.....CC...T.....  297
JOR/111/CK09.seq    ......................-A.T..G........T.....................G...........T.....CC...T.....  297
SPA/5742-3/CK09.seq ......................-..A..C.....................G.....................  296
IND/38825/CK05.seq  ......................-..A..C.....................G.....................  296
THA/3/CK08.seq      ......................-..T..G........T.....................G...........T......CG..T.....  297
THA/5/CK08.seq      ......................-..T..G........T.....................G...........T......CG..T.....  297
SA-WT#1.seq         ......................-..T..G.....................C.....  295
SA-WT#2.seq         ......................-..T..G.....................C.....  295
SA-WT#3.seq         ......................-..T..G........T.....................G...........T......C...T.....  297
SA-WT#4.seq         ......................-..T..G........T.....  297
SA-WT#6.seq         ......................-..T..G.....................C.....  295
SA-WT#7.seq         ......................-..T..G.....................C.....  295
SA-WT#8.seq         ......................-..T..G.....................-.....  294
SA-WT#9.seq         ......................-..T..G........T.....  297
SA-WT#10.seq        ......................-..T..G........T.....................G...........T......C...T.....  297
SA-WT#12.seq        ......................-..T..G.....................C.....  295
USA/A5969/CK55.seq  ......................-..A..C.....................G.....  296
USA/R/CK60.seq      ......................-..A..C.....................C.....................G.....  295
USA/F/CK58.seq      ......................A..A..C.....................................T.....  298
ts-11.seq           ......................-..T..G........T.....  297
6/85.seq            ......................-..T..G........T.....................C.....  297
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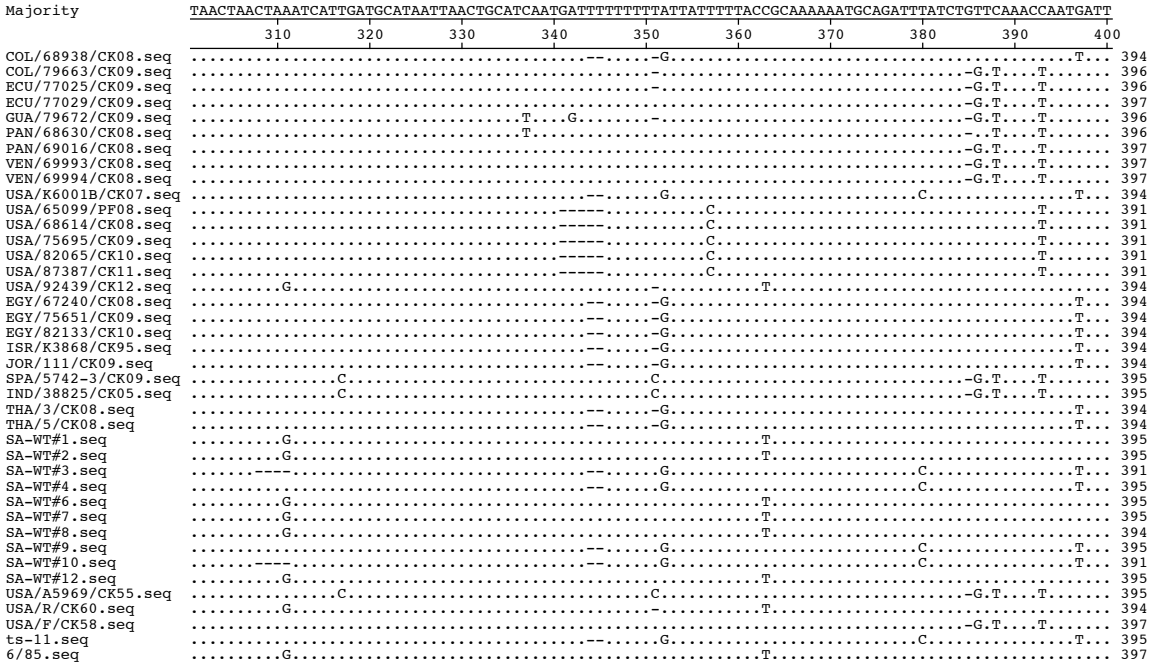
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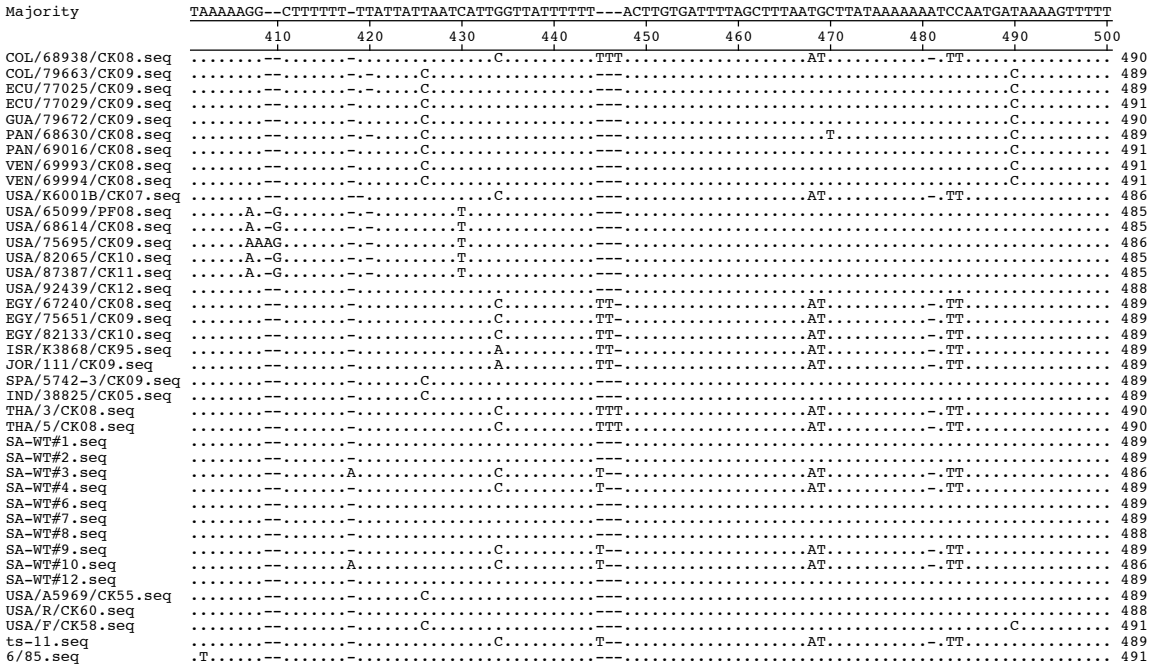
COL/79663/CK09.seq 396
ECU/77025/CK09.seq 396
ECU/77029/CK09.seq 397
GUA/79672/CK09.seq 396
PAN/68630/CK08.seq 396
PAN/69016/CK08.seq 397
VEN/69993/CK08.seq 397
VEN/69994/CK08.seq 397
USA/K6001B/CK07.seq 394
USA/65099/PF08.seq 391
USA/68614/CK08.seq 391
USA/75695/CK09.seq 391
USA/82065/CK10.seq 391
USA/87387/CK11.seq 391
USA/92439/CK12.seq 394
EGY/67240/CK08.seq 394
EGY/75651/CK09.seq 394
EGY/82133/CK10.seq 394
ISR/K3868/CK95.seq 394
JOR/111/CK09.seq 394
SPA/5742-3/CK09.seq 395
IND/38825/CK05.seq 395
THA/3/CK08.seq 394
THA/5/CK08.seq 394
SA-WT#1.seq 395
SA-WT#2.seq 395
SA-WT#3.seq 391
SA-WT#4.seq 395
SA-WT#6.seq 395
SA-WT#7.seq 395
SA-WT#8.seq 394
SA-WT#9.seq 395
SA-WT#10.seq 391
SA-WT#12.seq 395
USA/A5969/CK55.seq 395
USA/R/CK60.seq 394
USA/F/CK58.seq 397
ts-11.seq 395
6/85.seq 397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COL/68938/CK08.seq 490
COL/79663/CK09.seq 489
ECU/77025/CK09.seq 489
ECU/77029/CK09.seq 491
GUA/79672/CK09.seq 490
PAN/68630/CK08.seq 489
PAN/69016/CK08.seq 491
VEN/69993/CK08.seq 491
VEN/69994/CK08.seq 491
USA/K6001B/CK07.seq 486
USA/65099/PF08.seq 485
USA/68614/CK08.seq 485
USA/75695/CK09.seq 486
USA/82065/CK10.seq 485
USA/87387/CK11.seq 485
USA/92439/CK12.seq 488
EGY/67240/CK08.seq 489
EGY/75651/CK09.seq 489
EGY/82133/CK10.seq 489
ISR/K3868/CK95.seq 489
JOR/111/CK09.seq 489
SPA/5742-3/CK09.seq 489
IND/38825/CK05.seq 489
THA/3/CK08.seq 490
THA/5/CK08.seq 490
SA-WT#1.seq 489
SA-WT#2.seq 489
SA-WT#3.seq 486
SA-WT#4.seq 489
SA-WT#6.seq 489
SA-WT#7.seq 489
SA-WT#8.seq 488
SA-WT#9.seq 489
SA-WT#10.seq 486
SA-WT#12.seq 489
USA/A5969/CK55.seq 489
USA/R/CK60.seq 488
USA/F/CK58.seq 491
ts-11.seq 489
6/85.seq 491

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Majority                TT---AGAAAAGTGTTGTGCAAACTGATTTGTTGTGCTATAATCTTTAAGGCTTATATAGCTACATTGTTCTTTGAAAACTGAATACGACAAATCTTTCT
                                510       520       530       540       550       560       570       580       590       600
COL/68938/CK08.seq      ..T--..........................................................................................  588
COL/79663/CK09.seq      ..---.....................................A...........A.....................................  586
ECU/77025/CK09.seq      ..---.....................................A...........A.....................................  586
ECU/77029/CK09.seq      ..---.....................................A...........A.....................................  588
GUA/79672/CK09.seq      ..T--.....................................A...........A.....................................  588
PAN/68630/CK08.seq      ..---.....G...............................A...........A.....................................  586
PAN/69016/CK08.seq      ..---.....................................A...........A.....................................  588
VEN/69993/CK08.seq      ..---.....................................A...........A.....................................  588
VEN/69994/CK08.seq      ..---.....................................A...........A.....................................  588
USA/K6001B/CK07.seq     ..TTT.........................................................................................  586
USA/65099/PF08.seq      ..---.....................................A...........A.....................................  582
USA/68614/CK08.seq      ..---.....................................A...........A.....................................  582
USA/75695/CK09.seq      ..---.....................................A...........A.....................................  583
USA/82065/CK10.seq      ..T--.....................................A...........A.....................................  583
USA/87387/CK11.seq      ..T--.....................................A...........A.....................................  583
USA/92439/CK12.seq      ..---.....................................A...........A.....................................  585
EGY/67240/CK08.seq      ..---.....................................A...........A.....................................  586
EGY/75651/CK09.seq      ..---.....................................A...........A.....................................  586
EGY/82133/CK10.seq      ..---.....................................A...........A.....................................  586
ISR/K3868/CK95.seq      ..T--.....................................A...........A.....................................  587
JOR/111/CK09.seq        ..---.....................................A...........A.....................................  586
SPA/5742-3/CK09.seq     ..---.............A.......................A...........A.....................................  586
IND/38825/CK05.seq      ..---.............A.......................A...........A.....................................  586
THA/3/CK08.seq          ..T--.....................................A...........A.....................................  588
THA/5/CK08.seq          ..T--.....................................A...........A.....................................  588
SA-WT#1.seq             ..---.....................................A...........A.....................................  586
SA-WT#2.seq             ..---.....................................A...........A.....................................  586
SA-WT#3.seq             ..T--.....................................A...........A.....................................  584
SA-WT#4.seq             ..T--.....................................A...........A.....................................  587
SA-WT#6.seq             ..---.....................................A...........A.....................................  586
SA-WT#7.seq             ..---.....................................A...........A.....................................  586
SA-WT#8.seq             ..---.....................................A...........A.....................................  585
SA-WT#9.seq             ..T--.....................................A...........A.....................................  587
SA-WT#10.seq            ..T--.....................................A...........A.....................................  584
SA-WT#12.seq            ..---.....................................A...........A.....................................  586
USA/A5969/CK55.seq      ..---.............A.......................A...........A.....................................  586
USA/R/CK60.seq          ..---.....................................A...........A.....................................  585
USA/F/CK58.seq          ..---.....................................A...........A.....................................  588
ts-11.seq               ..T--.....................................A...........A.....................................  587
6/85.seq                ..---.....................................A...........A.....................................  588
```
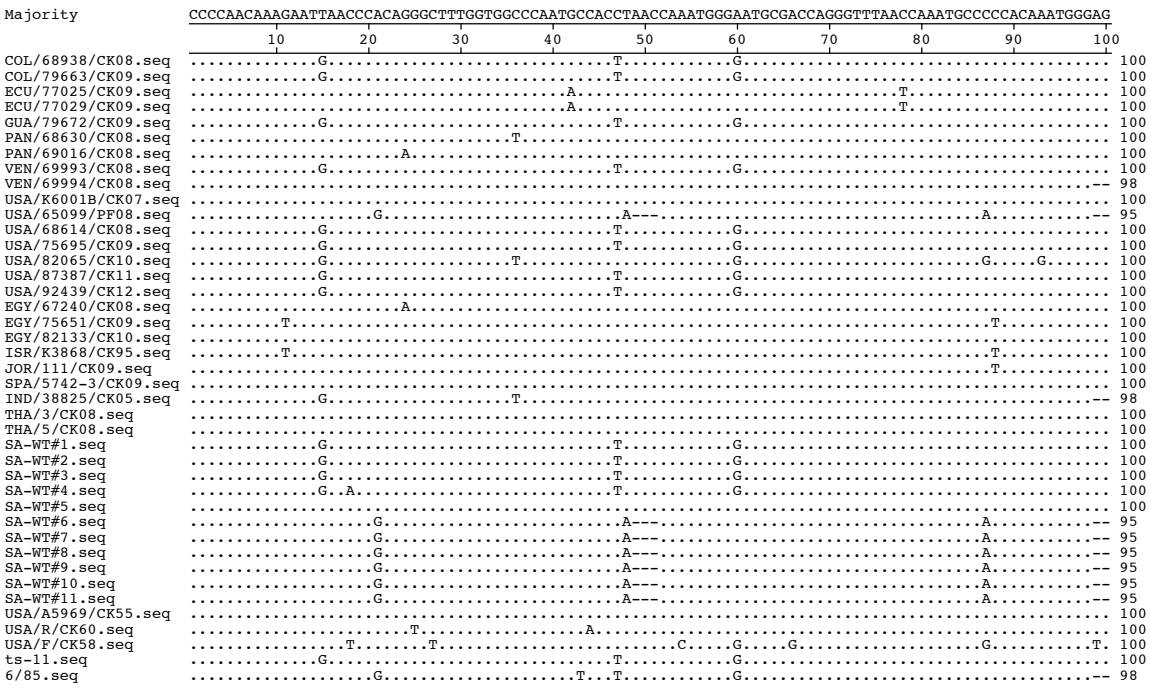
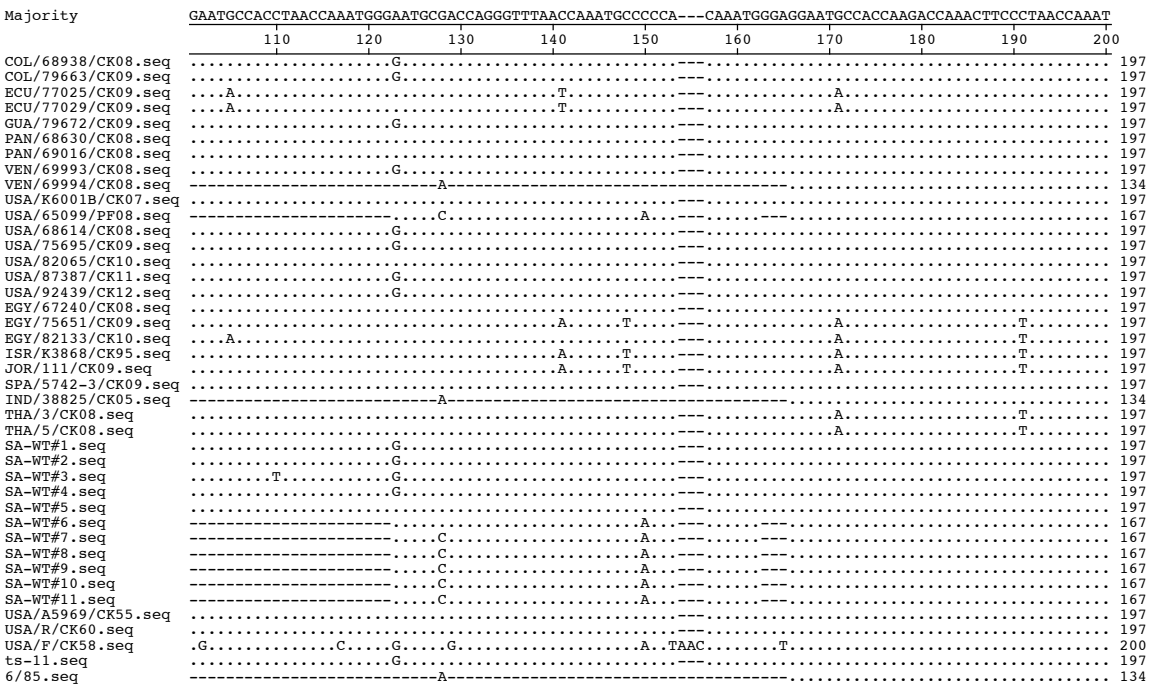
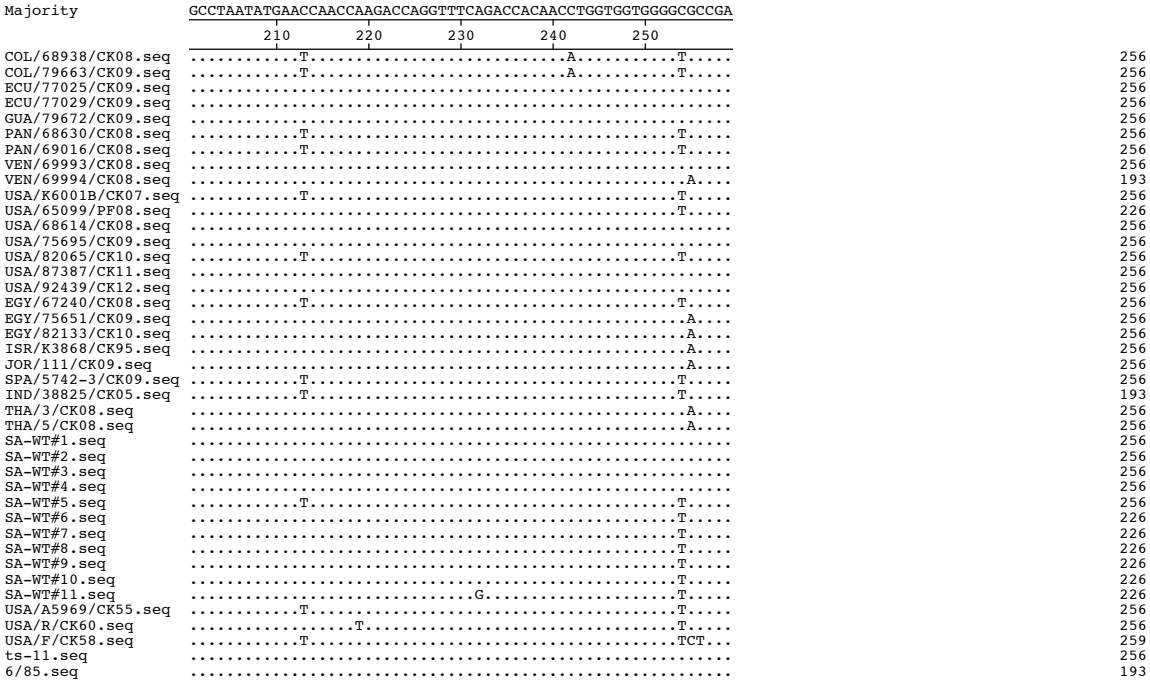

```
Majority                AGTCCGAAATTTGATTAAAGTATCAAATC-----AAATTTCATATAAT-ATAGATTCAATAAAAATAGCTAATGGATCAAATACATAAGTTACTA
                                610       620       630       640       650       660       670       680       690
COL/68938/CK08.seq      ..........................AAATC.............-.....................................................  682
COL/79663/CK09.seq      ..........................-----.............-.....................................................  675
ECU/77025/CK09.seq      ..........................-----.............-.....................................................  675
ECU/77029/CK09.seq      ..........................-----.............-.....................................................  677
GUA/79672/CK09.seq      ..........................-----.............-.....................................................  677
PAN/68630/CK08.seq      ..........................------..G..........-.....................................................  675
PAN/69016/CK08.seq      ..........................-----.............-.....................................................  677
VEN/69993/CK08.seq      ..........................-----.............-.....................................................  677
VEN/69994/CK08.seq      ..........................-----.............-.....................................................  677
USA/K6001B/CK07.seq     ..........................-----.............T.....................................................  676
USA/65099/PF08.seq      ..........................-----.............-.....................................................  671
USA/68614/CK08.seq      ..........................-----.............-.....................................................  671
USA/75695/CK09.seq      ..........................-----.............-.....................................................  672
USA/82065/CK10.seq      ..........................-----.............-.....................................................  672
USA/87387/CK11.seq      ..........................-----.............-.....................................................  672
USA/92439/CK12.seq      ..........................-----.............-.....................................................  674
EGY/67240/CK08.seq      ..........................-----.............-.....................................................  675
EGY/75651/CK09.seq      ..........................-----.............-.....................................................  675
EGY/82133/CK10.seq      ..........................-----.............-.....................................................  675
ISR/K3868/CK95.seq      ..........................-----.............-.....................................................  676
JOR/111/CK09.seq        ..........................-----.............-.....................................................  675
SPA/5742-3/CK09.seq     ..........................-----.............-.....................................................  675
IND/38825/CK05.seq      ..........................-----.............-.....................................................  675
THA/3/CK08.seq          ..........................AAATC.............-.....................................................  682
THA/5/CK08.seq          ..........................-----.............-.....................................................  677
SA-WT#1.seq             ..........................-----.............-.....................................................  675
SA-WT#2.seq             ..........................-----.............-.....................................................  675
SA-WT#3.seq             ..........................-----.............T.....................................................  674
SA-WT#4.seq             ..........................-----.............-.....................................................  676
SA-WT#6.seq             ..........................-----.............-.....................................................  675
SA-WT#7.seq             ..........................-----.............-.....................................................  675
SA-WT#8.seq             ..........................-----.............-.....................................................  674
SA-WT#9.seq             ..........................-----.............-.....................................................  676
SA-WT#10.seq            ..........................-----.............T.....................................................  674
SA-WT#12.seq            ..........................-----.............-.....................................................  675
USA/A5969/CK55.seq      ..........................-----.............-.....................................................  675
USA/R/CK60.seq          ..........................-----.............-.....................................................  674
USA/F/CK58.seq          ..........................-----.............-.....................................................  677
ts-11.seq               ..........................-----.............-.....................................................  676
6/85.seq                ..........................-----.............-.....................................................  677
```

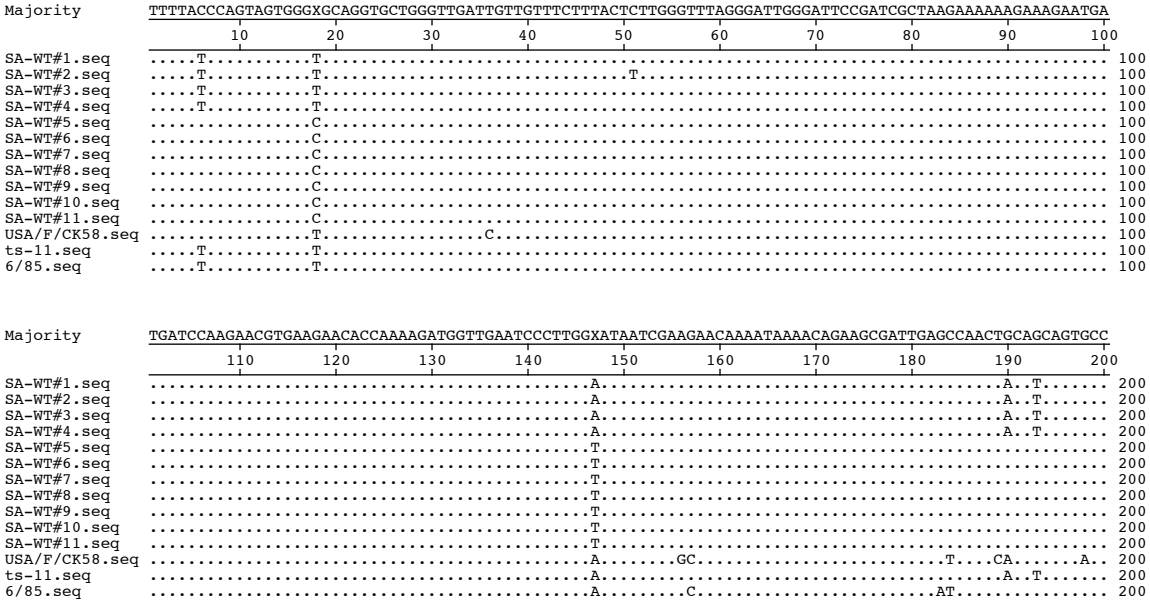
Supplementary Figure 2. Clustal-W alignment of the *mgc2* (fragment) nucleotide sequences of South African *M. gallisepticum* wild-types, international isolates and vaccine and reference strains. Nucleotides identical to the consensus sequence or absent are represented by '.' or '-' respectively.

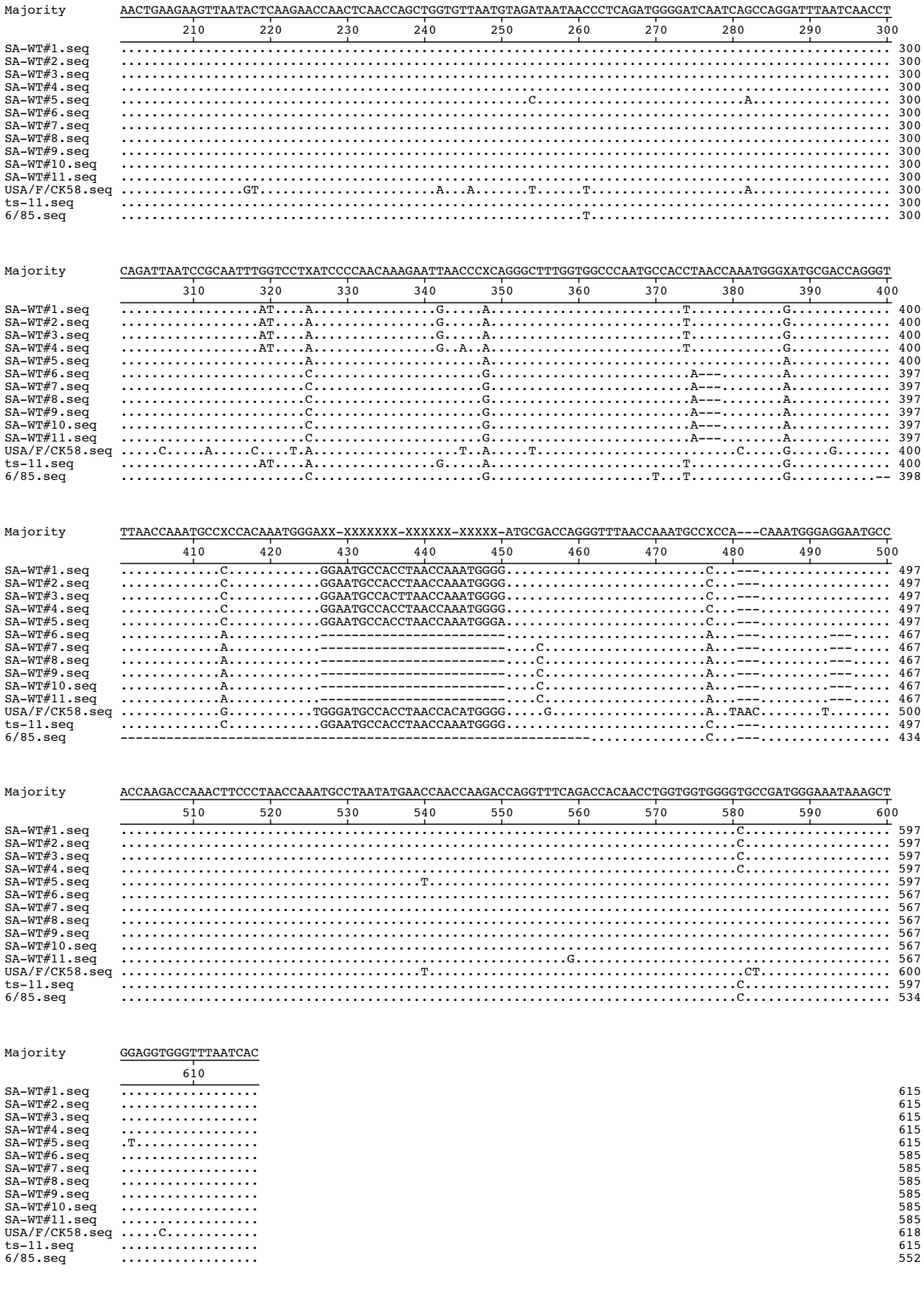





Supplementary Figure 3. Clustal-W alignment of the *mgc2* nucleotide sequences of South African *M. gallisepticum* wild-types and live *M. gallisepticum* vaccine strains. Nucleotides identical to the consensus sequence or absent are represented by '.' or '-' respectively.

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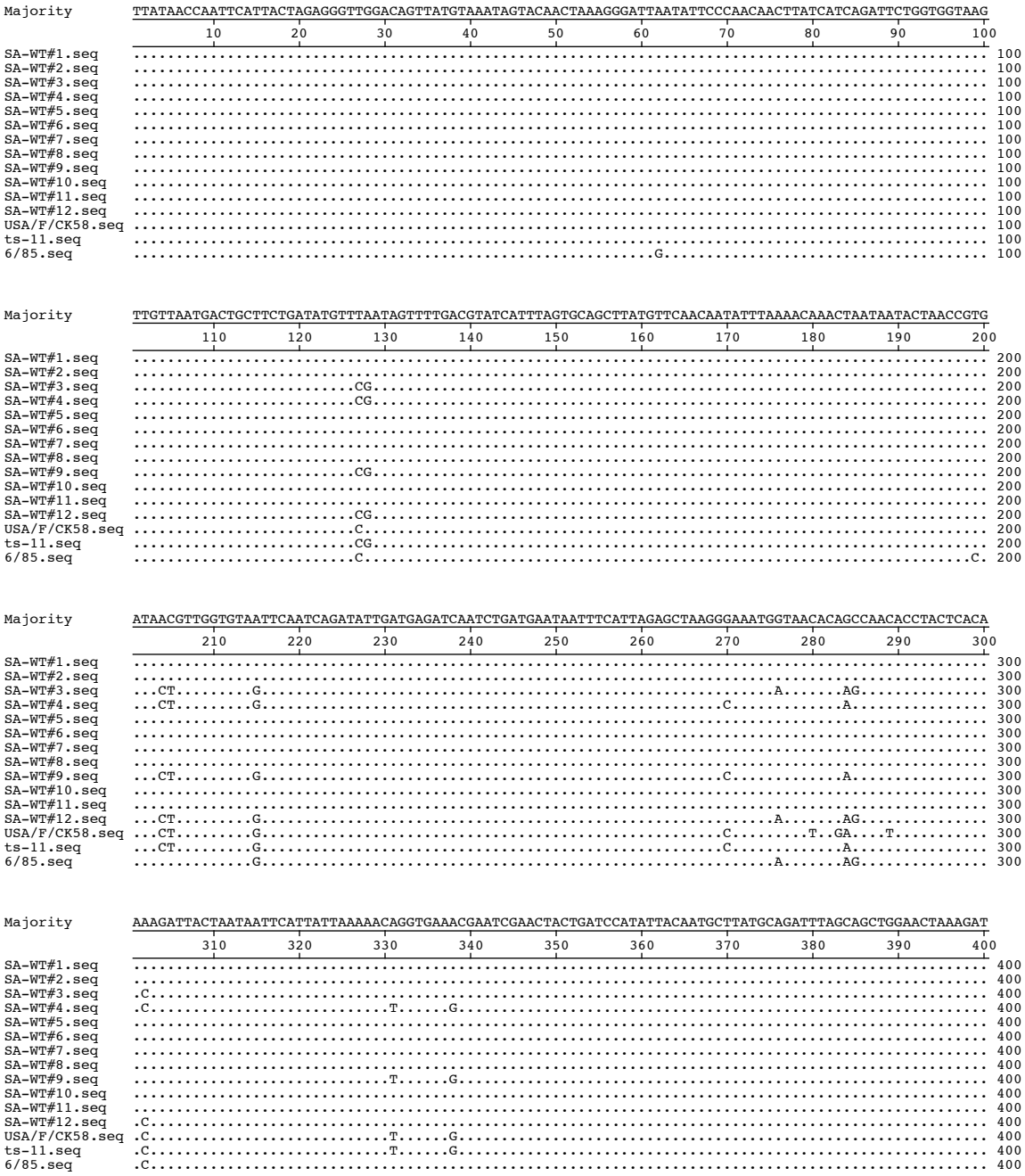
SA-WT#1.seq 300
SA-WT#2.seq 300
SA-WT#3.seq 300
SA-WT#4.seq 300
SA-WT#5.seq 300
SA-WT#6.seq 300
SA-WT#7.seq 300
SA-WT#8.seq 300
SA-WT#9.seq 300
SA-WT#10.seq 300
SA-WT#11.seq 300
USA/F/CK58.seq 300
ts-11.seq 300
6/85.seq 300
Majority CAGATTAATCCGCAATTTGGTCCTXATCCCCAACAAAGAATTAACCCXCAGGGCTTTGGTGGCCCAATGCCACCTAACCAAATGGGXATGCGACCAGGGT
310 320 330 340 350 360 370 380 390 400
SA-WT#1.seq 400
SA-WT#2.seq 400
SA-WT#3.seq 400
SA-WT#4.seq 400
SA-WT#5.seq 400
SA-WT#6.seq 397
SA-WT#7.seq 397
SA-WT#8.seq 397
SA-WT#9.seq 397
SA-WT#10.seq 397
SA-WT#11.seq 397
USA/F/CK58.seq 400
ts-11.seq 400
6/85.seq 398
Majority TTAACCAAATGCCXCCACAAATGGGAXX-XXXXXXX-XXXXXX-XXXXX-ATGCGACCAGGGTTTAACCAAATGCCXCCA---CAAATGGGAGGAATGCC
410 420 430 440 450 460 470 480 490 500
SA-WT#1.seq 497
SA-WT#2.seq 497
SA-WT#3.seq 497
SA-WT#4.seq 497
SA-WT#5.seq 497
SA-WT#6.seq 467
SA-WT#7.seq 467
SA-WT#8.seq 467
SA-WT#9.seq 467
SA-WT#10.seq 467
SA-WT#11.seq 467
USA/F/CK58.seq 500
ts-11.seq 497
6/85.seq 434
Majority ACCAAGACCAAACTTCCCTAACCAAATGCCTAATATGAACCAACCAAGACCAGGTTTCAGACCACAACCTGGTGGTGGGGTGCCGATGGGAAATAAAGCT
510 520 530 540 550 560 570 580 590 600
SA-WT#1.seq 597
SA-WT#2.seq 597
SA-WT#3.seq 597
SA-WT#4.seq 597
SA-WT#5.seq 597
SA-WT#6.seq 567
SA-WT#7.seq 567
SA-WT#8.seq 567
SA-WT#9.seq 567
SA-WT#10.seq 567
SA-WT#11.seq 567
USA/F/CK58.seq 600
ts-11.seq 597
6/85.seq 534
Majority GGAGGTGGGTTTAATCAC
610
SA-WT#1.seq 615
SA-WT#2.seq 615
SA-WT#3.seq 615
SA-WT#4.seq 615
SA-WT#5.seq 615
SA-WT#6.seq 585
SA-WT#7.seq 585
SA-WT#8.seq 585
SA-WT#9.seq 585
SA-WT#10.seq 585
SA-WT#11.seq 585
USA/F/CK58.seq 618
ts-11.seq 615
6/85.seq 552

Supplementary Figure 4. Clustal-W alignment of the MGA_0319 nucleotide sequences of South African *M. gallisepticum* wild-types and live *M. gallisepticum* vaccine strains. Nucleotides identical to the consensus sequence or absent are represented by '.' or '-' respectively.




Supplementary Figure 5. Clustal-W alignment of the *gapA* nucleotide sequences of South African *M. gallisepticum* wild-types and live *M. gallisepticum* vaccine strains. Nucleotides identical to the consensus sequence or absent are represented by '.' or '-' respectively.